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EDITORIAL OFFICE
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Xiu-Xia Song, Vice Director
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Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
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EDITORIAL

Withdrawal of anti-tumour necrosis factor α therapy in inflammatory bowel disease

Konstantinos Papamichael, Severine Vermeire

Konstantinos Papamichael, Severine Vermeire, Department of Clinical and Experimental Medicine, Translational Research Center for Gastrointestinal Disorders, 3000 Leuven, Belgium

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Correspondence to: Konstantinos Papamichael, MD, PhD, FEBGH, Department of Clinical and Experimental Medicine, Translational Research Center for Gastrointestinal Disorders, Herestraat 49, 3000 Leuven,
Belgium. konstantinos.papamichail@kuleuven.be
Telephone: +32-16-377557
Fax: +32-16-344419

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Abstract

Anti-tumour necrosis factor α (anti-TNFα) therapy is an established treatment in inflammatory bowel disease. However, this treatment is associated with high costs and the possibility of severe adverse events representing a true challenge for patients, clinicians and health care systems. Consequently, a crucial question is raised namely if therapy can be stopped once remission is achieved and if so, how and in whom. Additionally, in a real-life clinical setting, discontinuation may also be considered for other reasons such as the patient's preference, pregnancy, social reasons as moving to countries or continents with less access, or different local policy or reimbursement. In contrast to initiation of anti-TNFα therapy guidelines regarding stopping of this treatment are missing. As a result, the decision of discontinuation is still a challenging aspect in the use of anti-TNFα therapy. Currently this is typically based on an estimated, case-by-case, benefit-risk ratio. This editorial is intended to provide an overview of recent data on this topic and shed light on the proposed drug withdrawal strategies.

Key words: Inflammatory bowel disease; Anti-tumour necrosis factor α therapy; Withdrawal; Remission; Infliximab

Core tip: Anti-tumour necrosis factor α (anti-TNFα) therapy is an established treatment in inflammatory bowel disease. Although guidelines exist on initiation of anti-TNFα therapy in inflammatory bowel diseases, information on if, when, how and in whom therapy can be stopped is limited. This is nevertheless an important topic taking under consideration the cost and the possible adverse events associated with biological agents as well as the desire of patients to discontinue medication especially after a long maintained remission. Moreover, although drug discontinuation for reasons other than loss of response is very usual in real-life clinical practice, the optimal withdrawal strategy is still debated.

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INTRODUCTION

Anti-tumor necrosis factor α (anti-TNFα) therapy has greatly improved the management of patients with inflammatory bowel diseases (IBD) namely Crohn's disease (CD) and ulcerative colitis (UC)[1]. However, this treatment is associated with high costs and the possibility of severe adverse events such as opportunistic infections or risk for lymphoma. These aspects represent a true challenge not only for patients and clinicians but also for health care systems[2]. Consequently, a crucial question is raised: can therapy be stopped once remission is achieved and if so, when, how and in whom? Additionally, in a real-life clinical setting, discontinuation may also be considered for other reasons such as the patient's own preference, pregnancy, moving to places with less access to biological agents, local policy or different reimbursement systems[2].

Nowadays, as supporting data is lacking, there are no stopping rules for anti-TNFα therapy in IBD. There is even less information regarding prognostic factors that could predict relapse or sustained remission after anti-TNFα therapy discontinuation. The only provided evidence regarding CD comes from the landmark STORI trial[3] and a few retrospective observational[4-8] or small prospective studies[9-12], while for UC there are even less data available[7,9,13] (Table 1). As a result, the decision of discontinuation is currently made on the basis of an individual judgement of benefits versus risks and cost-effectiveness[14-18].

Another important issue when considering cessation of anti-TNFα therapy is whether the drug can safely be restarted when needed and whether efficacy will be similar. Possible lower response rates after re-initiation of biological therapy, limited alternative treatment options and/or immunogenicity concerns are all factors which constitute to the fear of stopping treatment[18,19].

WITHDRAWAL OF ANTI-TNFα THERAPY IN INFLAMMATORY BOWEL DISEASE. IS IT FEASIBLE?

Current guidelines suggest that anti-TNFα therapy should be started early in the course of the IBD to maximize its efficacy before irreversible bowel damage has occurred[20]. On the other hand, there are no rules and/or recommendations with respect to stopping, although it is often empirically proposed not to routinely stop anti-TNFα agents in IBD patients who respond, and especially in patients with disabling features of disease and/or at high-risk for relapse[14-18]. However, recent data indicate that a proportion of patients, in clinical remission can stop anti-TNFα therapy without a major impact on disease control even for a relatively long time period, while on immunomodulators (IMM) (Table 1). The pivotal STORI trial showed that it was possible to identify a subgroup of patients with only a 10% relapse risk 24 mo post-discontinuation[3].

Nevertheless, the results of the various studies may not always be comparable as the type of IBD (CD *vs* UC), of anti-TNFα therapy (infliximab *vs* adalimumab), the study design (prospective *vs* retrospective observational), the studied outcome (clinical *vs* endoscopic), the duration of remission before stopping anti-TNFα agents and the phenotypic and clinical characteristics of the patients often differ, as were the definition of relapse and the (median) follow up time after anti-TNFα cessation[21]. Moreover, CD patients are very heterogeneous in terms of disease type (luminal or fistulising), location (ileal, ileo-colonic or colonic) and behaviour (inflammatory, stricturing, or penetrating) and the consequences of relapse may widely vary, while establishment of complete mucosal healing and clinical remission is much more straightforward in UC than in CD.

One argument in favour of discontinuation of anti-TNFα therapy is the fact that all studies show that anti-TNFα therapy can be restarted without risking loss of response or adverse events in a large proportion of patients (Table 1). In a recent study by Baert *et al*[22] re-starting of infliximab therapy re-introduced response in 84.5% of patients at week 14, 70% at 1 year, and in 61% of patients at more than 4 years. Re-introduction of anti-TNFα therapy therefore seems possibly independent of the drug holiday[22], although Laharie *et al*[23] showed that retreatment with IFX in CD primary responders should be administered within 50 wk after induction, for better efficacy and tolerance.

Discontinuation of anti-TNFα therapy for clinical remission has been shown to be feasible in other autoimmune, chronic diseases such as rheumatoid arthritis[24], Behçet's uveitis[25], spondyloarthritis[26] and sarcoidosis[27].

WHEN SHOULD WE STOP?

Stopping of anti-TNFα therapy is more likely to succeed in terms of maintaining prolonged remission when complete clinical (CDAI < 150), endoscopic (complete mucosal healing) and serological (normal CRP) remission, were achieved prior to discontinuation[20]. However, the minimum time of remission before stopping anti-TNFα therapy has not been yet well defined. It is proposed that clinical remission for over than one year prior to discontinuation of anti-TNFα therapy is adequate[11,20] while others suggest to stop after a minimum of two years of clinical and endoscopic remission or longer if only clinical remission can be documented[28].

Table 1 Studies on the discontinuation of anti-tumor necrosis factor α therapy in inflammatory bowel disease

IBD type	Anti-TNFα therapy	*n*	Median follow up, mo	SCR at the end of follow up, %	Clinical benefit after re-introduction of anti-TNFα therapy for relapse, %	Ref.
CD	IFX	115	28	55	88	[3]
CD	IFX	48	49	35	ND	[4]
CD	IFX	53	18	12	96	[7]
UC	IFX	28	29	40	71	[7]
CD	IFX or ADM	121	12	55	55	[11]
UC	IFX	51	12	65	94	[13]
CD	IFX or ADM	37	1-44 (range)	26 (1 yr)	ND	[10]
CD	IFX or ADM	17	13	71	100	[9]
UC	IFX	34	13	65	90	[9]
CD	IFX	100	120	52	ND	[6]
CD	IFX or ADM	86	17	64 (1 yr)	93	[12]
CD	IFX	92	47	28	89	[5]

IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; IFX: Infliximab; ADM: Adalimumab; TNFα: Tumor necrosis factor α; ND: Not defined; SCR: Sustained clinical remission.

Moreover, preliminary evidence suggests that a long lasting and profound drug-free remission may be achieved in case of a short duration of disease from diagnosis to start of anti-TNFα therapy, probably before irreversible immunological aberrations and more intestinal tissue impairment have occurred[6,7].

HOW SHOULD WE STOP?

The optimal treatment following discontinuation of anti-TNFα therapy in IBD has not yet been clearly defined, as in all previous studies the majority of patients (67%-100%) maintained clinical remission after cessation of treatment, by a continuous administration of an IMM[2,21]. Consequently, it is unclear whether a sustained clinical remission can be achieved during a true drug-free period.

Most will agree that a close follow up is needed when anti-TNF is stopped but how to monitor these patients in the most optimal way is not clear. Monitoring of CRP and fecal calprotectin levels every 8-12 wk may be very useful for predicting early clinical relapse with endoscopic re-evaluation in case of a significant increase of these biomarkers (Figure 1)[29]. If and when endoscopic evaluation should be implemented in the follow up of patients who remain in full remission remains to be elucidated[28].

IN WHOM?

Another important issue, before applying a stopping strategy for anti-TNFα therapy, is to assess prediction of sustained remission after withdrawal of the drugs, in order to identify the ideal candidate for discontinuation of anti-TNFα treatment. We believe that the decision-making approach to stop anti-TNFα therapy is currently based on limited data (Table 2).

Although patients with (perianal) fistulising CD were excluded from many studies including the STORI trial[3] current data suggest that these patients relapse with the same rate as those with luminal disease[5,6,9,11,12]. However, in previous studies infliximab discontinuation led to a higher rate of relapse in patients with perianal fistulising compared to luminal CD[23,30,31]. One possible explanation could be that in most of these studies clinical remission and relapse for the majority of patients with (perianal) fistulising CD were not evaluated by imaging techniques or this information was missing. This is very important taking into account that perianal disease is often active despite external fistulae closure. Consequently, evaluation of CD patients with stricturing and/or penetrating disease, before discontinuation of anti-TNFα therapy, may include also imaging techniques. Patients with internal fistulas, an intestinal stenosis or a complex perianal fistulising disease may have a poor prognosis after discontinuation of the anti-TNFα therapy.

Moreover although complete mucosal healing at the time of IFX discontinuation for clinical remission in CD patients was predictive for sustained clinical remission after cessation of the drug[3,6], this was not confirmed by other studies[11,13,32].

Finally, regarding the role of therapeutic drug monitoring on the decision making of stopping anti-TNF therapy for clinical remission preliminary evidence points out that low IFX trough levels at the time of discontinuation are predictive of sustained remission after drug cessation in CD, while the role of antibodies to IFX has not been yet clearly defined[3,6]. It seems that these patients do not need the drug anymore to maintain remission which is in agreement with the results of the TAXIT trial where 9% of the patients being in remission had undetectable trough levels and were stopped successfully without relapse[33].

NEW CONCEPT OF INTERMITTENT ANTI-TNFα THERAPY IN INFLAMMATORY BOWEL DISEASE

Tailoring of anti-TNFα maintenance therapy for patients achieving remission is becoming nowadays a necessity

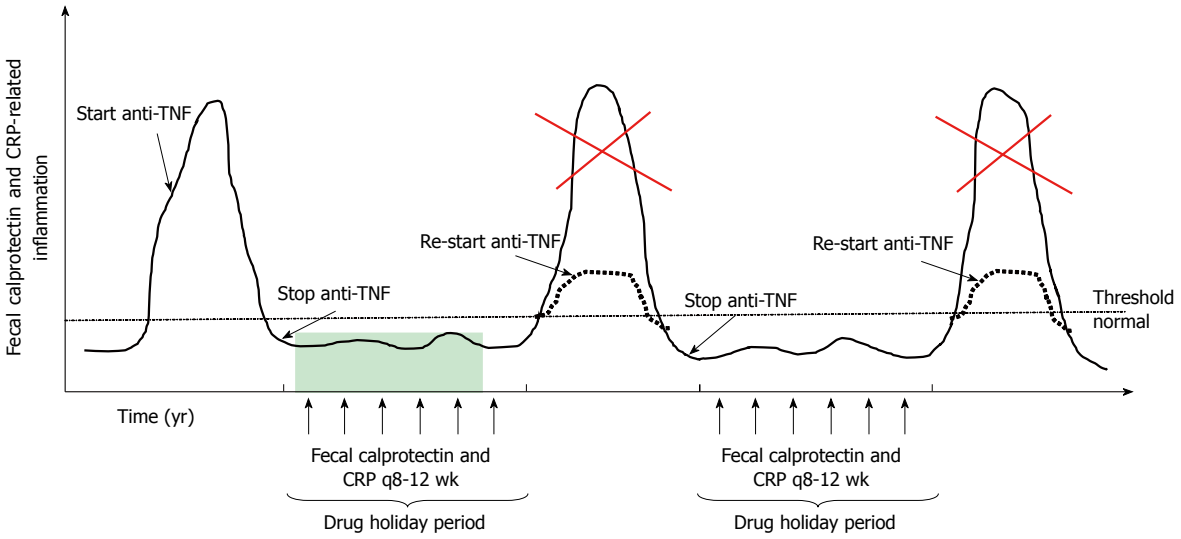

Figure 1 New concept of intermittent anti-tumor necrosis factor α therapy in inflammatory bowel disease. Stopping anti-TNFα agents after achieving a deep remission may result in prolonged clinical remission. Close monitoring of these patients with fecal calprotectin and CRP measurements (arrows) will allow early re-initiation of anti-TNFα therapy, when inflammation is starting to rise, which may result to a sustained clinical benefit (dotted line) preventing a disease flare (red cross). These patients may be considered as treated periodically and not episodically. TNF: Tumor necrosis factor; CRP: C-reactive protein.

Table 2 Risk factors for relapse after stopping anti-tumor necrosis factor α therapy for remission (clinical or endoscopic) in inflammatory bowel disease

Risk factors	Ref.
Clinical or phenotypic	
Corticosteroid use between 12 and 6 mo before baseline	[3]
Male gender	[3]
Absence of previous surgical resection	[3]
Longer disease duration from diagnosis to first infliximab	[7]
Previous biological therapy	[11-13]
Dose intensification during the first year of anti-TNFα therapy	[11]
Age at CD diagnosis ⩾ 25 yr	[6]
Ileocolonic disease at diagnosis	[12]
Active smoking	[5]
Previous antimetabolite failure	[5]
Perianal disease	[5]
Serological[1]	
Hemoglobin levels ⩽ 14.5 g/dL	[3]
White blood count > 6 × 10^9/L	[3]
High sensitive CRP ⩾ 5 mg/L	[3]
Infliximab trough levels ⩾ 2 μg/mL	[3]
Serum calprotectin > 5675 ng/mL	[37]
Endoscopic[1]	
CDEIS > 0	[3]
Mucosal[1]	
Lack of normalization of IL-17A and TNFα expression levels	[10]
Microbiological[1] (CD-associated dysbiosis)	
Low rate of Faecalibacterium prausnitzii in fecal samples	[38]
Low rate of Bacteroides in fecal samples	[38]
Fecal[1]	
Fecal calprotectin ⩾ 300 μg/g	[3]
Genetic	
Fc gamma receptor ⅢB-NA2/NA2 genotype (fistulising disease)	[39]

[1]At the time of anti-TNFα therapy discontinuation. CD: Crohn's disease; CRP: C-reactive protein; CDEIS: Crohn's Disease Endoscopic index of severity; IL: Interleukin; NA: Neutrophil antigen; TNFα: Tumor necrosis factor α.

as safety and costs issues may hinder the long-term, sustained clinical benefit deriving from this therapy. This could be achieved either by lowering the dose of these drugs[33], based on therapeutic drug monitoring[34] or by stopping them following the intermittent non-continuous pharmacological treatment approach[2]. Patients following the latter strategy may be considered as treated periodically rather than episodically (Figure 1)[35]. A paradigm of this therapeutic pharmacological approach from real-life clinical practice is described in Figure 2. We believe that prediction of sustained clinical remission after discontinuation of anti-TNFα therapy along with the close monitoring of these patients so as to avoid an upcoming disease flare by early re-introduction of these drugs may be a first step for optimizing maintenance anti-TNFα treatment in patients achieving remission.

CONCLUSION

Intentional cessation of anti-TNFα treatment will become a more prevalent practice in the future not only for safety and cost reasons but probably also due to newly available non TNFα neutralizing pharmaceutical therapeutics options, although with the introduction of biosimilars, costs will probably become less important. Anti-TNFα withdrawal strategy to achieve disease control based on an on-demand use of anti-TNFα therapy (when relapse is suspected after discontinuation of the drugs) and a continuous treatment with IMM, could be an option to reduce chronic exposure to biologics, at least to a highly selected IBD population.

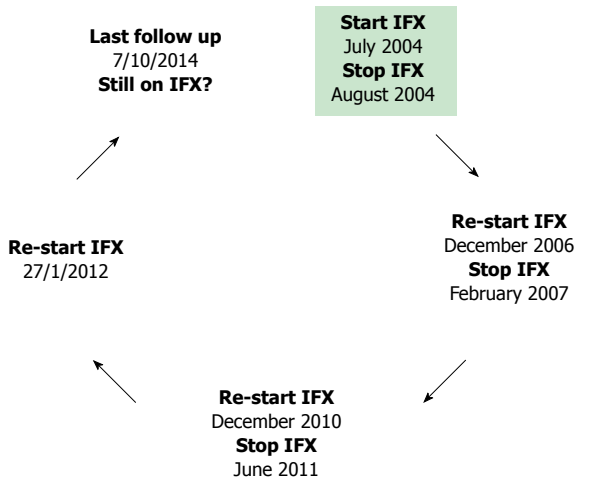

Figure 2 Successful intermittent infliximab therapy in a patient with ulcerative colitis: A paradigm from real-life clinical practice. This is an example of a UC patient (male, age at diagnosis 33 years) with pancolitis, treated in our center, who received successfully intermittent infliximab (IFX) therapy (black arrows represent time periods of clinical remission). He has been treated with IFX for relapse, after discontinuation of the drug on his own preference while in clinical remission continuing on azathioprine. At the last time of follow up he was still in clinical and biochemical (normal CRP) remission under IFX maintenance monotherapy. This patient has never developed antibodies to IFX despite receiving interrupted therapy, while the last available (25/2/2014) trough concentrations of IFX were 8.09 μg/mL (q5).

Nevertheless, in order to elucidate whether discontinuation of anti-TNFα therapy for remission will become a routine strategy in the future for the long-term management of IBD patients and to define the optimal withdrawal strategy more studies are needed. One of them would definitely be the SPARE study from the Groupe d'Etude Therapeutique des Affections Inflammatoires Digestives group, a Phase Ⅳ, prospective, open-label, randomized controlled trial comparing infliximab antimetabolites combination therapy to anti-metabolites monotherapy and infliximab monotherapy in CD patients in sustained steroid-free remission on combination therapy (ClinicalTrials.gov Identifier: NCT02177071)[36]. The main goal of this study is to demonstrate that infliximab scheduled maintenance with or without antimetabolites is superior to antimetabolites alone to maintain sustained steroid-free remission over 2 years, while the latter is non inferior with regards to the mean time spent in remission over the same duration[36].

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REVIEW

Challenges in animal modelling of mesenchymal stromal cell therapy for inflammatory bowel disease

Raghavan Chinnadurai, Spencer Ng, Vijayakumar Velu, Jacques Galipeau

Raghavan Chinnadurai, Spencer Ng, Vijayakumar Velu, Jacques Galipeau, Department of Hematology and Oncology, Winship Cancer Institute, Emory University, Atlanta, GA 30322, United States
Vijayakumar Velu, Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, Atlanta, GA 30329, United States
Jacques Galipeau, Department of Pediatrics, Emory University, Atlanta, GA 30322, United States
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Correspondence to: Jacques Galipeau, Professor, Department of Hematology and Oncology, Winship Cancer Institute, Emory University, 1365-C Clifton Road NE, Atlanta, GA 30322, United States. jgalipe@emory.edu
Telephone: +1-404-7781779
Fax: +1-404-7783965
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Abstract

Utilization of mesenchymal stromal cells (MSCs) for the treatment of Crohn's disease and ulcerative colitis is of translational interest. Safety of MSC therapy has been well demonstrated in early phase clinical trials but efficacy in randomized clinical trials needs to be demonstrated. Understanding MSC mechanisms of action to reduce gut injury and inflammation is necessary to improve current ongoing and future clinical trials. However, two major hurdles impede the direct translation of data derived from animal experiments to the clinical situation: (1) limitations of the currently available animal models of colitis that reflect human inflammatory bowel diseases (IBD). The etiology and progression of human IBD are multifactorial and hence a challenge to mimic in animal models; and (2) Species specific differences in the functionality of MSCs derived from mice versus humans. MSCs derived from mice and humans are not identical in their mechanisms of action in suppressing inflammation. Thus, preclinical animal studies with murine derived MSCs cannot be considered as an exact replica of human MSC based clinical trials. In the present review, we discuss the therapeutic properties of MSCs in preclinical and clinical studies of IBD. We also discuss the challenges and approaches of using appropriate animal models of colitis, not only to study putative MSC therapeutic efficacy and their mechanisms of action, but also the suitability of translating findings derived from such studies to the clinic.

Key words: Mesenchymal stromal cells; Inflammatory bowel disease; Colitis; Animal model; Crohn's disaese

Core tip: Several clinical trials have investigated the use of mesenchymal stromal cells (MSCs) for the treatment of inflammatory bowel disease. Although MSC therapy has proven to be safe, efficacy remains to be determined. Animal model studies are necessary to evaluate the efficacy and mechanism of action of MSCs, which will improve ongoing clinical trials. However, clinical translation is largely hampered by (1) variability

of colitis animal models available; and (2) differences in the biology of murine and human MSC counterparts. Here we discuss the challenges and approaches of translating animal studies to clinical trials.

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INTRODUCTION

Mesenchymal stromal cells (MSCs) are under intense clinical investigation worldwide for a number of regenerative and immune disorders. MSCs are attractive for cell therapy due to their immunomodulatory and regenerative properties and robust *in vitro* proliferative capacity[1,2]. Currently more than 300 clinical trials have been registered to test MSCs as therapeutics for various auto and alloimmune disorders such as inflammatory bowel diseases, graft *vs* host disease, multiple sclerosis, autoimmune rheumatic diseases, and autoimmune diabetes (clinicaltrials.gov). Isolation and expansion of MSCs for cell therapy can be achieved through harvesting various tissue sources such as bone marrow, adipose, umbilical cord and placenta[3,4]. The International Society for Cellular Therapy (ISCT) defined minimal criteria for the definition of multipotent MSCs such as (1) plastic adherence in standard *in vitro* culture conditions; (2) expression of CD105, CD73 and CD90, and lack of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR; and (3) differentiation to osteoblasts, adipocytes, and chondroblasts *in vitro*[5]. As an extension to these standards, ISCT has proposed further criteria for the immunological characterization of MSCs[6]. These include: (1) MSC response to the cytokines IFN-γ and TNF-α; (2) indoleamine 2,3-dioxygenase (IDO) response in cytokine licensing assays; (3) testing the functionality of the expanded cell product; (4) usage of purified immune responders in the functional assays; (5) analysis of mechanistic and efficacy studies of human MSCs in xenotransplantation models; (6) immune reaction to the infused MSCs; and (7) analysis of the lymphocyte populations of patients treated with MSCs[7]. Implementing some of these suggested analyses in preclinical studies involving animal models of tissue injury and inflammatory disorders will provide critical insight into MSC mechanisms of action and improve future clinical trials involving MSCs. Here we review the available data that utilize MSCs for mitigating colitis in animal models and highlight the challenges in translating these studies into effective clinical therapies.

CLINICAL TRIALS UTILIZING MSCs FOR INFLAMMATORY BOWEL DISEASES

Crohn's disease (CD) and ulcerative colitis (UC) are inflammatory diseases of the gastrointestinal system caused by the multiple factors such as genetic susceptibility, breakdown of mucosal immune tolerance, and self-immune activation to gut microbiota[8,9]. Current treatment approaches are predominately aimed at suppressing overt inflammation and include the use of pharmacological agents, biologicals, and surgery to remove sections of inflamed bowel. However, these treatment modalities have limitations due to non-adherence and relapse[10]. In the past decade, alternative cell-based immunosuppressive therapies utilizing MSCs have been tested in clinical trials for both luminal and fistulizing forms of IBD[11,12]. Immunosuppressive and differentiation properties of MSCs are thought to play a major role in ameliorating luminal and fistula conditions, respectively[13]. Almost all the early phase clinical trials are aimed at determining the safety and tolerability of utilizing autologous and allogeneic adipose- or bone marrow-derived sources of MSCs for the treatment of IBD[13]. The results proved MSC therapy is safe but efficacy data remains elusive and conflicting clinical benefit has been reported so far. A large Phase Ⅲ study for the treatment of complex cryptoglandular perianal fistulas using autologous adipose derived MSCs showed no efficacy[14]. In addition, a recent phase Ⅱ placebo-controlled, parallel group, multi-center study to investigate the safety and efficacy of allogeneic Multistem®, stem cells derived from adult bone marrow and non-embryonic tissue sources, in subjects with moderate to severe ulcerative colitis showed no clinical benefit (NCT01240915). However, another earlier phase Ⅱ clinical trial using allogeneic bone marrow-derived MSCs for refractory luminal CD demonstrated that 12 out of 15 patients showed reduction in CD activity index providing evidence of clinical efficacy[15]. Thus clinical trial provide conflicting results on the efficacy of MSCs, which is in contrast to the data derived from preclinical animal model studies. Most of the preclinical *in vivo* data with animal models of colitis demonstrated the consistent efficacy of MSC's therapeutic properties. Hence, variations in the clinical outcome could be due to two major factors: (1) discrepancies in MSC source, preparation, and handling methods, all of which may affect the quality of the cellular product. Our group and others demonstrated that thawed MSCs from cryopreservation showed attenuated biodistribution and immunosuppressive properties compared to actively growing MSCs in *in vitro* cultures. This observation may provide an explanation for variations in industry sponsored clinical trial outcomes[16-18]. Similarly prolonged expansion

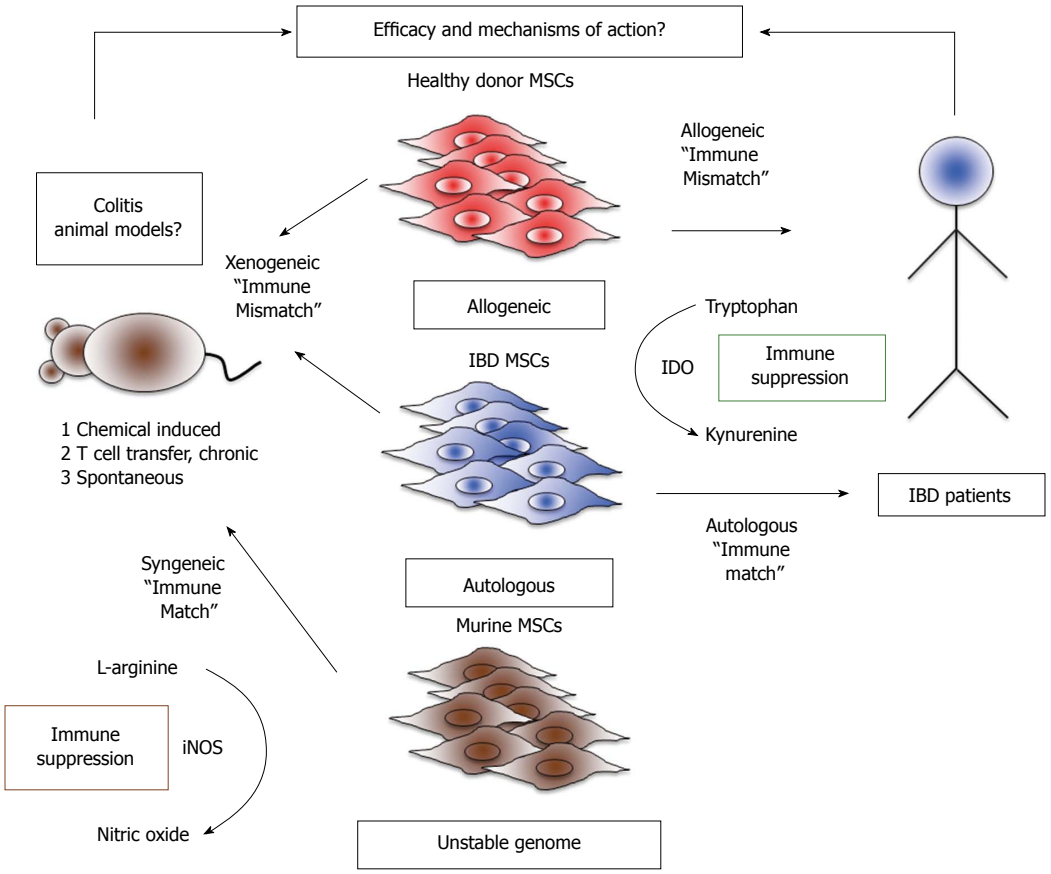

Figure 1 Challenges in modelling efficacy and mechanisms of action of mesenchymal stromal cells between preclinical and clinical studies. Right: Current clinical trials fall into two major groups: (1) allogeneic studies where inflammatory bowel diseases patients receive mesenchymal stromal cells (MSCs) from a random MHC unmatched randomdonor; and (2) autologous studies where patients are given their own MSCs. In human MSCs, immunosuppression is dependent on Indoleamine 2, 3 dioxygenase (IDO) activity, an enzyme that converts the essential amino acid tryptophan into the immunosuppressive catabolite, kynurenine. Left: Murine MSCs differ from human MSCs in their primary mechanism of immune suppression, utilizing inducible nitric oxide synthase (iNOS) to create nitric oxide as a product of L-arginine catabolism. The therapeutic effects of murine MSCs can be tested by engraftment into a colitic mouse, and is syngeneic if the MSCs are derived from a mouse with the same strain/background as the colitic mouse. However, murine MSCs may potentially transform after prolonged *in vitro* culture due to genomic instability. IBD: Inflammatory bowel diseases.

of MSCs lead to a senescent phenotype, resulting in their failure to mitigate lethal endotoxima in animals[19]. Thus, cellular preparation methods are one of the determining factors of clinical outcome; and (2) variability in immune cellular responses among patients, a complexity not often seen in controlled animal models of inflammation. Etiology and prognosis of human IBD are multifactorial and hence animal models of colitis do not fully represent human IBD. For this reason, translation of cellular or biological therapy from animal model of colitis in to human inflammatory bowel disease remains a challenge. This notion is supported by the failure of biologicals to show clinical benefit despite proven efficacy in animal model studies[10,20]. In addition, MSCs derived from mice and humans do not share identical immunobiology and hence data derived from murine MSCs do not entirely inform MSC based clinical trials in human (Figure 1). Thus, the use of an appropriate and standardized MSC cellular infusion product in animal models of colitis that most closely replicates human bowel inflammation should yield data most suited for clinical translation.

ANIMAL MODELS OF INFLAMMATORY BOWEL DISEASES

Animal models for colitis can be divided into: (1) chemical induced acute colitis; (2) adoptive T cell transfer mediated chronic colitis; and (3) spontaneous colitis (Figure 1 Left). Chemical induced colitis models commonly utilize 2,4,6, trinitrobenzene sulfonic acid (TNBS), oxazolone, and dextran sodium sulfate (DSS)[21]. In the TNBS model, colitis is induced in the rodents by intrarectal administration of alcohol containing TNBS. Alcohol serves not only as a carrier but also inflicts damage to the mucosal barrier. This aids TNBS in acting as a hapten for mucosal colonizing microbiota and subsequent Th1-dependent immune activation and infiltration of CD4+ T cells[22,23]. Studies in IFN$\gamma^{-/-}$ BALB/c mice demonstrated that TNBS induced colitis also involves the elaboration of Th2 cytokines, which is often seen in ulcerative colitis[24-26]. Although the TNBS model reflects human intestinal bowel inflammation to some degree, it is limited by the fact that it is a mouse strain dependent model

and it does not depict the spontaneous relapse often seen in human ulcerative colitis[21,27]. Thus, while the TNBS induced colitis model can be used to study the potential efficacy of MSCs, the mechanism behind these effects may be difficult to elucidate due to the lack of involvement of entire lympho myeloid cellular compartments and disease reoccurrence seen in human disease. Similar to the TNBS colitis model, intrarectal administration of oxazolone as a hapten induces colitis in the animals but the inflammation is largely mediated by Th2 cytokines[28]. Hence this model is used to study Th2 associated inflammation in colitis, an effect more pronounced in UC compared to CD[29]. Human bone marrow derived MSCs induce Th2-dependent immune responses and have been shown to have therapeutic benefits in the animal model of multiple sclerosis. Currently, it is unknown whether human MSCs exert such an effect in colitic animals[30]. Due to the confounding nature of Th2 polarizing responses with both human MSCs and oxozalone-induced colitis, this model is not well suited for studying the mechanistic underpinnings of MSC therapy.

In the widely used DSS colitis model, mice consume drinking water containing DSS to induce colitis. DSS causes severe injury in the intestinal epithelium leading to the infiltration of polymorphonuclear leukocytes, intestinal wall thickening, diarrhea, and decreases in body weight[31]. DSS mediated acute colitis models do not faithfully recapitulate T cell mediated gut injury seen in human IBD. Moreover, the secondary inflammation seen in DSS induced colitis is mediated by innate immune cells (as opposed to T-cells) and hence it is not equivalent to IBD of humans[32]. Due to these limitations, caution needs to be drawn on utilizing this model to study both efficacy and mechanisms of action of MSCs. Alternatively, in the T cell transfer colitis model, adoptive transfer of flow cytometry sorted naïve CD4+CD45RBhi+ cells induce chronic colitis in severe combined immunodeficient *(scid)* mice, lacking endogenous T and B cells[33]. This model has the advantage to study early and late immunological changes associated with IBD. Adoptive transfer of CD4+CD25+ regulatory T cells ameliorates colitis induced by CD4+CD45RBhi+ cells, suggesting that this model may be well suited to study cell therapy[34]. However, unlike regulatory T cells, MSCs possess a larger array of immunoregulatory properties and lack of the involvement complex systemic immune response in *scid* mice makes this model difficult to study MSC's mechanism of action.

An alternative approach to study chronic IBD progression is by examining the spontaneous colitis in genetic knockout animals lacking key immunoregulatory molecules. IL-10$^{-/-}$ mice are a perfect example for such studies, which develop spontaneous chronic colitis starting from week 4-6 to week 16-20[35,36]. Spontaneous colitis in IL-10$^{-/-}$ mice is analogous to chronic IBD seen in humans[37], which involves Th1 CD4+ T cells and the lack of mucosal immune tolerance due to the absence of immunosuppressive IL-10 cytokine[38]. Histopathological changes, total body weight loss, and IFN-γ and TNF-α upregulation have been reported in these animals during chronic colitis progression. The therapeutic effect of cytokines, antibodies, and chemical compounds have been tested in this model as a proof-of-concept in treating chronic IBD involving T cell mediated gut pathology[39-41]. Altogether, each colitis model has advantages and disadvantages for the study of MSC efficacy and mechanisms of action. Investigators may require the use of at least two of the models, for example an acute and a chronic colitis model, described above to validate their findings relevant to the clinical trials.

DIFFERENCES IN MSC BIOLOGY DERIVED FROM MICE AND HUMANS

MSC transplantation studies in the animal model of colitis can be divided into two categories (1) syngeneic/allogeneic; and (2) xenogeneic (Figure 1 Left). In the case of syngeneic/allogeneic studies, MSCs are derived from mouse species with/without difference in genetic background to the recipient animals. This is in contrast to xenogeneic studies, where human MSCs are used as a therapeutic agent in immune competent colitic animals. In syngeneic transplantation studies, MSCs are major histocompatibility complex (MHC) matched. These studies provide critical proof-of-concept data for clinical trials involving autologous MSC therapy. However, the major limitation for translating data from such studies to clinical trials is the differential characteristics of murine derived MSCs compared to human MSC counterparts. *In vitro* expansion and biological properties of MSCs are examples of such important differences. Human MSCs can readily be expanded *ex vivo* from the bone marrow or other tissues and can be cultured in basal growth medium. Indeed, robust *in vitro* proliferation properties of human MSCs make them an especially attractive cellular pharmaceutical for use in clinical trials. Despite their ability to proliferate *in vitro*, prolonged culture leads human MSCs to undergo cellular senescence[42]. This is in stark contrast to murine MSCs, which do not exhibit this property, as they do not proliferate *in vitro* like human MSCs. Moreover, murine MSCs are sensitive to oxygen and prolonged culture selects for hypoxia-resistant immortalized clones[43]. Immortalized MSCs with chromosomal aberrations show differential properties compared to primary MSCs[44], including reduced expressions of phenotype marker CD105, and go on to form osteosarcoma-like tumors when implanted into mice[45]. In addition, immortalized murine MSCs lose their multilineage differentiation potential *in vitro* and differentiate only into the osteoblastic lineage[44]. Hence, primary but not

immortalized murine MSCs need to be used in the cell therapy experiments, posing a hurdle for the use of murine MSCs in animal model studies.

In addition to the phenotypical differences, murine and human MSCs differ in their mechanisms of immunosuppression. Human MSCs suppresses T cell proliferation through indoleamine 2,3-dioxygenase (IDO)[46,47]. IFN-γ secreted by activated T cells upregulates IDO expression in MSCs, an enzyme that converts tryptophan to the immunosuppressive metabolite, kynurenine[46]. Blocking of IDO activity abolishes MSC's suppressive activity, which points to the immunological significance of this pathway in human MSCs[46-48]. Murine MSCs, on the other hand, do not suppress *via* IDO, alternatively utilizing nitric oxide (NO) to dampen inflammation[49,50]. Blocking of IFN-γ inducible nitric oxide synthase (iNOS) activity negates the suppressive capacity of murine MSCs[49,50]. Although, IDO knockout murine MSCs induce partial immune tolerance in mice with kidney allografts, IFN-γ does not induce IDO substantially in murine MSCs, suggesting a less important role for IDO in the immunobiology of murine derived MSCs[51]. While both of these enzymes exert their immunosuppressive effects differentially, studying the IDO pathway of human MSCs in a mouse model of colitis will likely yield the most relevant data for translation to clinical trials (Figure 1 Right).

Xenotransplantation methods afford an opportunity to study the effect of human MSCs in mice with colitis[52-58]. A recently published study from our group demonstrated that intravenously infused human MSCs in immune competent mice could be detected only up to 24 h in the lungs and not in the colon or any other organs in any time points tested[17]. Hence human MSCs get immune rejected rapidly in immune competent mice and studying their therapeutic effect despite of immune rejection is challenging. Another variable to be considered is the health status of the MSC donors. MSCs derived from patients with IBD or healthy individuals represent the cellular products used in autologous and allogeneic clinical trials, respectively. Currently, it is unclear if MSCs derived from IBD patients are functionally comparable to healthy donor MSCs. MSCs derived from patients with rheumatoid arthritis, immune thrombocytopenia, Gaucher's Disease, Sjögren's syndrome, Myelodysplastic syndromes, and systemic lupus erythematous show defective phenotype and function[59-63]. Although MSCs derived from Crohn's patients show intact phenotype and function in one study, the mechanism of suppression was not established[64]. Defects in the autophagy pathway have been shown to predispose Crohn's patients to disease progression, but whether such defects alter human MSC suppressive functions remain to be investigated[65]. Blocking of autophagy in murine MSCs actually enhances their immunosuppressive properties in T cell-mediated experimental autoimmune encephalomyelitis (EAE), a mouse model of the human demyelinating disease, multiple sclerosis[66]. It is unknown whether this observation can be translated to human MSCs and in patients with IBD. Further studies are required to understand the efficacy and mechanism of action of MSCs derived from patients with IBD, which inform insights for autologous MSC therapy for IBD (Figure 1 Right). In summary, both syngeneic and xenogeneic transplantation of MSCs from patients with IBD or random healthy donors in animal model of colitis have limitations, impeding the direct translation of the findings to the clinic.

EFFICACY AND MECHANISM OF ACTION OF MSCs IN MOUSE MODELS OF COLITIS

Efficacy of human MSCs derived from adipose tissue, umbilical cord and bone marrow has been shown for TNBS and DSS induced colitis in mice and rats[52-58]. Multiple mechanisms have been demonstrated such as induction of IL-10 secreting regulatory T cells[58], down regulation of IL-23/IL-17 regulated inflammatory reactions[57], and the modulation of Treg/Th17 cells[54], but the key factor in human MSCs responsible for therapeutic effect in colitic mice remains unknown. Hence, the efficacy of human MSCs in immune competent colitic mice is suggestive of the role of soluble factors produced by MSCs within a short period of time before clearance by the recipient immune system.

In an alternative approach in three independent studies, human MSCs preactivated *in vitro* with IL-1β, IFN-γ, and nucleotide-binding oligomerization domain 2 (NOD2)-activating ligands before engraftment showed enhanced therapeutic benefits[55,56,67]. Activation of NOD2 in human MSCs has been shown to suppress mononuclear cell proliferation through prostaglandin E2 production[55]. IL-1β primed MSCs express higher levels of CXCR4 on the surface and hence migrate to the inflammatory sites more efficiently[67]. Human MSCs pretreated with IFN-γ ameliorate colitis through the attenuation of Th1 inflammatory responses[56]. However, these studies still do not fully explain the mechanism of xenogeneic or allogeneic MSC mediated suppression of inflammation in colitic mice. Interestingly, IFN-γ pretreated syngeneic but not allogeneic MSCs ameliorate EAE[68]. In addition, allogeneic MSCs are immune rejected by MHC class Ⅰ- and class Ⅱ-mismatched recipient mice and hence they are not intrinsically immunoprivileged and cannot serve as a "universal donor" in immunocompetent MHC-mismatched recipients[69]. The therapeutic effect seen with xeno- and allogeneic transplantation studies in colitic mice can be described as a "hit and vanish" phenomenon of MSC action.

Biodistribution properties of MSCs are important for their therapeutic efficacy. MSC engraftment to the

colon may play a part in their therapeutic effects as topical administration of MSCs engraft to the inflamed colon and attenuates inflammation[70]. Castelo-Branco *et al*[71] demonstrated that intraperitoneal but not intravenous injected cryopreserved allogeneic MSCs migrate to the colon and ameliorate TNBS colitis This study suggested the defective distribution of intravenously infused cryopreserved, allogeneic MSCs, ultimately resulting in their failure to engraft into the colon. In this study, distribution was affected by two factors: (1) cryopreservation; and (2) allogeneic source of the MSCs. Industry sponsored clinical trials largely utilize thawed MSCs from cryopreservation while most preclinical studies utilize live MSCs from culture[72]. Our group has demonstrated that cryopreserved MSCs exhibit a heat shock response and defective actin polymerization, which affects their immunosuppressive and engraftment properties[16,17]. Altogether, cell preparation, handling methods, and the MHC matching of the cellular product likely affects the ensuing clinical outcome. How these factors mechanistically alter the disease course in colitic animals require further investigation.

APPROACHES TO STUDY THE MECHANISM OF ACTION OF MSCs IN MOUSE MODELS OF COLITIS

Two approaches can be employed to study the mechanism of action of MSCs in animal models of colitis, which can be translated to human MSC based clinical trials. (1) overexpression of key effector pathways specific to human MSCs in their murine counterparts; and (2) investigation of pathways shared by human and murine MSCs. Ling *et al*[73] pioneered the first approach by overexpressing human IDO protein in murine MSCs. The human IDO gene was cloned downstream of the murine iNOS promoter and transfected into MSCs derived from iNOS knockout animals. In this way, inflammatory stimuli induced transcription of human IDO transcription in place of iNOS. These IDO humanized murine MSCs were able to inhibit T cell proliferation and blockade of IDO activity with 1-methyl tryptophan (1-MT) abolished their suppressive effects. In addition, IDO overexpressing murine MSCs promoted tumor growth in melanoma and lymphoma models, an effect that was also reversed by 1-MT administration. While this is a novel approach to study human specific effector pathways in murine MSCs, caution needs to be taken when such MSCs are generated since the genome of murine MSCs is unstable and may result in the transformation of these primary MSCs into immortalized cells. In addition, transfection of primary cells and subsequent culture under selection pressure to generate clones may lead to the loss of key immunosuppressive and regenerative properties. Despite these shortcomings, such an approach is important for identifying the *in vivo* characteristics of a critical immunoregulatory pathway specific to human MSCs.

In support of the second approach, we studied the *in vivo* significance of an effector pathway shared between mouse and human MSCs. Both human and murine MSCs secrete high levels of IL-6. When BALB/c mice were sublethally irradiated and subsequently given single intraperitoneal injection of MHC mismatched C57Bl/6 MSCs, mortality was reduced in a dose dependent manner[74]. This effect was abrogated when similarly conditioned mice were given MSCs from IL-6$^{-/-}$ animals. MSCs accelerated the recovery of damaged intestinal epithelium by stimulating the proliferation of intestinal crypt cell pool. Thus, IL-6 produced by murine MSCs ameliorate gut injury in the absence of immune rejection in irradiated mice[74]. However MSCs contain a large array of immunoregulatory and regenerative molecules and some of these are specific to murine immunobiology (*e.g.*, iNOS), which could dominate the effect of other pathways that are shared by human and mice. The field of comparative MSC biology to identify shared and differentially operating pathways between mice and human is still in its infancy and requires further investigation. Regardless of the approach used in these mechanistic assays, *in vivo* colitic animal model studies are required for their validation as a companion to translation into clinical trials.

CONCLUSION

MSCs are an attractive agent for cell-based therapies not only for IBD, but also other auto- and allo-immune ailments. Lack of a comprehensive, comparative characterization of murine and human MSCs impede the direct translation of the preclinical animal model findings to clinical trials. Conflicting results seen in MSC efficacy studies are likely to reflect differences in MSC source, cell preparation and handling methods. Utilization of appropriate animal models and MSCs derived from animal and human origin to address these issues will help to understand their mechanism of action in inflammatory and regenerative settings.

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REVIEW

Metastatic pancreatic cancer: Is there a light at the end of the tunnel?

Vanja Vaccaro, Isabella Sperduti, Sabrina Vari, Emilio Bria, Davide Melisi, Carlo Garufi, Carmen Nuzzo, Aldo Scarpa, Giampaolo Tortora, Francesco Cognetti, Michele Reni, Michele Milella

Vanja Vaccaro, Sabrina Vari, Carlo Garufi, Carmen Nuzzo, Francesco Cognetti, Michele Milella, Medical Oncology A, Regina Elena National Cancer Institute, 00144 Rome, Italy
Isabella Sperduti, Medical Oncology A and Biostatistics, Regina Elena National Cancer Institute, 00144 Rome, Italy
Emilio Bria, Davide Melisi, Giampaolo Tortora, Medical Oncology, University of Verona, Azienda Ospedaliera Universitaria Integrata, 37100 Verona, Italy
Aldo Scarpa, ARC-Net Research Centre and Department of Pathology, Diagnostics and Surgery, University of Verona, 37100 Verona, Italy
Michele Reni, Medical Oncology Unit, Department of Oncology, San Raffaele Scientific Institute, 21132 Milan, Italy
Author contributions: Vaccaro V, Milella M, Bria E, Sperduti I, Garufi C, Nuzzo C and Vari S performed the literature research; Vaccaro V, Milella M and Reni M wrote the paper; Cognetti F, Scarpa A, Reni M, Milella M, Bria E, Melisi D, and Tortora G reiewed the paper; Vaccaro V and Milella M supervised the project.
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Correspondence to: Vanja Vaccaro, MD, Medical Oncology A, Regina Elena National Cancer Institute, Via Elio Chianesi 52, 00144 Rome, Italy. vanja.vaccaro@hotmail.it
Telephone: +39-6-52666919
Fax: +39-6-52665637
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Abstract

Due to extremely poor prognosis, pancreatic cancer (PDAC) represents the fourth leading cause of cancer-related death in Western countries. For more than a decade, gemcitabine (Gem) has been the mainstay of first-line PDAC treatment. Many efforts aimed at improving single-agent Gem efficacy by either combining it with a second cytotoxic/molecularly targeted agent or pharmacokinetic modulation provided disappointing results. Recently, the field of systemic therapy of advanced PDAC is finally moving forward. Polychemotherapy has shown promise over single-agent Gem: regimens like PEFG-PEXG-PDXG and GTX provide significant potential advantages in terms of survival and/or disease control, although sometimes at the cost of poor tolerability. The PRODIGE 4/ACCORD 11 was the first phase Ⅲ trial to provide unequivocal benefit using the polychemotherapy regimen FOLFIRINOX; however the less favorable safety profile and the characteristics of the enrolled population, restrict the use of FOLFIRINOX to young and fit PDAC patients. The nanoparticle albumin-bound paclitaxel (*nab*-Paclitaxel) formulation was developed to overcome resistance due to the desmoplastic stroma surrounding pancreatic cancer cells. Regardless of whether or not this is its main mechanisms of action, the combination of *nab*-Paclitaxel plus Gem showed a statistically and clinically significant survival advantage over single agent Gem and significantly improved all the secondary endpoints. Furthermore, recent findings on maintenance therapy are opening up potential new avenues in the treatment of advanced PDAC, particularly in a new era in which highly effective first-line regimens allow patients to experience prolonged disease control. Here, we provide an overview of recent advances in the systemic treatment of advanced PDAC, mostly focusing on recent findings that have set new standards in metastatic disease. Potential avenues for further development in the metastatic setting and current efforts to integrate

new effective chemotherapy regimens in earlier stages of disease (neoadjuvant, adjuvant, and multimodal approaches in both resectable and unresectable patients) are also briefly discussed.

Key words: Pancreatic cancer; Metastatic disease; Chemotherapy; Folfirinox; *nab*-Paclitaxel

Core tip: In this paper, we provide an overview on the latest progress in the systemic treatment of advanced pancreatic cancer, mostly focusing on recent findings that have set new standards in metastatic disease. Potential avenues for further development in the metastatic setting and current efforts to integrate new effective chemotherapy regimens in earlier stages of disease (neoadjuvant, adjuvant, and multimodal approaches in both resectable and unresectable patients) are also briefly discussed.

Vaccaro V, Sperduti I, Vari S, Bria E, Melisi D, Garufi C, Nuzzo C, Scarpa A, Tortora G, Cognetti F, Reni M, Milella M. Metastatic pancreatic cancer: Is there a light at the end of the tunnel? *World J Gastroenterol* 2015; 21(16): 4788-4801 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4788.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4788

INTRODUCTION

Pancreatic cancer ranks as the fourth leading cause of cancer-related deaths in the United States and in most Western countries[1]. With a 5-year survival rate of 6% and mortality closely approaching incidence (approximately 46000 new cases and 39000 deaths estimated in the United States in 2014), pancreatic adenocarcinoma (PDAC) remains arguably the most aggressive[1,2] and resistant among solid tumors, to either classic chemotherapeutics[3] or targeted agents[4].

Besides the dismal prognosis, PDAC patients are usually affected by a complex association of symptoms (obstructive jaundice, pain, and weight loss, *etc.*) that require prompt and frequent palliative measures in order to improve patient performance status (PS) and quality of life (QoL), regardless of the specific oncologic treatment adopted[5,6].

Before the advent of gemcitabine (Gem), fluorouracil (5-FU), in different doses, schedules, and combination regimens, has been considered the cornerstone in the palliative treatment of advanced PDAC. A Cochrane systematic review[7] demonstrated that 5-FU-based chemotherapy significantly prolongs 6- and 12-mo survival [odds ratio (OR) = 0.37, 95% confidence interval (CI): 0.25-0.57, *P* value < 0.00001 for the 12-mo comparison], compared to best supportive care, providing significant clinical benefits in at least one study[8]. However, no significant difference was found in one-year mortality for 5-FU alone *vs* 5-FU combinations (OR = 0.90, 95%CI: 0.62 -1.30)[7].

PAST: GEM AS THE CORNERSTONE OF SYSTEMIC THERAPY

Since the first demonstration of clinical benefit in 1997, Gem has been the cornerstone of first-line PDAC treatment. In a phase Ⅲ study, Burris *et al*[9] randomized 126 locally advanced or metastatic PDAC patients to receive Gem 1000 mg/m^2 (once weekly for 7 wk followed by a week of rest and then once weekly for 3 out of 4 wk) or 5-FU 600 mg/m^2 (once weekly). Patients had to be symptomatic at study entry (70% of the patients had a Karnofsky PS - KPS < 80%). Indeed, the primary study endpoint was clinical benefit response (CBR), a composite assessment of pain, analgesic consumption, KPS, and weight[10,11]. Gem demonstrated to be superior to 5-FU in terms of CBR (23.8% *vs* 4.8%, *P* = 0.0022), and relatively, unexpectedly in the secondary endpoint of overall survival (OS) (5.65 mo *vs* 4.41 mo, *P* = 0.0025). In addition, the 6-, 9-, and 12-mo survival rates were higher with Gem (46%, 24%, and 18%, respectively) than with 5-FU (31%, 6%, and 2%, respectively)[9], although the real impact of Gem, as compared to 5-FU, on OS has been questioned by subsequent meta-analyses[12].

One approach aimed at improving Gem activity has been pharmacokinetic modulation, achieved by prolonging the infusion time[13-16]. This approach is justified by the observation that deoxycytidine kinase, the enzyme that catalyzes the conversion of Gem to its active triphosphate metabolite, is rapidly saturated at plasma concentrations achieved with the standard 30-min infusion. Indeed, Gem doses of 300-350 mg/m^2 infused over 30 min have reportedly failed to result in increased intracellular accumulation of Gem triphosphate in peripheral blood mononuclear cells[17-19]. Conversely, infusion of the same Gem doses over a prolonged period at a constant dose rate of 10 mg/m^2 per minute would avoid enzyme saturation and permit greater intracellular accumulation, possibly increasing Gem antitumor activity. Fixed dose-rate (FDR) Gem infusion has proven feasible, well tolerated (even in patients with impaired liver function[20]), and has shown promising clinical activity[15,21,22]. Although, FDR-Gem failed to significantly extend survival over standard 30-min infusion in a randomized phase Ⅲ trial, pharmacokinetic Gem modulation did show a trend towards increased clinical activity and proved equivalent by adding a second chemotherapy drug (oxaliplatin) to a Gem backbone. However, FDR-Gem was administered at a higher (1500 mg/m^2) weekly dose, as compared to the standard 30-min infusion (administered at 1000 mg/m^2)[23].

Until recently, efforts to improve on single-agent Gem efficacy by combining it with either a second cytotoxic drug or a molecularly targeted agent have failed[24,25]. The addition of erlotinib, an oral epidermal growth factor receptor tyrosine kinase inhibitor, to Gem has produced a clinically negligible, albeit statistically significant, improvement in OS in advanced, inoperable PDAC[26]. Even though, the combination of Gem and erlotinib is not widely employed, particularly in Europe, currently available evidence suggests that PDAC patients who develop skin toxicity during treatment may derive substantial benefit from this approach[27-29].

The addition of oxaliplatin to Gem in the study by Louvet *et al*[30] improved response rate (ORR), progression-free survival (PFS) and CBR over single agent Gem, but no statically significant difference in OS was observed (9.0 mo *vs* 7.1 mo, respectively, $P = 0.13$). Similar results were shown in a second study on the combination of Gem/oxaliplatin, in which combination therapy was actually therapeutically equivalent to FDR-Gem alone[23]. Similarly, cisplatin plus Gem showed no statistically significant benefit in survival, with comparable tumor responses and PFS[31,32]. The Gem/capecitabine combination demonstrated a significant improvement in ORR (19.1% *vs* 12.4%, $P = 0.034$) and PFS [hazard ratio (HR) = 0.78, 95%CI: 0.66-0.93; $P = 0.004$)] but failed to increase OS (HR = 0.86, 95%CI: 0.72-1.02; $P = 0.08$)[33].

The overall negative results with Gem-based combinations have generally been attributed to a lack of statistical power to detect small differences in survival, thus prompting for cumulative analyses that could detect small survival differences with adequate statistical power. Several meta-analyses have been conducted with the aim of assessing the potential of Gem-based chemotherapy doublets to increase survival in advanced PDAC.

Our group conducted a literature-based meta-analysis on 6296 patients enrolled in 20 randomized clinical trials comparing the single agent Gem *vs* Gem-based combinations[24]. No survival benefits were observed with combination therapies [relative risk (RR) = 0.93, 95%CI: 0.84-1.03; $P = 0.17$). However, a statistically significant, albeit minimal, advantage for Gem-based combinations was found for PFS (RR = 0.91, 95%CI: 0.84-0.98; $P = 0.015$) and ORR (RR = 1.57, 95%CI: 1.31-1.86; $P < 0.0001$): this translates into a number of patients needed to treat for a single patient to benefit (NNT) of 39 patients for PFS (with a 2.6% absolute benefit) and 33 patients for ORR. None of the 4 different combination groups (Gem plus a platinum salt, Gem plus a fluoropyrimidine, Gem plus other classical cytotoxic agents, and Gem plus targeted drugs) demonstrated an OS benefit over single-agent Gem, while significant advantages in PFS (RR = 0.67, 95%CI: 0.53-0.83; $P = 0.0004$) and ORR were obtained for platinum-containing combinations[24].

Several other meta-analyses have been conducted, exploring whether adding a second drug to Gem would impact on survival of advanced PDAC patients. Sultana *et al*[12] conducted a meta-analysis on 19 studies, involving 4697 patients, and found a statistically significant, but clinically negligible, OS benefit for Gem-based combinations (HR= 0.91, 95%CI: 0.85-0.97; $P = 0.004$), particularly when Gem was combined with platinum salts. Heinemann *et al*[32] analyzed 15 randomized trials involving 4465 patients. Overall, they demonstrated a small, albeit statistically significant, survival advantage for Gem-based combinations, as compared to single-agent Gem (HR = 0.91, $P = 0.004$). The combined analysis of 5 randomized trials showed a significant prolongation in OS (HR = 0.85, $P = 0.010$) and a significant benefit in PFS and ORR for the combination of Gem with platinum salt. Meta-analytic results from 6 studies demonstrated a significant, albeit modest, improvement in survival (HR = 0.90, $P = 0.03$) for Gem/fluoropyrimidine combination, more pronounced when the association with capecitabine in 3 trials was considered (HR = 0.83, $P = 0.01$). In a subgroup analysis conducted on 1682 patients (38% of the overall population) for whom PS data were available, OS benefit for Gem-based combinations seemed to be confined to patients with good PS (HR = 0.76, $P < 0.001$)[34]. All these data are consistent with previous meta-analytic results showing no difference in 1-year mortality rate between Gem-combination and single agent Gem (OR = 0.88, 95%CI: 0.74-1.05) and a better 6-mo mortality rate for the subgroup of platinum/Gem schedules (OR = 0.59, 95%CI: 0.43-0.81; $P = 0.001$)[7]. Xie *et al*[35] evaluated 18 randomized trials involving 4237 patients and showed a reduction of 9% in the risk of death with Gem-based doublets at 6 mo (RR = 0.91, 95%CI: 0.85-0.97; $P = 0.005$) and of 4% at 1 year (RR = 0.96, 95%CI: 0.93-0.99; $P = 0.02$); Gem/capecitabine and Gem/oxaliplatin combinations significantly reduced the risk of death by 15% (RR = 0.85, 95%CI: 0.73-0.99; $P = 0.04$) and 20% (RR = 0.80, 95%CI: 0.70-0.91; $P = 0.001$), respectively. No survival benefit was shown for Gem-based combinations in the good PS group of patients and an increased risk of death was demonstrated for patients with poor PS. A further meta-analysis on thirty-five trials and a total of 9979 patients demonstrated that the Gem-based combination treatments achieved a significant benefit over single agent Gem (OS: OR = 1.15, $P = 0.011$; PFS: OR = 1.27, $P < 0.001$; ORR: OR = 1.58, $P < 0.001$). Improvement in terms of survival and ORR were especially evident when Gem was combined with fluoropyrimidines (OS: OR = 1.33, $P = 0.007$; PFS: OR = 1.53, $P < 0.001$; ORR: OR = 1.47, $P = 0.03$). Similar results were obtained for the combination with oxaliplatin (OS: OR = 1.33, $P = 0.019$; PFS: OR = 1.38, $P = 0.011$)[36]. A more recent meta-analysis provided a statistically significant, even though marginal, survival

improvement over single agent Gem (pooled HR = 0.93; 95%CI: 0.89-0.97; P = 0.001). As observed by the authors, such slight improvements were obtained at the price of a significantly greater incidence of toxic effects, notably diarrhea, nausea, neutropenia and thrombocytopenia[37]. Although slight differences were obtained in the results, overall all of these meta-analyses ultimately convey the very same message, that is summarized in the results of a recent pooled analysis, performed by our group: when the results of 7 randomized trials comparing Gem-monotherapy with the three most popular combination regimens (Gem/cisplatin, Gem/capecitabine and Gem/oxaliplatin) were pooled together, a clinically negligible, albeit statistically significant, absolute survival benefit (2%-3% at 1 year) was observed, ruling out the possibility that Gem-based combination regimens could improve 1-year survival by more than 5%[38]. Thus, the routine use of Gem-based doublets with either platinum salts or fluoropyrimidines in metastatic PDAC does not seem to be supported by available evidence.

PRESENT: FOLFIRINOX AND OTHER POLYCHEMOTHERAPY APPROACHES

The multicentre, randomized, phase Ⅱ-Ⅲ trial PRODIGE 4/ACCORD 11, comparing single-agent Gem with the polychemotherapy regimen FOLFIRINOX in patients with metastatic PDAC was published in 2011. Three hundred forty-two patients were randomly assigned to receive standard Gem 1000 mg/m^2 or FOLFIRINOX (oxaliplatin 85 mg/m^2, leucovorin 400 mg/m^2, irinotecan 180 mg/m^2, 5-FU 400 mg/m^2 administered by intravenous bolus, followed by a continuous intravenous infusion of 2400 mg/m^2 over a 46-h period), in cycles repeated every 2 wk. With a median follow-up of 26.6 mo, median OS was 11.1 mo (95%CI: 9.0-13.1) in the FOLFIRINOX group and 6.8 mo (95%CI: 5.5-7.6) in the Gem group (HR = 0.57; 95%CI: 0.45-0.73; P < 0.001); 1-year survival rate was 48.4% in the FOLFIRINOX group, as compared with 20.6% in the Gem group; HR for death remained significant when adjusted for independent adverse prognostic factors. A statistically significant difference was observed also for PFS (6.4 mo *vs* 3.3 mo, HR = 0.47; 95%CI: 0.37-0.59; P < 0.001). An impressive ORR of 31.8% in the FOLFIRINOX group was reported, as compared to 11.3% in the Gem group. Results were not influenced by second-line treatments, in fact approximately 45% of patients in both groups received second-line therapy. As expected, FOLFIRINOX's safety profile was less favorable, with a significantly higher incidence of grade 3 or 4 neutropenia, febrile neutropenia, thrombocytopenia, diarrhea, and sensory neuropathy, as well as grade 2 alopecia[39]. Nevertheless, health-related QoL analysis showed that FOLFIRINOX significantly reduces QoL impairment, as compared with Gem[40], highlighting the fact that in very aggressive diseases, such as advanced PDAC, QoL is influenced more by disease-related symptoms than treatment-related toxicity. One of the limits of Conroy's trial is that enrollment was restricted to patients younger than 76 years, with an Eastern Cooperative Oncology Group (ECOG) PS score of 0 or 1 and a bilirubin level ⩽ 1.5 times the upper normal limit; thus, the proportion of patients carrying a biliary stent was relatively low (14.3%). On these bases, the FOLFIRINOX regimen is currently recommended as a first-line treatment option for young and fit PDAC patients, with good hepatobiliary function, and dose/schedule modifications are applied by many groups, in order to avoid excessive toxicity[41-48].

Before the advent of FOLFIRINOX, other groups had shown potential advantages for polychemotherapy over single-agent Gem in advanced PDAC. In 2005, Reni *et al*[49] performed a randomized phase Ⅲ study in locally advanced or metastatic pancreatic cancer with the PEFG regimen, consisting of cisplatin 40 mg/m^2 and epirubicin 40 mg/m^2 given on day 1, Gem 600 mg/m^2 administered on days 1 and 8 and 5-fluorouracil 200 mg/m^2 per day as continuous infusion during the entire duration of chemotherapy treatment, with cycles repeated every 28 d. Primary endpoint was 4-mo PFS improvement with the four-drug combination over single-agent Gem. A previous phase Ⅱ study on the same regimen showed interesting ORR results (55% in the metastatic population), survival (median time to tumor progression 7 mo and median OS 9.5 mo), and safety profile[49]. In the phase Ⅲ trial the primary endpoint was met with a total of 99 patients enrolled (affected by either metastatic or locally advanced disease) and a median follow-up of 33.5 mo, more than 30% absolute difference in 4-mo progression-free survival was observed (4-mo PFS: 60%, 95%CI: 46-72, *vs* 28%, 95%CI: 17-42; HR = 0.46, 95%CI: 0.26-0.79; P = 0.001). Median PFS was 5.4 mo (95%CI: 2.0-9.6 mo) for the combination regimen *vs* 3.3 mo (95%CI: 2.2-5.3) for Gem (HR = 0.51, 95%CI: 0.33-0.78; P = 0.0033); the HR for death in the PEFG group compared with the Gem group was 0.65 (95%CI: 0.43-0.99; P = 0.047). Disease response was reported in 38.5% (95%CI: 25.3-51.7) in the combination group compared to 8.5% (95%CI: 0.5-16.5) in the monochemotherapy group (OR = 6.60, 95%CI: 2.11-20.60; P = 0.0008). PEFG was quite well tolerated, although more patients experienced grade 3-4 neutropenia and thrombocytopenia in the PEFG group (P < 0.0001)[50]. The small sample size and the choice of PFS as the primary endpoint may have constituted a weakness of the study, making the results difficult to generalize, leading to a general reluctance to widely adopt such a regimen as a possible standard in advanced PDAC[51]. Other concerns include the toxicity profile and the potential impairment in QoL in an already usually highly symptomatic population of patients[51]. A simplified schedule characterized by a better toxicity profile, more suitable for routine

clinical use, was indeed proposed by the authors[52,53]. Other attempts at making this regimen more easily manageable and even more active encompassed substituting the oral capecitabine for 5-FU (PEXG) and docetaxel for epirubicin (PDXG). The results of a randomized phase Ⅱ study comparing PEXG and PDXG confirmed a very high ORR (37% and 60%, respectively) and notable PFS (approximately 7.5 mo in both arms) and OS benefit (approximately 11 mo in both arms)[54].

Another multidrug regimen combining Gem, docetaxel, and capecitabine (GTX: capecitabine 750 mg/m^2 per day, days 1-14; Gem 750 mg/m^2 over 75 min on days 4 and 11 and docetaxel 30 mg/m^2 on days 4 and 11; cycles repeated every 21 d) was initially tested retrospectively in a metastatic PDAC population of 35 patients, with a reported ORR of 29% and disease stabilization in an additional 31%[55]. A subsequent analysis included 154 patients with locally advanced (73 patients; 24%) or metastatic PDAC (117 patients; 76%) treated with the GTX regimen where the majority of patients had an ECOG PS of 0 (29%) or 1 (66%) and 49% of patients had received previous chemotherapy treatment. Metastatic patients who received GTX as first-line treatment achieved a median survival of 11.3 mo; partial response and stable disease were observed in 11% and 62% of patients, respectively. Unfortunately, responses significantly correlated with toxicity, namely neutropenia, ALT elevation and hospitalizations: 9% of patients experienced grade 3-4 non-hematological toxicity and 41% experienced hematological toxicity (grade 3-4 anemia, neutropenia and thrombocytopenia in 12%, 34% and 13% of patients, respectively)[56].

PRESENT: GEM/*NAB*-PACLITAXEL

Nab-Paclitaxel is a nanoparticle albumin-bound (nab) paclitaxel characterized by a formulation of nanoparticle colloidal suspension, with an average size of 130 nm, prepared with human serum albumin. This formulation without solvents confers more favorable pharmacologic characteristics that allow the delivery of a higher dose of paclitaxel than Cremophor-paclitaxel, significantly lowering the risk of infusion hypersensitivity reactions and neutropenia and a faster recovery of peripheral neuropathy upon stopping the treatment[57]. *Nab*-Paclitaxel uptake into cells may be dependent on SPARC (secreted protein acidic and rich in cysteine) expression. SPARC is an albumin-binding protein that interacts with an extracellular matrix, influencing cell migration, proliferation, angiogenesis (especially during wound healing), matrix cell adhesion, and tissue remodeling. Pancreatic cancer is characterized by malignant epithelial cells surrounded by a rich stromal reaction, composed of extracellular matrix proteins (collagen, hyaluronic acid, SPARC) and cellular elements cancer-associated fibroblast, endothelial, immune, and inflammatory cells[58,59]. Analysis of pancreatic cancer tissue samples showed that SPARC is overexpressed[60] preferentially by stromal fibroblasts and epigenetically silenced in pancreatic cancer cells[61]. SPARC expression in peritumoral fibroblasts is a strong marker of poor prognosis in patients with resectable pancreatic adenocarcinoma, independent of common clinical parameters including tumor size, margin status, and lymph node metastasis[62-64]. As pancreatic cancer stroma may contribute to poor vascularisation and high intratumoural pressure, thereby causing decreased drug diffusion[65], SPARC represents an interesting stromal target[63] and the binding between SPARC and albumin may facilitate the tumor delivery of albumin-bound therapeutic agents[58]. Indeed, preclinical studies on PDAC stroma targeting strategies showed promising results and achieved decrease intratumor interstitial pressure, normalized vascularisation, and improved drug delivery[66-68].

This stimulated researchers' interest in testing *nab*-Paclitaxel in PDAC. A phase Ⅰ/Ⅱ study was conducted in metastatic PDAC patients that received Gem (1000 mg/m^2) with *nab*-Paclitaxel (100, 125, or 150 mg/m^2) on days 1, 8, and 15, every 28 d. In the 44 patients treated at the MTD of 125 mg/m^2 median PFS was 7.9 mo (95%CI: 5.8-1.0 mo), median OS was 12.2 mo (95%CI: 8.9-17.9 mo), and 1-year survival was 48%; ORR was 48%, with an overall disease control rate of 68%[60]. These promising results, along with the favorable safety profile prompted starting a phase Ⅲ study.

Eight-hundred-sixty-one metastatic pancreatic cancer patients were enrolled in a phase Ⅲ trial and randomized to receive *nab*-Paclitaxel at a dose of 125 mg/m^2 plus Gem (1000 mg/m^2) 3 wk on/1 wk off or single agent Gem (1000 mg/m^2). OS was significantly improved with *nab*-Paclitaxel plus Gem as compared to Gem monotherapy (8.7 mo *vs* 6.6 mo, HR = 0.72, 95%CI: 0.62-0.83; $P < 0.001$), as were the 1-year (35% *vs* 22%) and 2-year (10% *vs* 5%) survival rates. A significant improvement in PFS was also reported (5.5 mo *vs* 3.7 mo, HR = 0.69, 95%CI: 0.58-0.82; $P < 0.001$). The ORR was significantly higher with the combination treatment than with Gem (23% *vs* 7%; $P < 0.001$; response-rate ratio, 3.19). Disease control rate (DCR; confirmed response or stable disease for ⩾ 16 wk) was 48% in the *nab*-Paclitaxel/Gem population and 33% in the Gem group (ratio for disease control 1.46; 95%CI: 1.23-1.72). The difference between treatment groups could not be attributed to the use of second-line therapy[69-71]. The most common adverse events related to *nab*-Paclitaxel/Gem combination were fatigue (54%), alopecia (50%), and nausea (49%). Grade 3 or higher adverse events were neutropenia (38% in the combination group *vs* 27% in the Gem group, with 3% *vs* 1% of febrile neutropenia, respectively), fatigue (17% *vs* 7%), and peripheral

Table 1 Selected adjuvant or "strategy" (neoadjuvant *vs* adjuvant) clinical trials employing contemporary treatment regimens for resectable pancreatic adenocarcinoma

Study ID[1]	Design	Phase	Estimated accrual	Status
NCT00882310	Adjuvant Gem, Taxotere, and Xeloda	Ⅱ	32	Active, not recruiting
NCT00960284	Post-operative Gemcitabine *vs* PEFG Followed by Chemoradiation	Ⅱ/Ⅲ	102	Completed
NCT01150630	Adjuvant Gem *vs* Adjuvant PEXG *vs* Neoadjuvant/Adjuvant PEXG	Ⅱ/Ⅲ	370	Recruiting
NCT01526135	Adjuvant Gem *vs* modified FOLFIRINOX[2]	Ⅲ	490	Recruiting
NCT01660711	Neoadjuvant/Adjuvant modified FOLFIRINOX[3]	Ⅱ	21	Recruiting
NCT01845805	Adjuvant *nab*-Paclitaxel/Gem/Azacitidine	Ⅱ	80	Recruiting
NCT01964430	Adjuvant Gem *vs* *nab*-Paclitaxel/Gem (APACT)	Ⅲ	800	Recruiting
NCT02023021	Adjuvant *nab*-Paclitaxel/Gem	Ⅱ	80	Recruiting
NCT02047474	Neoadjuvant/Adjuvant modified FOLFIRINOX[4]	Ⅱ	46	Recruiting
NCT02047513	Adjuvant only *vs* Neoadjuvant/Adjuvant *nab*-Paclitaxel/Gem (NEONAX)	RⅡ[5]	166	Not yet recruiting
NCT02172976	Adjuvant Gemcitabine *vs* Neoadjuvant/Adjuvant FOLFIRINOX	Ⅱ/Ⅲ	126	Not yet recruiting

[1]ClinicalTrials.gov Identifier; [2]Oxaliplatin 85 mg/m^2, Irinotecan 165 mg/m^2, Leucovorin 400 mg/m^2, fluorouracil (5-FU) 2400 mg/m^2 c.i.v.i. over 48 h (no 5-FU bolus); [3]Oxaliplatin 85 mg/m^2, Irinotecan 180 mg/m^2, Leucovorin 400 mg/m^2, 5-FU 2400 mg/m^2 c.i.v.i. over 48 h (no 5-FU bolus); [4]Dose/schedule modifications not specified; [5]Randomized phase Ⅱ.

neuropathy (17% *vs* 1%). Median time to occurrence of G3 peripheral neuropathy in the combination group was 140 d, with a median time to recovery of such toxicity to G1 or lower of 29 d. 44% of patients could then resume combined treatment and the median OS of patients experiencing G3 peripheral neuropathy was strikingly longer (14.9 mo), as compared to that observed in the ITT population (8.5 mo).

Stromal SPARC expression, evaluated in 36 patients enrolled in the phase Ⅰ/Ⅱ trial by Von Hoff *et al*[61] was demonstrated to be a predictor factor for OS. Patients with high-SPARC expression showed a prolonged OS compared with low-SPARC expression group (median OS: 17.8 mo *vs* 8.1 mo, respectively; P = 0.0431). No significant correlation with OS was reported for the expression of SPARC in tumor cells[60]. Conversely, the exploratory analysis of the MPACT study on the prognostic significance of SPARC expression did not show any correlation with OS. Stromal SPARC was neither a prognostic factor, nor a significant, independent predictive factor for OS or secondary endpoints, such as PFS, TTF, ORR and DCR. In an additional analysis, tumor epithelial SPARC, baseline and change from baseline of plasma SPARC were similarly not predictive for OS. However, only 256 patients (30% of the ITT population) were evaluable for stromal SPARC expression, among the 860 patients enrolled in the MPACT study, and the IHC assay was different from that employed in the phase Ⅰ/Ⅱ trial, although it showed 86% concordance[72]. These two aspects, together with differences in patient characteristics and tissue of origin, may explain to some extent the failure of such exploratory analysis to highlight a significant predictive value of SPARC expression in this setting. However, a closer look at survival curves according to SPARC expression does reveal differences that may have potential biological and clinical meaning, although they do not reach statistical significance, thus supporting the continued exploration of a potential role of SPARC expression in regulating sensitivity to *nab*-Paclitaxel/Gem combinations.

FUTURE: IS THERE A RATIONALE FOR MAINTENANCE THERAPY?

Maintenance therapy refers to systemic therapy given to cancer patients who have achieved an objective response or disease stabilization after first line treatment, with the aim to extend responses or delay recurrence, eventually prolonging OS. The maintenance approach is largely used for hematologic malignancies and has been recently investigated in solid tumors, providing conflicting results. In breast cancer this strategy seems to improve PFS without OS benefit; in colorectal cancer no evidence exists in favor of continuous treatment[73]. A PFS advantage can be obtained with maintenance paclitaxel or maintenance bevacizumab in ovarian cancer, while maintenance therapy has been approved by the US Food and Drug Administration for advanced NSCLC[74-76]. With regard to pancreatic cancer, the trial recently published by Reni *et al*[77] was the first to address the role of a maintenance strategy in this disease. They performed a multicentre phase Ⅱ study in which 56 metastatic PDAC patients, who were progression-free after 6 mo from the start of first line chemotherapy, were randomized to receive sunitinib 37.5 mg/d continuously or observation only, with the primary endpoint of a 20% improvement in 6-mo PFS (PFS-6). The study met its primary endpoint: PFS-6 was 3.6% (95%CI: 0%-10.6%) in the observation group and 22.2% (95%CI: 6.2%-38.2%) in patients receiving sunitinib. Median PFS were 2.0 and 3.2 mo, respectively (P < 0.01, HR = 0.51, 95%CI: 0.29-0.89). Although differences in OS did not reach statistical significance (HR = 0.71, 95%CI: 0.40-1.26; P = 0.11), the proportion of patients who were alive at two years was tripled in the sunitinib maintenance arm (22.9% *vs* 7.1%), as compared with the observation arm. Most common grade 3-4 adverse events in the experimental arm were: thrombocytopenia, neutropenia and hand-foot syndrome (12%) and diarrhea (8%). Although

Table 2 Selected ongoing studies of contemporary "nanoparticle albumin-paclitaxel-based" systemic therapy in borderline resectable, locally advanced and metastatic pancreatic cancer

Title (Study ID[1])	Phase	Stage	Status
Randomized Phase Ⅱ Trial of Pre-Operative Gemcitabine and *nab*-Paclitaxel With or With Out Hydroxychloroquine (NCT01978184)	Phase 2	Potentially resectable	Recruiting
Phase 1/2 Safety and Feasibility of Gemcitabine and *nab*-Paclitaxel in Combination With LDE-225 as Neoadjuvant Therapy in Patients With Borderline Resectable Pancreatic Adenocarcinoma (NCT01431794)	Phase 1 Phase 2	Resectable	Recruiting
A Pilot Phase Ⅱ Multi Center Study of Gemcitabine and *nab*-Paclitaxel (Abraxane) as Preoperative Therapy for Potentially Operable Pancreatic Cancer (NCT01298011)	Phase 2	Resectable	Active, not recruiting
Phase Ⅱ Study of Preoperative FOLFIRINOX Versus Gemcitabine/*Nab*-Paclitaxel in Patients With Resectable Pancreatic Cancer (NCT02243007)	Phase 2	Resectable	Not yet recruiting
Phase Ⅱ Neoadjuvant Chemotheraphy (Gemcitabine and *nab*-Paclitaxel *vs* mFOLFIRINOX) and Sterotatic Body Radiation Therapy for Borderline Resectable Pancreatic Cancer (NCT02241551)	Phase 2	BR	Not yet recruiting
Nab-Paclitaxel Plus Gemcitabine With Concurrent MR-Guided IMRT in Patients With Locally Advanced and Borderline Resectable Pancreatic Cancer (NCT02283372)	Phase 1	BR or LA	Not yet recruiting
A Phase Ⅰ Dual Dose Escalation Study of Radiation and *nab*-Paclitaxel in Patients With Unresectable and Borderline Resectable Pancreatic Cancer (NCT02207465)	Phase 1	BR or LA unresectable	Recruiting
A Phase 2 Trial of Gemcitabine Plus *nab*-Paclitaxel With or Without FG-3019 as Neoadjuvant Chemotherapy in Locally Advanced, Unresectable Pancreatic Cancer (NCT02210559)	Phase 2	LA, unresectable	Recruiting
A Phase Ⅰ Study of Chemoradiotherapy Using Gemcitabine Plus *nab*-Paclitaxel for Unresectable Locally Advanced Pancreatic Adenocarcinoma (NCT02272738)	Phase 1	LA, unresectable	Recruiting
A Phase 1, Multicenter, Open-label, Dose-escalation Study to Investigate the Safety and Pharmacokinetics of Nab® -Paclitaxel (ABI-007) Plus Gemcitabine in Subjects With Advanced Pancreatic Cancer Who Have Cholestatic Hyperbilirubenemia Secondary to Bile Duct Obstruction (NCT02267707)	Phase 1	LA unresectable or metastatic	Not yet recruiting
Evaluation of Tumoral Perfusion Modification by Dynamic Imaging After Chemotherapy Combining Gemcitabine and *nab*-Paclitaxel (Abraxane) in Patients With Potentially Operable, Locally Advanced or Metastatic Pancreatic Adenocarcinoma (NCT01715142)	Phase 0	Stage Ⅰ-Ⅱ-Ⅲ-Ⅳ	Not yet recruiting
A Phase Ⅱ Randomized Trial Comparing a Combination of Abraxane and Gemcitabine Versus Gemcitabine Alone as First Line Treatment in Locally Advanced Unresectable Pancreatic Cancer. GAP (Gemcitabine Abraxane Pancreas) Trial (NCT02043730)	Phase 2	Stage Ⅲ	Active, not recruiting
A Multicenter Phase Ⅰ/Ⅱ Randomized Phase Ⅱ Study of Gemcitabine and *nab*-Paclitaxel With or Without NPC-1C in Patients With Metastatic or Locally Advanced Pancreatic Cancer (NCT01834235)	Phase 1 Phase 2	Stage Ⅲ-Ⅳ	Recruiting
A Phase IB Study of Erlotinib in Combination With Gemcitabine and *nab*-Paclitaxel in Patients With Previously Untreated Advanced Pancreatic Cancer (NCT01010945)	Phase 1	Stage Ⅲ-Ⅳ	Completed
A Phase 1 Study to Assess Safety, Pharmacokinetics, and Pharmacodynamics of PLX7486 as a Single Agent and in Combination With Gemcitabine and *nab*-Paclitaxel in Patients With Advanced Solid Tumors (NCT01804530)	Phase 1	Stage Ⅲ-Ⅳ	Recruiting
An Open-Label, Phase Ⅰ Dose Escalation Trial of TH-302 in Combination With Gemcitabine and *Nab*-Paclitaxel in Previously Untreated Subjects With Metastatic or Locally Advanced Unresectable Pancreatic Adenocarcinoma (NCT02047500)	Phase 1	Stage Ⅲ-Ⅳ	Recruiting
Phase Ⅱ Study Evaluating Bi-weekly Dosing of Gemcitabine Plus *nab*-Paclitaxel in the Treatment of Surgically Unresectable/Metastatic Pancreatic Cancer (NCT01851174)	Phase 2	Stage Ⅲ-Ⅳ	Recruiting
Phase 1B Trial of ADI-PEG 20 Plus *nab*-Paclitaxel and Gemcitabine in Subjects With Advanced Pancreatic Cancer (NCT02101580)	Phase 1	Stage Ⅲ-Ⅳ	Not yet recruiting
Phase Ⅱ Trial of Abraxane® in the Treatment of Patients With Pancreatic Cancer Who Have Failed First-Line Treatment With Gemcitabine-Based Therapy (NCT00691054)	Phase 2	Stage Ⅲ-Ⅳ	Completed
A Phase Ⅰ/Ⅱ/Pharmacodynamic Study of Hydroxychloroquine in Combination With Gemcitabine/Abraxane to Inhibit Autophagy in Pancreatic Cancer (NCT01506973)	Phase 1 Phase 2	Stage Ⅲ-Ⅳ	Recruiting
BYL719 in Combination With Gemcitabine and (Nab)-Paclitaxel in Locally Advanced and Metastatic Pancreatic Cancer (NCT02155088)	Phase 1	Stage Ⅲ-Ⅳ	Recruiting
A Phase Ⅰ and Randomized, Double-Blinded Phase Ⅱ Study of *nab*-paclitaxel/Gemcitabine Plus AZD1775 or Placebo in Treatment-Naïve Metastatic Adenocarcinoma of the Pancreas (NCT02194829)	Phase 1 Phase 2	Stage Ⅲ-Ⅳ	Suspended
A Phase Ib Study of Dovitinib in Combination With Gemcitabine and *nab*-Paclitaxel in Patients With Advanced Solid Tumors and Pancreatic Cancer (NCT02048943)	Phase 1	Stage Ⅲ-Ⅳ-recurrent	Not yet recruiting
Phase Ⅰ-Ⅱ Trial of Gemcitabine Plus *nab*-Paclitaxel (GemBrax) Followed by Folfirinox as First Line Treatment of Patients With Metastatic Pancreatic Adenocarcinoma (NCT01964287)	Phase 1 Phase 2	Stage Ⅳ	Recruiting
A Phase 1b Dose Escalation Study of Vantictumab (OMP-18R5) in Combination With *nab*-Paclitaxel and Gemcitabine in Patients With Previously Untreated Stage Ⅳ Pancreatic Cancer (NCT02005315)	Phase 1	Stage Ⅳ	Recruiting
A Phase 1b Dose Escalation Study of OMP-54F28 in Combination With *nab*-Paclitaxel and Gemcitabine in Patients With Previously Untreated Stage Ⅳ Pancreatic Cancer (NCT02050178)	Phase 1	Stage Ⅳ	Recruiting
A Phase 2, Randomized, Multicenter Study of PEGPH20 (PEGylated Recombinant Human Hyaluronidase) Combined With *nab*-Paclitaxel Plus Gemcitabine Compared With *nab*-Paclitaxel Plus Gemcitabine in Subjects With Stage Ⅳ Previously Untreated Pancreatic Cancer (NCT01839487)	Phase 2	Stage Ⅳ	Recruiting
A Phase Ⅰ/Ⅱ Study of Indoximod in Combination With Gemcitabine and *nab*-Paclitaxel in Patients With Metastatic Adenocarcinoma of the Pancreas (NCT02077881)	Phase 1 Phase 2	Stage Ⅳ	Not yet recruiting
A Phase 1b/2 Study of OMP-59R5 in Combination With *nab*-Paclitaxel and Gemcitabine in Subjects With Previously Untreated Stage Ⅳ Pancreatic Cancer (NCT01647828)	Phase 1 Phase 2	Stage Ⅳ	Recruiting
Phase Ⅰ Trial of the Proapoptotic Agonist, LCL161, and Gemcitabine Plus *nab*-Paclitaxel in Patients With Metastatic Pancreatic Cancer (NCT01934634)	Phase 1	Stage Ⅳ	Recruiting

A Phase Ⅰ Study of *nab*-paclitaxel (Abraxane), Gemcitabine, and Capecitabine (Xeloda) (AGX) in Patients With Previously Untreated, Metastatic Pancreatic Adenocarcinoma (NCT01161186)	Phase 1	Stage Ⅳ	Completed
A Phase 1b/2 Pilot Trial of *nab*-Paclitaxel Plus Cisplatin Plus Gemcitabine (Nabplagem) in Patients With Previously Untreated Metastatic Pancreatic Ductal Adenocarcinoma (PDA) (NCT01893801)	Phase 1 Phase 2	Stage Ⅳ	Recruiting
A Phase Ⅰ/Ⅱ, Two-Part, Multicenter Study to Evaluate the Safety and Efficacy of M402 in Combination With *nab*-Paclitaxel and Gemcitabine in Patients With Metastatic Pancreatic Cancer (NCT01621243)	Phase 1 Phase 2	Stage Ⅳ	Recruiting
A Phase 2, Randomized, Double-blind Study of Gemcitabine And *nab*-Paclitaxel Combined With Momelotinib in Subjects With Previously Untreated Metastatic Pancreatic Ductal Adenocarcinoma Preceded by a Dose-finding, Lead-in Phase (NCT02101021)	Phase 2	Stage Ⅳ	Not yet recruiting
Phase 1/2 Study Of PF-03084014 In Combination With Gemcitabine And *nab*-Paclitaxel In Patients With Previously Untreated Metastatic Pancreatic Ductal Adenocarcinoma (NCT02109445)	Phase 1 Phase 2	Stage Ⅳ	Not yet recruiting
Phase Ⅰ/Ⅱ Study to Evaluate *nab*-Paclitaxel in Substitution of CPT11 or Oxaliplatin in FOLFIRINOX Schedule as First Line Treatment in Metastatic Pancreatic Cancer (NCT02109341)	Phase 1 Phase 2	Stage Ⅳ	Recruiting
A Randomized, Double-Blinded, Placebo-Controlled Phase Ⅱ Trial Of Gemcitabine Plus *nab*-Paclitaxel Combined With OGX-427 Or Placebo In Patients With Metastatic Pancreatic Cancer (The Rainier Trial) (NCT01844817)	Phase 2	Stage Ⅳ	Recruiting
A Phase Ⅱ Study of Gemcitabine and Nanoparticle-Bound Paclitaxel as Second Line Therapy in Patients With Metastatic Pancreatic Cancer (NCT02242409)	Phase 2	Stage Ⅳ	Recruiting
Enzalutamide in Combination With Gemcitabine and *nab*-Paclitaxel for the Treatment of Advanced Pancreatic Cancer (NCT02138383)	Phase 1	Stage Ⅳ	Recruiting
A Phase Ib/Ⅱ Study of the Selective Inhibitor of Nuclear Export (SINE) KPT-330, Gemcitabine and *nab*-Paclitaxel in Patients With Metastatic Pancreatic Cancer (NCT02178436)	Phase 1 Phase 2	Stage Ⅳ	Not yet recruiting
Phase Ⅱ Trial of *nab*-Paclitaxel Plus S-1 in First-line Treatment of Patients With Advanced Pancreatic Cancer (NCT02124317)	Phase 2	Stage Ⅳ	Recruiting
Biological Effect of *nab*-Paclitaxel Combined to Gemcitabine in Metastatic Pancreatic Cancer (NCT02174887)	Phase 1	Stage Ⅳ	Not yet recruiting
A 3-Arm Phase 2 Double-Blind Randomized Study of Gemcitabine, Abraxane® Plus Placebo Versus Gemcitabine, Abraxane® Plus 1 or 2 Truncated Courses of Demcizumab in Subjects With 1st-Line Metastatic Pancreatic Ductal Adenocarcinoma (NCT02289898)	Phase 2	Stage Ⅳ	Not yet recruiting
A Phase Ib Clinical Study of BBI608 in Combination With Gemcitabine and *nab*-Paclitaxel in Adult Patients With Metastatic Pancreatic Adenocarcinoma (NCT02231723)	Phase 1	Stage Ⅳ	Recruiting
Nab-paclitaxel Plus Gemcitabine in Chinese Patients With Advanced Pancreatic Cancer (NCT02135822)	Phase 2	Advanced	Recruiting

[1]ClinicalTrials.gov Identifier. *nab*-Paclitaxel: Nanoparticle albumin-bound paclitaxel.

the study has obvious limitations (small sample size above all), it does provide a proof of the principle that switch maintenance in appropriately selected patients may indeed be beneficial in advanced PDAC; this becomes all the more relevant now that the proportion of advanced PDAC patients who reach the 6-mo landmark without experiencing progression of their disease is up to approximately 50% with contemporary first-line chemotherapy (such as FOLFIRINOX or *nab*-Paclitaxel/Gem). Such provocative findings open up an entirely new field in the treatment of PDAC, which clearly deserves further investigation.

WHERE DO WE GO FROM HERE?

Despite twenty years of well-deserved therapeutic nihilism[24,78,79], the field of systemic therapy for advanced/metastatic pancreatic cancer is finally moving forward: in only three years the median and 1-year OS have almost doubled from the 6 mo and 20% of the Gem era to the 9-11 mo and 35%-48% achieved with *nab*-Paclitaxel/Gem and FOLFIRINOX. Such evidence clearly sets new standard(s) of systemic therapy in metastatic pancreatic cancer, so that the use of Gem monotherapy appears nowadays justified only in a minority of patients, characterized by suboptimal PS (KPS < 70%), advanced age, and/or significant comorbidities. These results also substantially raise the bar for the design of present and future clinical trials, where the use of Gem monotherapy as the standard comparator arm is no longer acceptable, except, perhaps, in specific subpopulations of unfit patients.

The first burning question is: how will such advances in systemic therapy impact on outcomes in earlier stages of disease [locally advanced (LAPC), borderline resectable (BRPC), and frankly resectable disease], where there is, theoretically, much more to be gained and patients may be rendered NED and potentially cured? FOLFIRINOX is being actively pursued as a neoadjuvant/induction chemotherapy regimen in the multimodal management of LAPC and BRPC[45,46,80-83], most often with dose modifications aimed at improving tolerability and reducing the risk of serious toxicity: with ORR ranging from 27% to 50% and resectability rates of 12%-51%, such approach appears promising, although its ultimate impact on survival endpoints (median PFS ranging from 8 to 13 mo and median OS approximately 22 mo), as compared with more traditional regimens, will need to be judged on more congruous numbers of patients with adequate follow up. The combination of *nab*-Paclitaxel and Gem is also an attractive neoadjuvant treatment strategy for LAPC and BRPC. Although, currently available data in this setting are still anecdotal (case reports and preliminary reports of small case series)[84-88], such combination is extremely interesting, particularly because of the peculiar mechanism of action, which may encompass stromal depletion, arguably much more relevant in the primary pancreatic tumor than in metastatic lesions[58]. Even more importantly, both

Table 3 Selected ongoing studies of "folfirinox-based"and other contemporary regimens in borderline resectable, locally advanced and metastatic pancreatic cancer

Title (Study ID[1])	Phase	Stage	Status
A Phase Ⅱ Study of Neoadjuvant FOLFIRINOX in Patients With Resectable Pancreatic Ductal Adenocarcinoma With Tissue Collection (NCT02178709)	Phase 2	Resectable	Recruiting
Phase Ⅱ Study of Preoperative FOLFIRINOX Versus Gemcitabine/*nab*-Paclitaxel in Patients With Resectable Pancreatic Cancer (NCT02243007)	Phase 2	Resectable	Not yet recruiting
Neoadjuvant CAPOXIRI Chemotherapy in the Treatment of Resectable, Borderline Resectable and Locally Advanced Pancreatic Adenocarcinoma Protocol (NCT01760252)	Phase 2	Resectable, BR and LA	Recruiting
GTX-RT in Borderline Resectable Pancreatic Cancer (NCT01754623)	Phase 2	BR	Active, not recruiting
Neoadjuvant FOLFIRINOX for Borderline Resectable Pancreatic Cancer - a Pilot Study (NCT02148549)	Phase 1	BR	Recruiting
Phase Ⅱ Neoadjuvant Chemotheraphy (Gemcitabine and *nab*-Paclitaxel *vs* mFOLFIRINOX) and Sterotatic Body Radiation Therapy for Borderline Resectable Pancreatic Cancer (NCT02241551)	Phase 2	BR	Not yet recruiting
Phase IB Study of FOLFIRINOX Plus PF-04136309 in Patients With Borderline Resectable and Locally Advanced Pancreatic Adenocarcinoma (NCT01413022)	Phase 1	BR or LA	Recruiting
Phase Ⅱ Single Arm Clinical Trial of FOLFIRINOX for Unresectable Locally Advanced and Borderline Resectable Pancreatic Cancer (NCT01688336)	Phase 2	BR or LA unresectable	Recruiting
Prospective Randomized Multicenter Phase Ⅱ Trial to Investigate Intensified Neoadjuvant Chemotherapy in Locally Advanced Pancreatic Cancer (NEOLAP) (NCT02125136)	Phase 2	LA	Not yet recruiting
The Effect of FOLFIRINOX and Stereotactic Body Radiation Therapy for Locally Advanced, Non-Resectable Pancreatic Cancer (BCC-RAD-13) (NCT02128100)	Phase 2	LA, unresectable	Not yet recruiting
A Phase Ⅱ, Randomized, Open Label Study of Single Dose siG12D LODER in Combination With Chemotherapy in Patients With Unresectable Locally Advanced Pancreatic Cancer (NCT01676259)	Phase 2	LA, unresectable	Not yet recruiting
A Prospective Evaluation of Neoadjuvant FOLFIRINOX Regimen in Patients With Non-metastatic Pancreas Cancer (Baylor University Medical Center and Texas Oncology Experience) (NCT01771146)	Not provided	Localized, Non-metastatic	Recruiting
Phase 1b Clinical Trial of LDE225 in Combination With Fluorouracil, Leucovorin, Oxaliplatin and Irinotecan (FOLFIRINOX) in Previously Untreated Locally Advanced or Metastatic Pancreatic Adenocarcinoma, With an Expansion Cohort at the Recommended Phase 2 Dose (NCT01485744)	Phase 1	LA unresectable or metastatic	Recruiting
A Phase Ⅰ Study of FOLFIRINOX Plus IPI-926 for Advanced Pancreatic Adenocarcinoma (NCT01383538)	Phase 1	Not provided	Active, not recruiting
Phase Ⅱ Study: Neoadjuvant Gemcitabine, Docetaxel and Capecitabine Followed by Neoadjuvant Radiation Therapy With Gemcitabine and Capecitabine in the Treatment of Stage Ⅱ and Ⅲ Pancreatic Adenocarcinoma (NCT01065870)	Phase 2 Phase 3	Stage Ⅱ-Ⅲ	Recruiting
A Phase Ⅰ-Ⅱ Study of PAXG in Stage Ⅲ-Ⅳ Pancreatic Adenocarcinoma (NCT01730222)	Phase 1-2	Stage Ⅲ-Ⅳ	Recruiting
Phase Ⅱ Study of Modified FOLFIRINOX in Advanced Pancreatic Cancer (NCT01523457)	Phase 2	Stage Ⅲ-Ⅳ	Recruiting
Ceritinib and Combination Chemotherapy in Treating Patients With Advanced Solid Tumors or Locally Advanced or Metastatic Pancreatic Cancer (NCT02227940)	Phase 1	Stage Ⅲ-Ⅳ, recurrent	Not yet recruiting
Phase 2, Multicenter Study of FOLFIRINOX Followed by Ipilimumab in Combination With Allogeneic GM-CSF Transfected Pancreatic Tumor Vaccine (GVAX) in the Treatment of Metastatic Pancreatic Cancer (NCT01896869)	Phase 2	Stage Ⅳ	Recruiting
A Phase Ⅱ Study of Induction Consolidation and Maintenance Approach for Patients With Advanced Pancreatic Cancer (NCT01488552)	Phase 1-2	Stage Ⅳ	Recruiting
A Phase Ⅰ Open-Label Dose-Escalation Clinical Trial of CPI-613 in Combination With Modified FOLFIRINOX in Patients With Metastatic Pancreatic Cancer and Good Performance Status (NCT01835041)	Phase 1	Stage Ⅳ	Recruiting
Phase Ⅰ B/Randomized Phase Ⅱ Study of Folfirinox Plus AMG-479 (Ganitumab) or Placebo in Patients With Previously Untreated, Metastatic Pancreatic Adenocarcinoma (NCT01473303)	Phase 1 Phase 2	Stage Ⅳ	Withdrawn
Phase Ⅱ Trial to Investigate the Efficacy and Safety of mFOLFIRINOX in Patients With Metastatic Pancreatic Cancer in China (NCT02028806)	Phase 2	Stage Ⅳ	Recruiting
S1313, A Phase IB/Ⅱ Randomized Study of Modified FOLFIRINOX + Pegylated Recombinant Human Hyaluronidase (PEGPH20) Versus Modified FOLFIRINOX Alone in Patients With Good Performance Status Metastatic Pancreatic Adenocarcinoma (NCT01959139)	Phase 1 Phase 2	Stage Ⅳ	Recruiting
Phase I-Ⅱ Trial of Gemcitabine Plus *nab*-Paclitaxel (GemBrax) Followed by Folfirinox as First Line Treatment of Patients With Metastatic Pancreatic Adenocarcinoma (NCT01964287)	Phase 1 Phase 2	Stage Ⅳ	Recruiting
Phase Ⅱ Study of Modified FOLFIRINOX in Advanced Pancreatic Cancer (NCT01523457)	Phase 2	Stage Ⅳ	Recruiting
Phase Ⅰ/Ⅱ Study to Evaluate *nab*-Paclitaxel in Substitution of CPT11 or Oxaliplatin in FOLFIRINOX Schedule as First Line Treatment in Metastatic Pancreatic Cancer (NCT02109341)	Phase 1 Phase 2	Stage Ⅳ	Recruiting
Phase Ⅱ Study for Inoperable Non-Metastatic Pancreatic Cancer (Stage ⅣA) With Neoadjuvant Gemzar, Taxotere and Xeloda (GTX), and Radiation With Gemzar (NCT00869258)	Phase 2	Stage Ⅳ	Active, not recruiting
Phase Ⅱ Study of Gemcitabine/Taxotere/Xeloda (GTX) in Combination With Cisplatin in Subjects With Metastatic Pancreatic Cancer (NCT01459614)	Phase 2	Stage Ⅳ	Active, not recruiting
Phase-2 Study Evaluating Overall Response Rate (Efficacy) and Autonomy Daily Living Preservation (Tolerance) of "FOLFIRINOX " Pharmacogenetic Dose Adjusted, in Elderly Patients (70 yr or Older) With a Metastatic Pancreatic Adenocarcinoma (NCT02143219)	Phase 2	Stage Ⅳ	Not yet recruiting

[1]ClinicalTrials.gov Identifier. BR: Borderline resectable; LA: Locally advanced; *nab*-Paclitaxel: Nanoparticle albumin-bound paclitaxel.

strategies (aggressive polychemotherapy with PEFG- or FOLFIRINOX-like regimens and *nab*-Paclitaxel/Gem combinations) are being tested in the adjuvant setting or in "strategy studies", comparing neoadjuvant *vs* adjuvant chemotherapy with such novel regimens, in an attempt to improve the "cure" rate obtained by surgery in resectable PDAC patients (Table 1).

The second question is: how do we build on current standards to push forward survival of advanced PDAC patients even further? In relation to this, is the question of if, and how, we can integrate promising molecularly targeted agents into current treatment paradigms. Many ongoing phase Ⅰ-Ⅱ trials (Tables 2 and 3) are aimed at making the most of both worlds (polychemotherapy approaches and *nab*-Paclitaxel) and try to incorporate *nab*-Paclitaxel into PEFG- or FOLFIRINOX-like backbones, mostly by substituting *nab*-Paclitaxel for one of the components of the original regimen[89] (see also NCT01730222 and NCT02109341, Tables 2 and 3). An "add on" strategy is also the preferred design to try incorporating biological agents into first-line treatment (Tables 2 and 3). Such a strategy, however, has many potential pitfalls in our opinion: (1) if we are to learn from past experience, twenty years of negative studies using the Gem *vs* Gem + an additional agent (either a second chemotherapy drug or a targeted agent) paradigm should have taught us that such an "add on to current standard" strategy does not pay off (*nab*-Paclitaxel/Gem being the only notable exception)[24,38]; (2) while the combination of *nab*-Paclitaxel/Gem may still constitute a reasonable backbone for "add on" strategies, FOLFIRINOX-like regimens have substantial toxicity issues (so that most groups have adopted "modified" schedules), making it very unlikely that other agents may be simply added, without modifying the original regimen (and thereby potentially diminishing its efficacy); and (3) one of the most important clinical consequences of finally having "active" first-line regimens is that an increasing proportion of patients experience prolonged disease control, which enables them to receive a second or subsequent lines of therapy with clinical benefit. Thus, if the aim is to prolong disease control across multiple lines of treatment using all or most active agents upfront may actually turn out to be detrimental over the entire course of the disease.

Although there is no easy solution to the challenge of identifying optimal development strategies in advanced PDAC, one possibility is to exploit different disease settings, as an alternative to the classical "all in first-line" or "at relapse" strategies, to test the activity of new agents. In this respect, data recently obtained in the maintenance setting, even with a relatively inactive (in first- or second-line) class of agents such as VEGF/VEGFR inhibitors[90-93], is extremely provocative. Indeed, these results raise the interesting hypothesis that targeting the VEGFR pathway, which may be of marginal relevance and insufficient to alter the natural history of the disease against a bulky and rapidly growing tumor, could still be effective against progression under conditions of maximum cytoreduction and chemotherapy-induced tumour damage As more patients achieve disease control at 6 mo and as more active agents against pancreatic cancer become available, the maintenance setting may potentially achieve even more exciting results. Another disease setting that is currently relatively unexplored as a testing arena for new drugs is neoadjuvant treatment, which would also have the advantage of being able to truly (histologically) assess response and get access to post-treatment cancer tissue and tumor microenvironment, to look for drug effects on specific pathways/tissue components.

Last, but not least, thanks to novel technologies and "omics"-based characterization efforts, the molecular classification of pancreatic cancer(s) is evolving, as in many other malignancies, towards the clusterization of individual cases in discrete subgroups, characterized by alterations in common pathways. Some of these "driver" alterations could already be exploited therapeutically, while some other will require more preclinical modeling efforts, in order to make them suitable therapeutic targets. In addition to yielding novel therapeutic targets, such efforts are expected to rapidly lead to the identification of potentially predictive biomarkers, which would help select populations of pancreatic cancer patients who would derive the most benefit from specific therapeutic approaches. While eagerly awaiting this "new wave" of biology-based improvements in pancreatic cancer care, we will continue to work together with our patients and look at the therapeutic options that have been made recently available with renewed hope.

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REVIEW

Vanek's tumor of the small bowel in adults

Bassam Abboud

Bassam Abboud, Department of General Surgery, Hotel Dieu de France Hospital, Faculty of Medicine, Saint-Joseph University, Beirut, Lebanon
Author contributions: Abboud B designed and performed the research, analysed the data and wrote the paper.
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Correspondence to: Bassam Abboud, MD, Department of General Surgery, Hotel Dieu de France Hospital, Alfred Naccache Street 16-6830, Beirut, Lebanon. dbabboud@yahoo.fr
Telephone: +961-1-15300
Fax: +961-1-615295
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Abstract

Inflammatory fibroid polyps (IFPs), or Vanek's tumor, are one of the least common benign small bowel tumors. IFP affects both sexes and all age groups, with a peak of incidence in the fifth and seventh decades. They can be found throughout the gastrointestinal tract but most commonly in the gastric antrum or ileum. The underlying cause of IFPs is still unknown. Genetic study of IFP showed mutations in platelet derived growth factor alpha in some cases. At the time of diagnosis most IFPs have a diameter of 3 to 4 cm. The lesions have always been recorded as solitary polyps. Symptoms depend on the location and the size of the lesion, including abdominal pain, vomiting, altered small bowel movements, gastrointestinal bleeding and loss of weight. IFPs arising below the Treitz ligament can present with an acute abdomen, usually due to intussusceptions. Abdominal computed tomography is currently considered the most sensitive radiological method to show the polyp or to confirm intussusceptions. Most inflammatory fibroid polyps can be removed by endoscopy. Surgery is rarely needed. Exploratory laparoscopy or laparotomy is frequently recommended as the best treatment for intussusceptions caused by IFP. The operation should be performed as early as possible in order to prevent the intussusceptions from leading to ischemia, necrosis and subsequent perforation of the invaginated bowel segment. This report aims at reviewing the diagnosis, etiology, genetics, clinical presentation, endoscopy, radiology, and best treatment of IFP.

Key words: Small bowel; Inflammatory fibroid polyps; Abdominal pain; Intussusception; Computed tomography scan; Surgery

Core tip: Inflammatory fibroid polyps, or Vanek's tumor, are one of the least common benign small bowel tumors. Abdominal computed tomography is currently considered the most sensitive radiological method to show the polyp or to confirm complications. This report aims at reviewing the etiology, diagnosis, and treatment options of this entity, with emphasis on the success rate of radiologic investigations, the need for laparoscopic diagnosis and the role of surgery. The debate arises over the importance of the differential diagnosis. Moreover, if surgery is performed, consideration needs to be given to what operation should be undertaken and in which patients.

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INTRODUCTION

Inflammatory fibroid polyps (IFPs), or Vanek's tumor, are one of the least common benign small bowel tumors. They fall under the classification of submucosal connective tissue tumors. IFP affects both sexes (with slight predominance of the condition in men) and all age groups, with a peak of incidence in the fifth and seventh decades[1-11]. The mean age of patients with gastric tumors is significantly higher compared with IFP of the small bowel (72 years *vs* 53 years). IFP account for 0.1%-3.0% of all gastric polyps[11]. They can be found throughout the gastrointestinal (GI) tract-most commonly in the gastric antrum or ileum, but rarely in the duodenum and jejunum. The underlying cause of IFPs is still unknown. Many have suggested etiologies possibly related to chemical, physical, or metabolic triggers. Genetic study of IFP showed mutations in platelet derived growth factor alpha (PDGFRA) in some cases[12-16]. At the time of diagnosis most IFPs have a diameter of 3 to 4 cm; however, there is also a report of a case with an IFP 20 cm in size[3]. The lesions have always been recorded as solitary polyps. Symptoms depend on the location and the size of the lesion, and include abdominal pain, vomiting, altered small bowel movements, GI bleeding, loss of weight and intestinal intussusceptions. Abdominal computed tomography (CT) is currently considered the most sensitive radiological method to show the polyp or to confirm intussusceptions[17,18]. Most inflammatory fibroid polyps can be removed by endoscopy. Only rarely is surgery necessary. IFPs arising below the Treitz ligament can present with an acute abdomen (obstructive ileus) usually due to intussusceptions. Exploratory laparoscopy or laparotomy is frequently recommended as the best treatment for intussusceptions caused by IFP. The operation should be performed as early as possible in order to prevent the intussusceptions from leading to ischemia, necrosis and subsequent perforation of the invaginated bowel segment[19-21].

DEFINITIONS

IFPs are rare, idiopathic, localized, pseudotumor, benign neoplastic lesions originating in the submucosa of the gastrointestinal tract. The disease was first described in the stomach by Vanek in 1949[4] as a benign, non-encapsulated, submucosal granuloma, composed mainly of loose connective tissues, vessels and with an eosinophilic inflammatory component. At the same time the German pathologist Franz Bolck noticed these lesions, too, and referred to them as granuloblastoma of the stomach. The involvement of the small intestine and colon by IFPs is rare[4-9]. Various names for IFPs have been suggested, including eosinophilic granuloma, submucosal fibroma, hemangiopericytoma, inflammatory pseudotumor and fibroma. However, the term IFP first proposed by Helwig and Ranier[22] in 1953 for gastric polyps has gained acceptance for similar lesions throughout the GI tract. Since then several hundred reports, mainly case series and reports on single cases, have been published which predominantly focused on clinical and morphologic aspects. Notably, inflammatory fibroid polyps have been regarded as reactive lesions for decades. In 2008, however, the neoplastic nature of IFP became evident by the detection of activating PDGFRA mutations in these tumors[14]. IFPs can develop in many different locations in the GI tract. The most common site is the gastric antrum (66%-75%), followed by the small bowel (18%-20%), colorectal region (4%-7%), gallbladder (1%), esophagus (1%), duodenum (1%), and appendix (< 1%). However, the ileal segment is the most common site where these polyps cause intussusception[22-35].

GENETICS

The gene *PDGFRA* is located at 4q12.

The first genetic study of IFP showed mutations in PDGFRA[14]. The frequency of mutations among case series ranges from 21.7% to 69.6%. Activating PDGFRA mutations occur in exons 12, 14 and 18. A genotype-phenotype correlation could be established in terms of tumor location as gastric IFP harbors significantly more frequent exon 18 mutations. Exon 12 mutations are, however, associated with small bowel lesions. So far, only two cases with exon 14 mutations have been described, one originating from the small bowel the other from the stomach[12-16].

ETIOLOGY

Historically, IFP have been thought to represent a reactive inflammatory lesion. It was assumed that IFP might be associated for example with Helicobacter infection or type A gastritis. After discovery of activating PDGFRA mutations[14] in these tumors it became apparent that IFP represent true neoplasms which are obviously driven by activating mutations in the PDGFRA gene. The precise etiopathogenesis of IFPs remains unknown, but trauma, allergic reaction, genetic tendency, bacterial, physical, chemical and even metabolic stimuli have been suggested as initiators of the process. For some authors, it could be a consequence of chronic irritation and inflammation or a consequence of extreme reaction of the body to an intestinal trauma or a localized variant of eosinophilic gastroenteritis, given that it has a marked eosinophilic infiltration. The more common occurrence of these lesions in the stomach, with its coarse food content and active muscular contractions, supports a traumatic etiology, but it is difficult to account for lesions in the lower ileum on this basis[2]. Some cases of association between IFP and Crohn's disease were reported[36-38].

HISTOLOGY

IFP are benign. So far local recurrences have been described only anecdotally. No convincing case with invasive growth or aggressive outcome has been reported. It was suggested that IFP with concentric stromal proliferation may originate from a population of dendritic interstitial cells. Macroscopically, these tumors are pedunculated or sessile, measure 0.2-20 cm in diameter[39-42] and project into the bowel lumen. The mucosal surface is usually ulcerated and pale. Histologically IFP arise from the submucosa and are characterized by vascular and fibroblast proliferation and an inflammatory response, dominated by eosinophils. Microscopically, it is composed of mononuclear, spindle-shaped cells, which are characteristically arranged in whorls or in an onion skin like fashion around blood vessels or mucosal glands, forming a confused or whirl-like structure. The inflammatory infiltration also includes blood vessels, eosinophils, lymphocytes, macrophages, and mastocytes. The matrix consists of fine fibrillar collagen but might also be collagen-rich. The "classic" (or gastric) type which was originally described by Josef Vanek is characterized by a heavy inflammatory infiltrate which is rich in eosinophilic granulocytes. These lesions show many spindle cells but only little collagen. Some authors have shown that there is another morphological subtype ("intestinal type") which, in contrast, is paucicellular and collagen-rich. Both cellular elements, fibroblastic spindle cells and inflammatory infiltrate, are less numerous. These tumors tend to be larger than those of the gastric type[15]. Outside of ulcerations no necroses are found. There is no considerable proliferative activity as mitoses of the spindle cell are almost never seen and Ki67 is below 1%. Immunohistochemically, the spindle cells are mostly positive for CD34 but this feature may be absent especially in the intestinal type. PDGFRA expression is frequently found[14]. Immunostains for KIT, DOG-1, S100 and EMA are consistently negative. This may be important in the differential diagnosis of gastrointestinal stromal tumors, perineurioma and other spindle cell lesions of the gastrointestinal tract. IFP are clearly distinct from gastrointestinal stromal tumors by their morphology, submucosal origin and clinical behavior, although both entities share common mutational subtypes of the PDGFRA gene[16]. An immunohistochemical study of IFP showed a strong positivity for vimentin and the absence of staining for macrophages (HAM-56) expression in all cases evaluated, which suggests a major component of spindle cells best recognizable as fibroblasts. Further immunohistochemical analysis can demonstrate variable reactivity for actin, CD34, desmin, CD117 and S100[23,43-46].

CLINICAL PRESENTATION

IFPs are usually asymptomatic, remain undiagnosed for a long time or are incidental findings at endoscopy and laparotomy. When they are symptomatic, the clinical symptoms depend on the location and size of the tumor[2]. Abdominal pain is the main symptom in patients with lesions in the stomach. Intussusception and obstruction are the most frequent initial symptoms when the polyp is located in the small intestine[8,47-61]. Other GI symptoms, such as vomiting, diarrhea, bloody stools (larger polyps tend to erode and ulcerate superficially), tenesmus, and alterations in bowel habits, are also seen although their frequencies are low. Patients with IFPs in the small bowel are most likely to present with chronic episodes of colicky abdominal pain, lower GI bleeding, anemia and, more rarely, intestinal obstruction due to episodes of intestinal intussusception and rarely with necrosis and perforation[58,62-67]. Although malignant diseases represent the major causes of intussusceptions in adults, there are few reports of intestinal obstruction and perforation caused by IFPs. Pre-operative diagnosis of intussusceptions is rare but can occur in finding a palpable mass on the abdomen or with the use of imaging techniques.

DIAGNOSIS

An accurate diagnosis is based on a good medical history, thorough physical examination, and specific imaging modalities, such as X-ray, ultrasonography (US), CT, magnetic resonance imaging (MRI), enteroclysis, endoscopic procedures, angiography, and capsule endoscopy[17,18,69-72]. Diagnostic laparoscopy (DL) may assist in the diagnosis of intussusception in cases in which the diagnosis is suspected but not confirmed by preoperative workup. Moreover, DL can help to establish the cause and is less traumatic than an exploratory laparotomy[48].

RADIOLOGICAL APPEARANCE

Typically, abdominal X-ray examination[2,17,18] is the first diagnostic tool used, because obstructive symptoms dominate the clinical picture in most cases. Barium enema was the gold standard for diagnosis of intussusception until the mid-1980s. Around the same time, it was found that air could be used to diagnose and treat intussusception. Today, enteroclysis is rarely used in the diagnosis of intussusception. This invasive double-contrast imaging method is performed under fluoroscopy, MRI, or CT imaging. Although enteroclysis shows not only the inside of the lumen but also has high sensitivity and specificity for revealing small and mucosal lesions, its invasive nature limits its use. US is considered a useful tool in the diagnosis of intussusceptions. The primary imaging modality of choice is ultrasound scanning, which enables the diagnosis or exclusion of intussusception with a sensitivity of 98% and 100%, respectively, a specificity of 88%, and a negative predictive value

of 100%. Its classical imaging features include the target or doughnut sign in the transverse view and the pseudokidney, sandwich, or hayfork sign in longitudinal view. However, obesity and the presence of a large amount of air in the distended bowel loops can limit image quality and subsequent diagnostic accuracy[70,71]. Abdominal CT is currently considered the most sensitive radiological method for confirming intussusception, with a reported diagnostic accuracy of 58%-100%. In contrast to US, CT is unaffected by the presence of gas in the bowel lumen. With CT scan the mean sensitivity, specificity, positive predictive value, and negative predictive value for diagnosing surgical enteroenteric lesions using a measured lesion length of > 3.5 cm were 100%, 57.3%, 5.7,% and 100%, respectively. Similarly, the figures for polyps with a measured axial diameter > 3 cm were 100%, 32.9%, 3.7% and 100%, respectively. MRI can contribute to the radiological diagnosis of intussusception by demonstrating the "bowel-within-bowel" or "coiled-spring" appearance. A polyp can be detected as a leading point using a combination of breathing-independent T2-weighted MRI and gadolinium-enhanced breath-hold T1-weighted imaging. A study comparing MRI and CT for the diagnosis of intestinal obstruction showed that the cause of obstruction was correctly diagnosed by CT in 71% and by MRI in 95% of cases. The sensitivity, specificity and accuracy for MRI imaging was 95%, 100% and 96%, respectively as compared to 71%, 71% and 71% for helical CT[17,18,48,50,69-72].

ENDOSCOPIC EVALUATION

The gastric IFP may be diagnosed and treated by gastroscopy[73]. Double-balloon enteroscopy, also known as "push-and-pull" enteroscopy, can be used to examine approximately 70-150 cm of the small bowel, and double balloon enteroscopy can examine the full length of the small bowel, both antegrade and retrograde[74]. Capsule endoscopy and digital balloon endoscopy are newer means of diagnosing various gastrointestinal disorders. Capsule endoscopy is a noninvasive diagnostic test used to locate the source of gastrointestinal bleeding and to identify the causes of other intestinal disorders, including intussusception and various tumors. On capsule endoscopy, intussusception has been reported to appear as a mass lesion of the small bowel. Although obstructive symptoms are contraindicated for capsule endoscopy, this new method for evaluating the small bowel could be helpful in cases with long-standing abdominal pain and negative results on radiological examination, CT, or barium studies, to exclude the possibility of malignancy. Colonoscopy is useful only in cases in which colonic involvement is strongly suspected, and it allows the lesion to be diagnosed and biopsied. On colonoscopy, intussusception is seen as an intraluminal mass directed centrally and distally. However, diagnosis is rarely made by colonoscopy, and the diagnosis is usually made during surgery[2,48].

DIFFERENTIAL DIAGNOSIS

The underlying cause in most cases of enteroenteric intussusceptions was benign, whereas it was mostly malignant in the ileocolic and colocolic cases. That is, the potential for malignancy increased from proximal to distal intussusception. Causes of intestinal obstruction in adult patients include adhesion bands, malignant tumors and hernia in descending order of appearance. Most lead points in the GI tract involve primary or metastatic malignancy, lipomas, leiomyomas, adenomas, neurofibromas, postoperative adhesions, Meckel's diverticulum, foreign bodies, vascular anomalies, lymphoid hyperplasia, trauma, celiac disease, cytomegalovirus colitis, lymphoid hyperplasia secondary to lupus, Henoch-Schönlein purpura, Wiskott-Aldrich syndrome, appendiceal stump, or IFP. The etiologies of intussusceptions in the small bowel and the colon are quite different. In the small intestine there is a predominance of benign processes, with up to 90% of cases including hamartomas, lipomas, leiomyoma neurofibromas, adenomas, Peutz-Jeghers syndrome, IFP, adhesions, Meckel's diverticulum, lymphoid hyperplasia, trauma, celiac disease, intestinal duplication, Henoch-Schönlein purpura, appendiceal stump, and tuberculosis and more rarely IFPs. The ileum segment is the most common site where these polyps cause intussusception. Malignant lesions (either primary or metastatic) account for 14%-47% of cases of intussusception in the small intestine. On the other hand, intussusception occurring in the large bowel is more likely to have a malignant etiology and accounts for 43%-80% of cases[2,40,48]. Colon adenocarcinoma is the most important cause of malignant large bowel intussusception. Benign lesions provoking large bowel intussusception include lipomas, leiomyomas, adenomatous polyps and endometriosis. IFPs can mimic several other tumor and non-tumor processes of the gastrointestinal tract. Differential histopathological diagnoses include spindle cell lesions, such as inflammatory fibrosarcoma, spindle-cell carcinoids, and gastrointestinal stromal tumors (GISTs). Differentiation can be difficult, especially between IFPs and GISTs. GISTs are common in the stomach and frequently present as polypoid masses. In the intestine, these tumors can present with intussusception similar to IFP. Immunohistochemistry is used to distinguish between IFPs and GISTs. Both tumors are positive for CD34 and vimentin, but GISTs are positive for CD117, while IFPs are not[2].

TREATMENT AND PROGNOSIS

Most inflammatory fibroid polyps can be removed by endoscopy (endoscopic submucosal dissection is required)[73,75]. Only rarely is surgery necessary.

Inflammatory fibroid polyps are benign and do not recur nor metastasize. Exploratory laparoscopy or laparotomy is frequently recommended as the best treatment for intussusceptions caused by IFP. The lesion seems to lack malignant potential and recurrence of the polyp has been reported very rarely. The operation should be performed as early as possible in order to prevent the intussusceptions from leading to ischemia, necrosis and subsequent perforation of the invaginated bowel segment. When surgery is delayed and intestinal perforation with peritonitis occurs, there is a considerable increase in morbidity and mortality. The appropriate management of adult intussusceptions remains controversial, with the debate focusing mostly on the issue of primary *en bloc* resection *vs* initial reduction followed by more limited resection. Thus, the most important factors in the surgical decision process in an adult patient without a histopathological diagnosis in the preoperative period and intussusception was detected during a surgical operation, and are the location and size of the mass and viability of the invaginated segment[2,47-50]. Reduction by surgery before resection may theoretically permit more limited resection; however, the risk of potential intraluminal seeding or venous tumor dissemination during the manipulation of a malignant lesion should also be taken into consideration. The incidence of malignancy as the cause of small intestinal intussusceptions ranges from 1% to 47%, and the majority of lesions are metastatic. Therefore, recent reports have recommended initial reduction of externally viable small bowel prior to resection. The likelihood of cancer in ileocolic and colocolic intussusception is 43%-100%. The vast majority of these lesions arise as a primary lesion, in which resection without reduction is recommended[47-50].

CONCLUSION

The debate arises over the importance of the differential diagnosis. Moreover, if surgery is performed, consideration needs to be given to what operation should be undertaken and in which patients.

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REVIEW

New endoscopic ultrasound techniques for digestive tract diseases: A comprehensive review

Fan-Sheng Meng, Zhao-Hong Zhang, Feng Ji

Fan-Sheng Meng, Feng Ji, Department of Gastroenterology, the First Affiliated Hospital, Zhejiang University, Hangzhou 310000, Zhejiang Province, China
Zhao-Hong Zhang, Department of Hematology, People's Hospital of Linyi, Linyi 276300, Shandong Province, China
Author contributions: Meng FS and Zhang ZH searched the literature and wrote the manuscript; Ji F critically revised the manuscript; Meng FS and Zhang ZH contributed equally to this manuscript.
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Correspondence to: Feng Ji, MD, Department of Gastroenterology, the First Affiliated Hospital, Zhejiang University, 79 Qingchun Road, Hangzhou 310000, Zhejiang Province, China. jifeng1126@sina.com
Telephone: +86-571-87236586
Fax: +86-571-87236611
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Abstract

Endoscopic ultrasound (EUS) is one of the most important modalities for the diagnosis of digestive tract diseases. EUS has been evolving ever since it was introduced. New techniques such as elastography and contrast enhancement have emerged, increasing the accuracy, sensitivity and specificity of EUS for the diagnosis of digestive tract diseases including pancreatic masses and lymphadenopathy. EUS-elastography evaluates tissue elasticity and therefore, can be used to differentiate various lesions. Contrast-enhanced EUS can distinguish benign from malignant pancreatic lesions and lymphadenopathy using the intravenous injection of contrast agents. This review discusses the principles and types of these new techniques, as well as their clinical applications and limitations.

Key words: Endoscopic ultrasound; Elastography; Contrast-enhanced; New techniques; Digestive tract diseases

Core tip: This article primarily focuses on emerging techniques such as elastography and contrast-enhanced endoscopic ultrasound. Principles, types and clinical applications are discussed. These emerging techniques have high accuracy, sensitivity and specificity in differential diagnosis between benign and malignant lesions.

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INTRODUCTION

Endoscopic ultrasound (EUS) has continuously evolved since its initial introduction. With the development of accessories and technologies, EUS-guided fine-needle aspiration (FNA) has emerged as the gold standard for the diagnosis of gastrointestinal lesions. However, EUS-FNA is technically demanding and is associated

with a low (but not negligible) risk of complications. EUS-elastography and contrast-enhanced EUS have emerged as non-invasive techniques in diagnosis of digestive disorders. Recently, 3-D EUS technology and EUS-guided interventions such as biliary and pancreatic fluid collection drainage and fine-needle injections have been introduced and are rapidly gaining in popularity. EUS-guided interventions will be discussed elsewhere.

Recently, many studies have demonstrated that elastography and contrast-enhanced EUS have high accuracy, sensitivity and specificity in discriminating between benign and malignant lesions (Table 1).

EUS-ELASTOGRAPHY

Principle

Elasticity varies in different types of tissues and in the same tissue affected by different pathologic states[1]. Elastography can evaluate the hardness of tissue by measuring its elasticity[2]. The principle of elastography is that tissue compression produces strain; alterations in strain can be detected and displayed in real time alongside conventional B-mode images with special software[3,4]. Elastography was developed in order to complement conventional EUS for the assessment of previously hard-to-reach tumors near the gastrointestinal tract, such as pancreatic masses[5,6] and lymph nodes[1,7].

Categories

Qualitative elastography: Less tissue deformation is caused by compression of hard tissue than of soft tissue[4]. The degree of deformation is represented by different colors[4,8]. Hard tissue is blue and soft tissue is red; tissues with an intermediate elasticity are in the green-yellow spectrum[6,9].

Quantitative elastography

Hue/SH analysis: A histogram is used to represent the digital color distribution. Specialized software (Image J or SH) analyzes the color of the pixels inside the target lesions and each pixel color is represented by a value from 0 to 255 (soft to hard)[4,8]. Histograms produce an average value that represents the overall elasticity of tissues[6].

Strain ratio: Strain ratio (SR) is based on a different principle from histograms. The elasticity of the target tissue is expressed not as an absolute value, but as a relative ratio compared to the reference value provided by these tissues[2]. Two non-overlapping areas inside the region of interest (ROI) are selected: The lesion (area A) and the reference zone (area B). The B/A quotient yields the SR[10,11].

Elastography has been used to evaluate several organs including the breast, thyroid, prostate, cervix, liver and others[12,13]. Studies have demonstrated that primarily blue masses are malignant, whereas red and green masses are considered to be benign.

CONTRAST-ENHANCED EUS

Principle

The contrast agents used in this new technique are gas-containing microbubbles that are covered by a protective shell[14]. The principles of contrast-enhanced EUS are as follows: when subjected to an ultrasonic signal, the microbubbles oscillate or break and generate components that can be detected and reconstructed on an ultrasound image[15,16], and components of a higher frequency are required for EUS enhancement[17].

Two generations of contrast agents have been developed. The first-generation agent was Levovist, which is composed of microbubbles of air covered by galactose and palmitic acid[18]. However, Levovist requires high acoustic power to oscillate the microbubbles. Second-generation contrast agents, such as Sonovue, Sonazoid and Definity, can be oscillated or broken by lower acoustic power[19,20]. The development of these contrast agents promoted the use of harmonic imaging in EUS[21].

The contrast microbubbles are restricted to the vascular system and do not lead to enhancement of the entire circulatory system[21]. They are generally safe, and adverse events have rarely been observed.

Categories

Contrast-enhanced color and power Doppler sonography (CD-EUS): CD-EUS allows the detection of intra-tumoral vasculature through the enhancement of tumor vessels[22,23]; it increases the sensitivity to signals from vessels by producing pseudo-Doppler signals from microbubbles[24]. However, CD-EUS technique has a limited ability to detect slow blood flow and it suffers from Doppler-related artifacts such as motion and blooming[14,25].

Contrast-enhanced harmonic EUS (CH-EUS): CH-EUS has been developed to overcome the limitations of CD-EUS. This technique allows microvessels and parenchymal perfusion to be visualized[26]. Moreover, by measuring the time-course of changes in the intensity of echogenicity (time-intensity curve), vascularity can be quantitatively analyzed[27,28].

EUS-GUIDED CONFOCAL MICROSCOPY

Confocal endomicroscopy is an emerging technique and allows real-time optical biopsies to be perfo-rmed in the gastrointestinal tract. The technique uses a EUS puncture needle in which the stylet is replaced by a confocal mini-probe. The mini-probe, which is preloaded into the EUS needle, is guided

Table 1 Summary of studies with new endoscopic ultrasound techniques

Ref.	No. of cases	Target lesions	Techniques	Accuracy	Specificity	Sensitivity
König *et al*[13]	151	Prostatic lesions	RTE	84.10%	N/A	N/A
Kanamori *et al*[48]	46	LNs lesions	CE	82.10%	77.30%	88.20%
Alam *et al*[12]	85	LNs lesions	RTE	84%	59%	98%
Kamoi *et al*[54]	107	Prostatic Lesions	RTE	76%	81%	68%
Ohno *et al*[44]	87	IPMNs	CE	75.90%	92.90%	60%
Giovannini *et al*[33]	222	LNs and PLs	RTE	N/A	82.5% (LN) 80.0% (PL)	91.8% (LN) 92.3% (PL)
Săftoiu *et al*[22]	54	Pancreatic masses	CE and RTE	83.30%	95.20%	75.80%
Napoleon *et al*[42]	35	Pancreatic masses	CE	86%	88%	89%
Xia *et al*[49]	43	Intra-abdominal lesions	CE	97.60%	100%	96.30%
Săftoiu *et al*[6]	258	Pancreatic masses	RTE	85.40%	66%	93.40%
Xu *et al*[7]	368	LNs lesions	RTE	N/A	91%	85%
Sakamoto *et al*[51]	76	GISTs	CH	83%	63%	100%
Kapoor *et al*[55]	50	Prostatic lesions	RTE	N/A	86.80%	91.70%
Waage *et al*[56]	69	Rectal lesions	RTE	94%	96%	93%
Hocke *et al*[5]	58	Pancreatic lesions	RTE CE	N/A	94.7%(RTE) 89.5%(CE)	33.4%(RTE) 92.3%(CE)
Dawwas *et al*[31]	104	Pancreatic masses	RTE	86.50%	16.70%	100%
Kitano *et al*[39]	277	Pancreatic lesions	CH	N/A	94.40%	91.20%
Gong *et al*[41]	1139	Pancreatic masses	CE	N/A	93%	93%
Knabe *et al*[3]	40	LN lesions	RTE	51.5	86.70%	88.90%
Lee *et al*[43]	37	Pancreatic lesions	CH	92%	N/A	93%
Havre *et al*[34]	39	Pancreatic lesions	RTE	N/A	71%	67%
Imazu *et al*[59]	36	GB lesions	CH	94.40%	98%	89.60%

LN: Lymph node; PL: Pancreatic lesion; RTE: Real-time elastography; CE: Contrast-enhanced; CH: Contrast-enhanced harmonic; IPMN: Intraductal papillary mucinous neoplasm; GIST: Gastrointestinal stromal tumor; GB: Gallbladder; N/A: Not available.

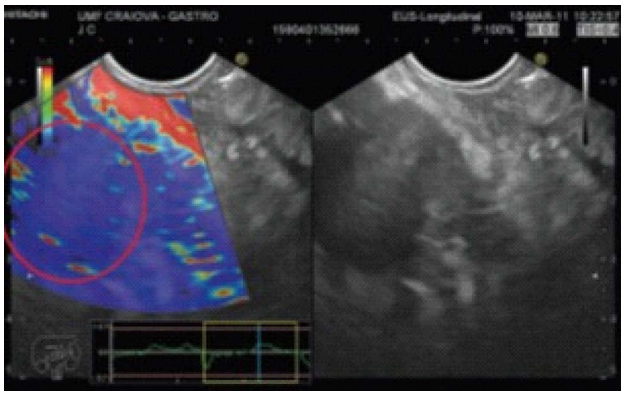

Figure 1 A patient with a malignant pancreatic tumor. The elastography image in the left panel shows a homogeneous blue mass (red circle). The B-mode reference image is shown in the right panel (Popescu *et al*[4]).

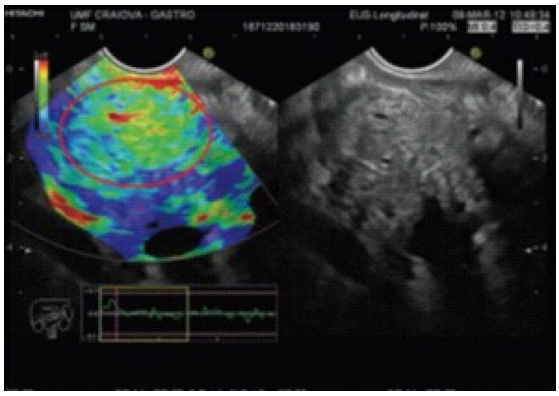

Figure 2 A patient with chronic pancreatitis. The elastography image in the left panel shows a heterogeneous green mass (red circle). The B-mode reference image is shown in the right panel (Popescu *et al*[4]).

endosonographically into the target lesion. The intratumoral CM examination begins after the injection of fluorescein[29,30].

CLINICAL APPLICATIONS

EUS-elastography and CH-EUS for solid pancreatic lesions

Many published studies have reported that a EUS-elastography finding of a blue (*i.e.,* hard) pancreatic lesion is highly sensitive and specific for adenocarcinoma (Figure 1). Chronic pancreatitis is an intermediately soft (green) mass (Figure 2), and normal pancreatic tissue is homogeneously soft on EUS-elastography.

A prospective study conducted by Dawwas *et al*[31] which used elastography to differentiate pancreatic masses revealed that quantitative and qualitative EUS elastography techniques had a sensitivity of 100.0% and 95.7%, a specificity of 16.7% and 22.2%, a positive predictive value (PPV) of 86.1% and 86.4%, a negative predictive value (NPV) of 100.0% and 50.0%, and an overall accuracy of 86.5% and 83.8%, respectively. A recent meta-analysis that reviewed six studies showed that using the qualitative color pattern as the diagnostic standard, the pooled sensitivity was 99% (95%CI: 98%-100%) and the specificity was

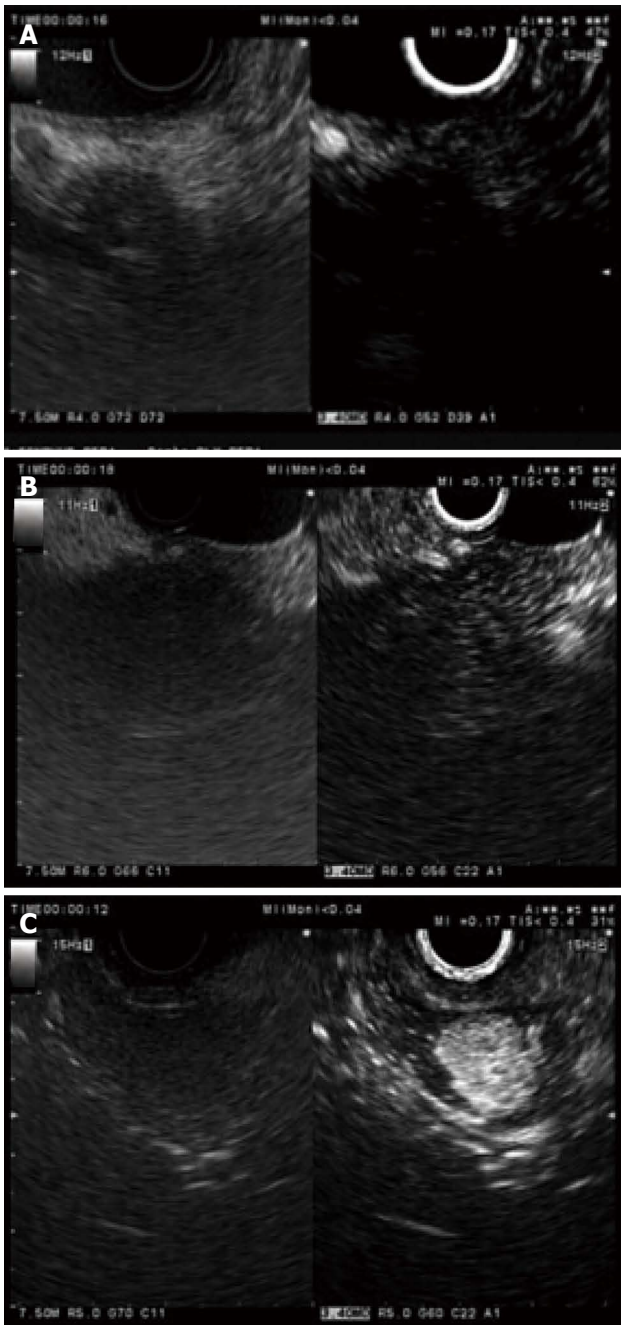

Figure 3 Typical contrast-enhanced harmonic endoscopic ultrasound images of pancreatic tumors. A: Pancreatic carcinoma with hypoenhancement. Conventional EUS (left) shows a hypoechoic mass at the pancreas tail. Contrast-enhanced harmonic endoscopic ultrasound (CH-EUS) (right) indicates that the mass has hypoenhancement compared with the surrounding tissue; B: Chronic pancreatitis with isoenhancement. Conventional EUS (left) shows a hypoechoic mass at the pancreas body. CH-EUS (right) indicates homogeneous enhancement mass similar to the surrounding tissue; a margin is not observed; C: Neuroendocrine tumor with hyperenhancement. Conventional EUS (left) shows a hypoechoic mass at the pancreas body. CH-EUS (right) indicates that enhancement in the mass is higher than in the surrounding tissue (Kwek *et al*[65]).

74% (95%CI: 65%-82%)[32].

More recent studies have focused on quantitative elastography. A European multicenter study conducted by Săftoiu *et al*[6] demonstrated that Hue histogram elastography using 175 as the cut-off value had a sensitivity of 93.4%, a specificity of 66.0%, a PPV of 92.5%, an NPV of 68.9%, and an overall accuracy of 85.4 %. Another multicenter study conducted by Giovannini *et al*[33] yielded similar results. A study conducted by Havre *et al*[34] showed that the median SR in malignant lesions was 7.05 (3.02-27.57) and was 1.56 (0.07-35.55) ($P < 0.001$) in benign lesions. Iglesias-Garcia *et al*[8] reported that the SR was significantly higher among patients with pancreatic cancers than in those with inflammatory masses. An earlier study conducted by Săftoiu *et al*[35] in 2008 investigated the ability of quantitative EUS elastography to differentiate between benign and malignant pancreatic masses, and its sensitivity, specificity, PPV, NPV and accuracy were 91.4%, 87.9%, 88.9%, 90.6%, and 89.7%, respectively.

Ying *et al*[36] analyzed 10 studies including 893 pancreatic masses and found that the pooled sensitivity and specificity for the diagnosis of malignant pancreatic masses were 0.98 (95%CI: 0.93-1.00) and 0.69 (95%CI: 0.52-0.82) for qualitative EUS elastography, and 0.96 (95%CI: 0.86-0.99) and 0.76 (95%CI: 0.58-0.87) for quantitative EUS elastography, respectively. Another meta-analysis conducted by Li *et al*[37] yielded similar conclusions.

However, other elastography studies have reported less promising results. One study found overly similar color patterns between cancerous masses and pancreatitis[38]. One recently published large single-center study reported that quantitative elastography was not as accurate as was described in previous studies and meta-analyses[31].

There are four types of enhancement patterns in CH-EUS: non-enhancement, hypo-enhancement, iso-enhancement and hyper-enhancement[39]. A hypo-enhancing pattern has been considered to be one of the most common distinguishing characteristics of pancreatic adenocarcinoma (Figure 3), and is more diagnostically accurate than the finding of a hypoechoic lesion on conventional EUS ($P < 0.001$)[40]. A recent meta-analysis of CE-EUS showed that this method can identify pancreatic adenocarcinomas with a pooled sensitivity and specificity of 94% and 89%, respectively[41]. Hypo-vascularity which is a sign of ductal carcinomas in CH-EUS yielded a sensitivity of 89%-95% and a specificity of 64%-89%[36,40,42]. In particular, CH-EUS was significantly more accurate than CT in diagnosing small ductal carcinomas $\leqslant$ 2 cm ($P < 0.034$)[39].

Lee *et al*[43] demonstrated that pancreatic carcinomas and pancreatic neuroendocrine tumors showed different enhancement patterns on CE-EUS, suggesting that the enhancement pattern may be an important characteristic for diagnosis.

CH-EUS for cystic pancreatic lesions

Differentiating between benign and malignant intraductal papillary mucinous neoplasms of the pancreas is challenging. Mural nodules have been

identified as one of the most important signs predicting for malignancy. An earlier study conducted by Ohno *et al*[44] analyzed the enhancement pattern of mural nodules and found that papillary and invasive nodular patterns were more frequently related to invasive cancer. A recent study of CE-EUS in the differentiation of pancreatic cystic lesions showed that CE-EUS considerably increases the sensitivity of displaying cystic wall vascularization[45].

EUS-elastography and CH-EUS for lymph nodes

At present, the established standards indicating malignant involvement of lymph nodes (LN) include the following: round shape, hypo-echogenicity, diameter > 1 cm and distinguishing margin. However, all four features of malignant involvement are present in only one-fourth of malignant LNs[46] and the specificity of these findings is poor[8].

A recent meta-analysis conducted by Xu *et al*[7] found that EUS elastography demonstrated a pooled sensitivity of 88% and specificity of 85% for differentiating between benign and malignant LNs. A study conducted by Okasha *et al*[1] reached similar conclusions. However, a recent study by Larsen *et al*[47] delivered a disappointing result. The investigators concluded that EUS-elastography was not better than conventional EUS in differentiating between malignant and benign LNs.

On CD-EUS, the presence of a filling defect is a typical characteristic of malignant lymphadenopathy, with a sensitivity of 100% and a specificity of 86.4%[48]. In a study conducted by Xia *et al*[49], the sensitivity, specificity and accuracy rates of CD-EUS in diagnosing LN lesions with unknown origin were 96.3%, 100% and 97.6%,respectively.

EUS-elastography and CH-EUS for gastrointestinal submucosal lesions

The risk classifications for GISTs are based on size and the number of mitoses/50 high power fields. Immunohistochemical analysis should also be performed. Therefore, elastographic evaluation of malignancy in such lesions may be difficult.

A recent study conducted by Kannengiesser *et al*[50] demonstrated that the enhancement pattern of CH-EUS was able to distinguish between GISTs and other benign submucosal tumors such as leiomyoma or lipoma by the enhancement pattern. All histologically proven GISTs showed hyper-enhancement, while lipoma and leiomyoma both showed hypo-enhancement. A study conducted by Sakamoto *et al*[51] demonstrated that the overall sensitivity, specificity and accuracy of CH-EUS in prediction of malignant GISTs were 100%, 63% and 83%, respectively.

EUS-elastography and CH-EUS guided FNA

Elastography can help the user to select a site where FNA can be performed with improved diagnostic yield, particularly in patients with either necrotic tumors or possible cancers within diffuse inflammatory lesions.

CH-EUS clearly depicts subtle lesions that conventional EUS is unable to identify and, can be used to select targets for EUS-FNA[52]. Real-time CH-EUS-FNA can identify and avoid an avascular site, helping to prevent sampling of necrotic areas and allowing the selection of more suitable sites for biopsy[53].

OTHER CLINICAL APPLICATIONS

The use of EUS-elastography has been investigated for the diagnosis and evaluation of prostate cancer, rectal cancer, and inflammatory bowel disease. In prostate cancer, EUS-elastography has been demonstrated to be better than conventional EUS[54], and it increases the specificity of prostate biopsies by highlighting areas that are highly suspicious for malignancy[55]. A study of transrectal elastography conducted by Waage *et al*[56] showed that the sensitivity, specificity and accuracy rates of SR were 93%, 96% and 94%, respectively. Dietrich *et al*[57] reported that left hepatic tumors can be differentiated by EUS-elastography.

Elastography of the hepatobiliary system is particularly useful for evaluation of the papilla of Vater and staging papillary carcinoma and papillomatosis[58].

A recent study of CH-EUS for the differential diagnosis of gallbladder wall thickening, which was conducted by Imazu *et al*[59], reported that the overall sensitivity, specificity and accuracy rates of CH-EUS for diagnosing malignant GB wall thickening were 89.6%, 98% and 94.4%, respectively.

CE-EUS has also been used in other gastrointestinal diseases, such as inflammatory bowel disease. A study published in 2012 showed that CE-EUS had excellent sensitivity and specificity for the diagnosis of postoperative recurrence in Crohn's disease[60].

EUS-confocal microscopy for pancreatic cystic lesions

Studies of EUS-confocal microscopy are rare. A recent study conducted by Giovannini *et al*[61] demonstrated that EUS-confocal microscopy can effectively distinguish different pancreatic cystic lesions.

LIMITATIONS AND FUTURE DEVELOPMENT

EUS-elastography is an operator-dependent technique, with a high image selection bias and, in some cases, a lack of reproducibility. Excessive compression of the tissue can artificially cause more deformation. The presence of certain tissues (*e.g.,* vessels, cysts, and bone) in the ROI significantly influences elasticity measurements. Furthermore, the appropriate cut-off values for quantitative elastography remain controversial. Some authors have reported promising findings, while others noted disappointing results. Consequently, most authors have indicated that elastography is not ready to replace EUS-FNA, but

may be a supplementary procedure in patients with negative or inconclusive EUS-FNA findings, if a strong suspicion of malignancy still exists[4].

CE-EUS has been criticized for its qualitative nature, and quantitative methods have been proposed to improve its reliability[62].

The therapeutic potential of CE-EUS is to selectively deliver medications and reduce side-effects using contrast microbubbles as carriers[63,64].

CONCLUSION

EUS-elastography and CH-EUS are emerging techniques. These techniques are simple and easy to perform (using a touch of a button for elastography), do not require extensive training and costly devices, have a low cost and low complication rate, do not add extra time to EUS procedures, and can provide valuable information regarding the characteristics of focal masses. Therefore, both are effective supplemental techniques in EUS-FNA and should be implemented in clinical practice. A combination of these emerging techniques can further increase the ability of EUS to diagnose pancreatic masses. However, these techniques should be performed in tertiary centers by experienced operators with expertise in EUS and EUS-FNA.

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ORIGINAL ARTICLE

Basic Study

Murine study of portal hypertension associated endothelin-1 hypo-response

Nicholas Theodorakis, Mary Maluccio, Nicholas Skill

Nicholas Theodorakis, Mary Maluccio, Nicholas Skill, Department of Surgery, Division of Transplant Surgery, Indiana School of Medicine, Indiana University, Indianapolis, IN 46202, United States

Author contributions: Theodorakis N and Skill N performed the research; Skill N and Maluccio M designed the research; Skill N and Maluccio M wrote the paper.

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Correspondence to: Nicholas Skill, PhD, Assistant Research Professor, Department of Surgery, Division of Transplant Surgery, Indiana School of Medicine, Indiana University, Walthur Cancer Research Building, Rm C519. 980 W. Walnut Street, Indianapolis, IN 46202, United States. nskill@iupui.edu
Telephone: +1-317-2744532
Fax: +1-317-2748046

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Abstract

AIM: To investigate endothelin-1 hypo-responsive associated with portal hypertension in order to improve patient treatment outcomes.

METHODS: Wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ mice received partial portal vein ligation surgery to induce portal hypertension or sham surgery. Development of portal hypertension was determined by measuring the splenic pulp pressure, abdominal aortic flow and portal systemic shunting. To measure splenic pulp pressure, a microtip pressure transducer was inserted into the spleen pulp. Abdominal aortic flow was measured by placing an ultrasonic Doppler flow probe around the abdominal aorta between the diaphragm and celiac artery. Portal systemic shunting was calculated by injection of fluorescent microspheres in to the splenic vein and determining the percentage accumulation of spheres in liver and pulmonary beds. Endothelin-1 hypo-response was evaluated by measuring the change in abdominal aortic flow in response to endothelin-1 intravenous administration. In addition, thoracic aorta endothelin-1 contraction was measured in 5 mm isolated thoracic aorta rings *ex-vivo* using an ADI small vessel myograph.

RESULTS: In wild type and iNOS$^{-/-}$ mice splenic pulp pressure increased from 7.5 ± 1.1 mmHg and 7.2 ± 1 mmHg to 25.4 ± 3.1 mmHg and 22 ± 4 mmHg respectively. In eNOS$^{-/-}$ mice splenic pulp pressure was increased after 1 d (P = NS), after which it decreased and by 7 d was not significantly elevated when compared to 7 d sham operated controls (6.9 ± 0.6 mmHg and 7.3 ± 0.8 mmHg respectively, P = 0.3). Abdominal aortic flow was increased by 80% and 73% in 7 d portal vein ligated wild type and iNOS when compared to shams, whereas there was no significant difference in 7 d portal vein ligated eNOS$^{-/-}$ mice when compared to shams. Endothelin-1 induced a rapid reduction in abdominal aortic blood flow in wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ sham mice (50% ± 8%, 73% ± 9% and 47% ± 9% respectively). Following portal vein ligation endothelin-1 reduction in blood flow was significantly diminished in each mouse group. Abdominal aortic flow was reduced by 19% ± 9%, 32% ± 10% and 9% ± 9% in wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ mice respectively.

CONCLUSION: Aberrant endothelin-1 response in murine portal hypertension is NOS isoform independent. Moreover, portal hypertension in the portal vein ligation model is independent of ET-1 function.

Key words: Liver disease; Portal hypertension; Hyperdynamic circulation; Endothelin-1; Nitric oxide synthase isoforms

Core tip: Portal hypertension (PHT) is a complication associated with vascular derangements in response to liver disease and fibrosis. Perturbations of nitric oxide (NO) and endothelin-1 are believed to be interrelated and play a key role in PHT vasculopathy. This study investigates the importance of NO biosynthesis in endothelin-1 vasoconstriction hypo-response seen in patients with PHT. PHT was induced in wild type, $eNOS^{-/-}$ and $iNOS^{-/-}$ mice by partial portal vein ligation (PVL) and endothelin-1 contractile response was determined. Endothelin-1 (ET-1) induced contraction was significantly reduced following PVL in all mouse groups. Aberrant ET-1 function associated with PHT is NO independent.

Theodorakis N, Maluccio M, Skill N. Murine study of portal hypertension associated endothelin-1 hypo-response. *World J Gastroenterol* 2015; 21(16): 4817-4828 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4817.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4817

INTRODUCTION

Portal hypertension (PHT) is a life threatening complication of liver cirrhosis. Elevated portal venous pressure increases morbidity and mortality by promoting the formation and potential hemorrhage of gastric and esophageal varices[1,2]. PHT typically originates from underlying hepatic disease and is exacerbated by systemic and splanchnic vascular deregulation[3]. Hepatic injury stimulates liver stellate cell differentiation to adopt a smooth muscle cell phenotype resulting in sinusoidal contraction, increased sinusoidal perfusion resistance and increased portal pressure[4]. In addition, dilation of systemic and splanchnic vessels causes a hyperdynamic circulatory dysfunction characterized by increased cardiac output and hyperemia[5-8]. Consequently, although the underlying etiology of PHT is usually hepatic, clinical manifestations and intervention pertain more to vascular and cardiac control[9].

Vascular dilators and constrictors play a significant role in controlling blood flow and pressure *via* modulation of vascular resistance to flow[10-13]. Increased vascular resistance reduces flow and increases pressure whereas decreased resistance increases flow and lowers pressure. In patients with PHT reduced vascular resistance increases blood supply to the portal system, which is congested because of increased hepatic resistance, and increases portal pressure. Consequently, reducing cardiac output and increasing vascular resistance has the potential to reduce portal pressure and lower the risk of esophageal variceal hemorrhage. Primarily prophylaxis using β-blockers (*e.g.*, propranolol) to prevent/treat variceal bleeding in cirrhotic patients by the reduction of heart rate by 25% is the mainstay of treatment but does not work for all[14-16]. Moreover, the use of vasoconstrictors such as octreotide demonstrate the potential for vasoconstrictors despite limited reductions in bleeding and no improvement in overall outcomes when compared to direct endoscopic treatments to the varices (banding and sclerotherapy)[17,18]. The introduction of long-acting octreotide analogs may provide better results although care should be taken to avoid renal complications caused by renal hypertension[19,20].

Therefore, to date, options for patients with PHT, whom are at risk of variceal formation and hemorrhage, are limited. One reason for this deficiency in treatment options is due to our lack of knowledge relating to vascular aberrancies concomitant with PHT. Previous studies have demonstrated that PHT is associated with a significant diminution of reactivity to specific vasoconstrictors[21]. Endothelin-1 (ET1), a potent vasoconstrictor, is associated with vascular resistance and blood pressure[22]. Previous studies indicate that synthesis of the potent vasoconstrictor ET1 is increased contemporaneously with PHT[23,24]. Plasma ET1 concentrations are three times higher in patients with cirrhosis than in healthy controls[25]. Subsequent studies showed that despite increased ET1 levels vessels from PHT animals exhibited a markedly reduced contractile response to exogenous ET1[26,27]. In cirrhotic rats mesenteric arterial response to endothelin-1 is markedly reduced concomitant with a 5-fold reduction in ET1 cellular signaling[28]. This reduction in vasoconstrictor response has been linked to elevations in expression of vasodilators prostacyclin (PGI_2) and nitric oxide (NO) which counter ET1 induced vasoconstriction[29-32] because disruption of NO or PGI_2 biosynthesis reduces portal pressure in experimental models of PHT[33-35]. Suggesting that inhibition of vasodilators NO or PGI2 would also ameliorate ET-1 vasoconstriction in PHT animals.

Therein this manuscript examines the importance and etiology of NO biosynthesis in impaired ET1 vasoconstriction associated with PHT. This initial study utilizes the pre-hepatic murine portal vein ligation (PVL) model because PHT develops rapidly and vascular aberrancies have been reported in this model[30]. At present we do not fully know the importance and etiology of ET1 hypo-response in PHT. Previous studies have suggested that NO plays a significant role in ET1 hypo response[36,37]. This is based on data

that shows ET1 hypo-response is not observed when NO synthesis is inhibited[38-41] and the connection between ET1 signaling, *via* endothelin receptor A (ETA) and endothelin receptor B (ETB) receptors, and vasodilatory compounds. (Figure 1)[42] Vascular smooth muscle cell ETA and ETB promotes vasoconstriction *via* phospholipase C, phosphoinositide metabolism and increased Ca^{2+}[43]. Whereas, endothelial cell ETB are functionally coupled with NO and PGI2 biosynthesis and promote vasodilation[44-48]. Therefore, increased levels of NO biosynthesis *via* nitric oxide synthase isoforms could explain ET1 hypo-contractile response. In experimental animal studies ETB antagonism prevents hyperemia and PHT following portal vein ligation[49]. However, testing the role of ETA and ETB in PHT murine models is hindered because, homozygous ETA or ETB receptor gene knock out results in lethal developmental phenotypes in the mouse[50]. In contrast, $eNOS^{-/-}$ and $iNOS^{-/-}$ mice are viable and have previously been used to better understand the pathophysiology of PHT[34,35].

To examine the relationship between eNOS and ET-1 hypo-response this manuscript examines the development of ET1 hypo response and PHT in a murine PVL model of pre-hepatic PHT using $eNOS^{-/-}$ mice. If the hypothesis that eNOS is important to the development of ET1 hyper-response is correct then *eNOS* gene deletion will prevent aberrant ET1 function following PVL. In contrast, we observed that *eNOS* gene deletion enhanced ET1 contraction in sham mice and did not prevent ET1 hypo-response following PVL. In addition we found that aberrant ET1 contractility is not central to the development of PHT in the PVL model and that eNOS mediated hyperemia is key. This data does not negate the role and importance of ET1 in portal hypertension. Normalized ET1 contractility, potentially, would reduce portal venous flow, pressure and bleeding. Our data suggests that targeting of NO biosynthesis would not mediate this affect and alternate targeting and study is required.

MATERIALS AND METHODS

Pre-hepatic PHT model: Partial portal vein ligation

All studies were approved by the Indiana University committee for animal research and adhered to AAALC and federal guidelines for the humane care and treatment of animals. Mice were maintained in sterilized isolette cages on a 12-h light/dark cycle and were allowed access to food and water *ad libitum*. Mice were anesthetized using halothane inhalation. A midline laparotomy was performed and the portal vein was exposed. A blunt-ended 27-gauge needle was placed alongside the portal vein and a 4-0 silk suture was tied around the vein and needle, after which the needle was withdrawn, producing a standardized stenosis. In sham animals the procedure consisted of dissection and visual inspection of the portal vein without ligature. The abdomen was closed and the animals were allowed to recover under a heat lamp.

Physiological measurements

Physiological measurements were performed as previously described by Theodorakis *et al*[34] 2003. At the indicated times post sham-operation or PVL, animals were anesthetized and subjected to laparotomy to allow physiological measurements to be taken. Portal pressure was determined by measuring the splenic pulp pressure (SPP). We have previously shown that portal venous pressure and splenic pulp pressure are directly proportional[34]. To measure SPP, a microtip pressure transducer (ADI, CO) was inserted into the spleen pulp. Abdominal aortic flow (Qao) was measured by placing an ultrasonic Doppler flow probe (Transonic #11RB) around the abdominal aorta between the diaphragm and celiac artery. Flow rates were obtained with a Transonic T206 Blood Flow Meter (Transonic Instruments, NY) and recorded using ADI Chart 5 software. Aortic blood flows were standardized per gram of body weight. Fluorescent microspheres were used to assess the degree of portosystemic shunting as described previously[34]. 0-7 d after sham operation or PVL, mice were anesthetized and a laparotomy was performed as described earlier. Approximately 15 x 10^6 μm red polystyrene fluorescent microspheres (Molecular Probes, Eugene, OR) were injected into the spleen (red spheres). The liver and lungs were collected and placed in 20 mL of 2% sodium dodecyl sulfate, 0.1 mol/L EDTA, 10 mmol/L Tris, pH 8.0, and the tissue was homogenized. Proteinase K was added to 0.1 mg/mL, and the proteins were digested overnight at 45 ℃. Microspheres were collected by centrifugation at 1000 × *g*, washed in 0.2% Tween-80, centrifuged again, and re-suspended in 0.1 mL of 0.2% Tween-80. Microspheres were counted using a hemocytometer and a Nikon TE300 inverted microscope equipped for epifluorescence. The degree of shunting was calculated as the percentage of microspheres in the lungs compared to lung and liver combined.

Gene-deficient mice

Mice containing targeted mutations in the *nos2* gene (iNOS; strain B6,129P-*Nos2*^{tm1Lau},and the *nos3* gene (eNOS, strain C57BL/6J-*Nos3*^{tm1Unc}, were purchased from The Jackson Laboratory, ME. Age-matched mice from congeneic strains (B6, 129P or C57BL/6J) were used as wild type controls. Mice genotypes were confirmed by PCR on DNA isolated from tail samples using Qiagen Dneasy kit (Qiagen Inc, Stamford, CA), as per manufactures instructions. Gene-specific primers: nos3: 5'gtgtgaaggcaaccattctg 3'actcatccatgcacaggacc and nos2: 5'ggcttcacgggtcagagcca 3'tgcccattgctgggacagtc (cycle = 1 min each of 94 ℃, 60 ℃ and 74 ℃ x 25) are complementary to the site specific mutations previously published by Shesely *et al*[51] (1996) and Laubach *et al*[52] (1995).

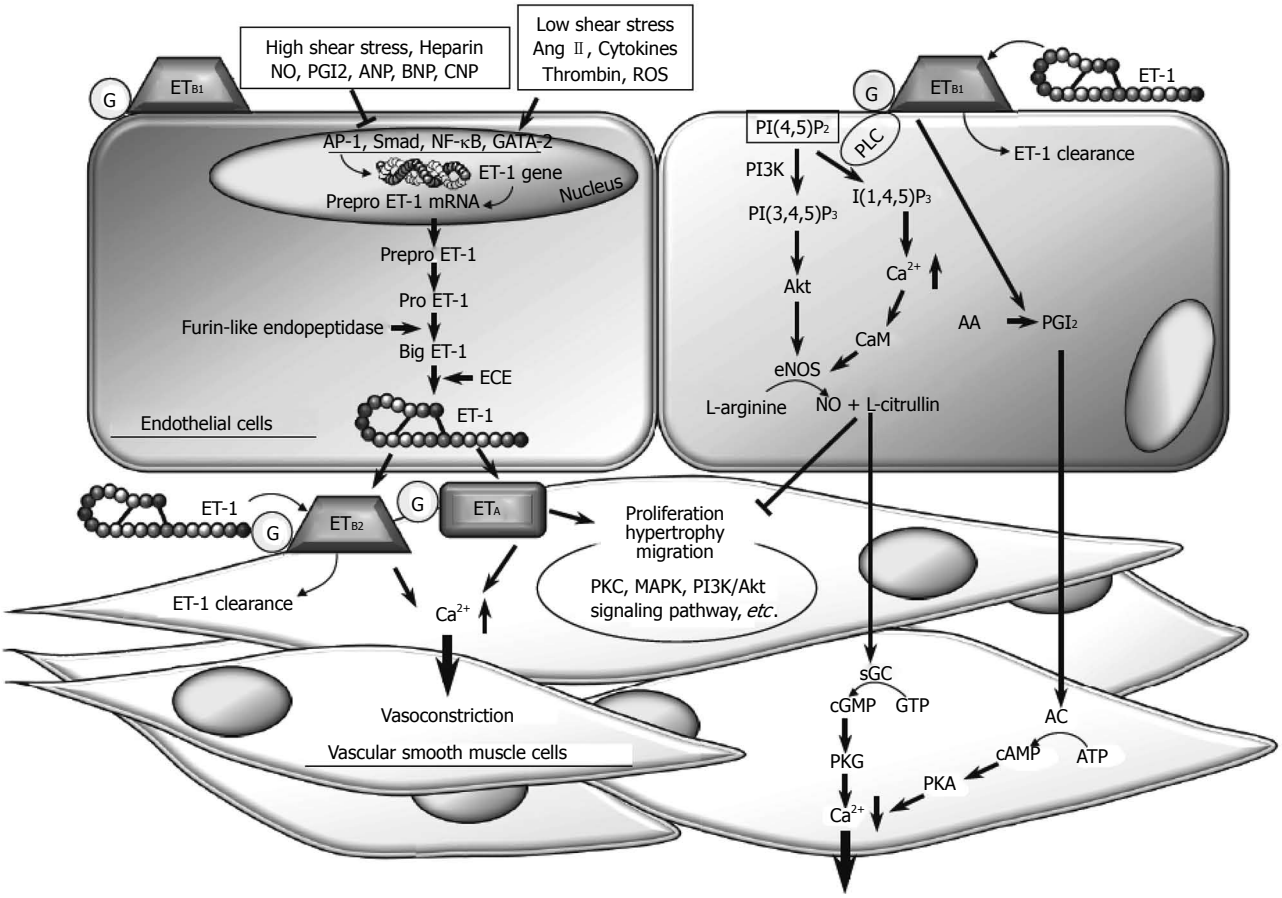


Figure 1 Illustration of interrelationship between Endothelin-1 induced vasoconstriction and nitric oxide mediated vasodilation. Endothelin-1, PGI2 and NO are closely related in relation to vascular smooth muscle cell tone. (Reprinted with permission Ohkita *et al*[42] 2002).

Endothelin-1 function

***In-vivo*:** Seven days Sham and PVL mice were anesthetized using halothane inhalation. A midline laparotomy was performed and the abdominal aorta was exposed. A Doppler flow probe was placed on the aorta between the diaphragm and celiac artery. Flow rates were allowed to stabilize and were monitored while the femoral vein was cannulated. Mice were discarded if Qao decreased by more than 20% during the cannulation. 10 μg/L (4 nmol/L) ET1 bolus (50 μL) was injected in to the femoral vein and the abdominal aortic flow was constantly recorded. The dose of ET1 (5 pmol/kg) was similar to that used in human studies[53]. Except ET1 was given as a bolus rather than *via* a peristaltic pump. Preliminary studies showed that 1hr following 5 pmol/kg ET1 injection plasma nitrite/nitrate (NOx) was increased 41%. Moreover, 4 nmol/L is significantly greater than normal murine plasma ET1 levels (1 pmol/L)[54]. This high dose will equalize out any differences in endogenous ET1 levels between PVL and sham mice.

***Ex-vivo*:** Seven days Sham and PVL mice were anesthetized using halothane inhalation. The abdominal aorta was carefully dissected and placed in oxygenated Krebs-Ringer solution (119 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl, 1.17 mmol/L $MgSO_4$, 25 mmol/L $NaHCO_3$, 1.18 mmol/L KH_2PO_4, 27 μmol/L EDTA, 5.5 mmol/L glucose). ET1 contractility was measured on a small vessel myograph (610 M, ADI, CO) as per manufacturer's instructions. This instrument provides a temperature controlled oxygenated environment to measure vessel contractility/dilation. Vessels are attached to two wires (0.2 μm diameter). One wire is an anchor while the other is attached to a strain gauge. Segments were incrementally pre-tensioned by separating the two wires to 100 mmHg, as previously described and according to manufacturer's detailed instructions[55]. Vessel contractility was measured in response to 10^{-8}-10^{-4} mol/L ET1. Four vessel segments were analyzed per mouse, five mice per group.

Statistical analysis

The data shown are mean ± SE, with 5-7 animals per experimental group. Statistical significance was estimated using one-way ANOVA statistical analysis. A value of $P < 0.05$ was considered significant.

RESULTS

Plasma NOx

Plasma NOx and ET-1 were determined using commercially available assays (Oxford Biomedical, Oxford, MI) (R&D systems, Minneapolis, MN). Plasma

NOx and ET-1 were not significantly different between unadulterated C57B/6J (wild type), iNOS$^{-/-}$ and eNOS$^{-/-}$ mice. This is similar to previous studies[34,56]. Plasma NOx was increased 1 d following PVL in wild type (41%, P = 0.03) and iNOS$^{-/-}$ (35%, P = 0.02) but was not altered in eNOS$^{-/-}$ mice (11.7 ± 0.9 *vs* 9.9 ± 0.6 μmol/L, 1 d sham and 1 d PVL respectively, P = 0.31). Similarly serum ET-1 increased 1 d following PVL (1 ± 0.7 pg/mL *vs* 12 ± 3 pg/mL 1 d sham and PVL respectively, P = 0.02). After which levels returned to normal (2 ± 1.1 P = 0.23) and were not different to shams. In a similar manner serum ET-1 was increased 9 and 10 fold in eNOS$^{-/-}$ and iNOS$^{-/-}$ mice respectively 1 d following PVL.

Hemodynamics following PVL

The development of PHT was evaluated by recording (1) splenic pulp pressure; (2) aortic flow; and (3) portal systemic shunting as indices of portal pressure, hyperdynamic and collateral circulation respectively.

Splenic pulp pressure was quantified by placing a micro tip pressure transducer into the spleen: The spleen/body weight ratio was significantly increased in all wild type, iNOS, and eNOS mice 7 d following PVL (85%, 90% and 111% respectively). In wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ mice splenic pulp pressure was increased immediately following ligation of the portal vein. In wild type and iNOS$^{-/-}$ mice splenic pulp pressure increased from 7.5 ± 1.1 mmHg and 7.2 ± 1 mmHg to 25.4 ± 3.1 mmHg and 22 ± 4 mmHg respectively (Figure 2A and B). In eNOS$^{-/-}$ mice splenic pulp pressure was increased after 1 d (P = NS), after which it decreased and by 7 d was not significantly elevated when compared to 7 d sham operated controls (6.9 ± 0.6 mmHg and 7.3 ± 0.8 mmHg respectively, P = 0.3) (Figure 2C). This is similar to data reported previously[34].

Abdominal aortic flow (Qao) was measured by placing a doppler flow probe around the aorta between the diaphragm and celiac artery: Heart rate was not significantly altered by PVL in any mouse groups (data not shown). Immediately after portal vein ligation Qao reduced rapidly (0.15 ± 0.02 mL/min per gram *vs* 0.12 ± 0.01 mL/min per gram BW 1 d wild type sham and PVL respectively), decreasing by 20%, 22% and 30% in wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ mice respectively. Two days following PVL Qao had recovered and was greater in PVL mice when compared to shams in wild type and iNOS$^{-/-}$ mice (0.17 ± 0.02 mL/min per gram and 0.18 ± 0.04 mL/min per gram BW). By 7 d Qao had increased by 80% and 73% in wild type and iNOS (Figure 2D and E), whereas there was no significant difference in eNOS$^{-/-}$ mice between 7 d sham and 7 d PVL (Figure 2F).

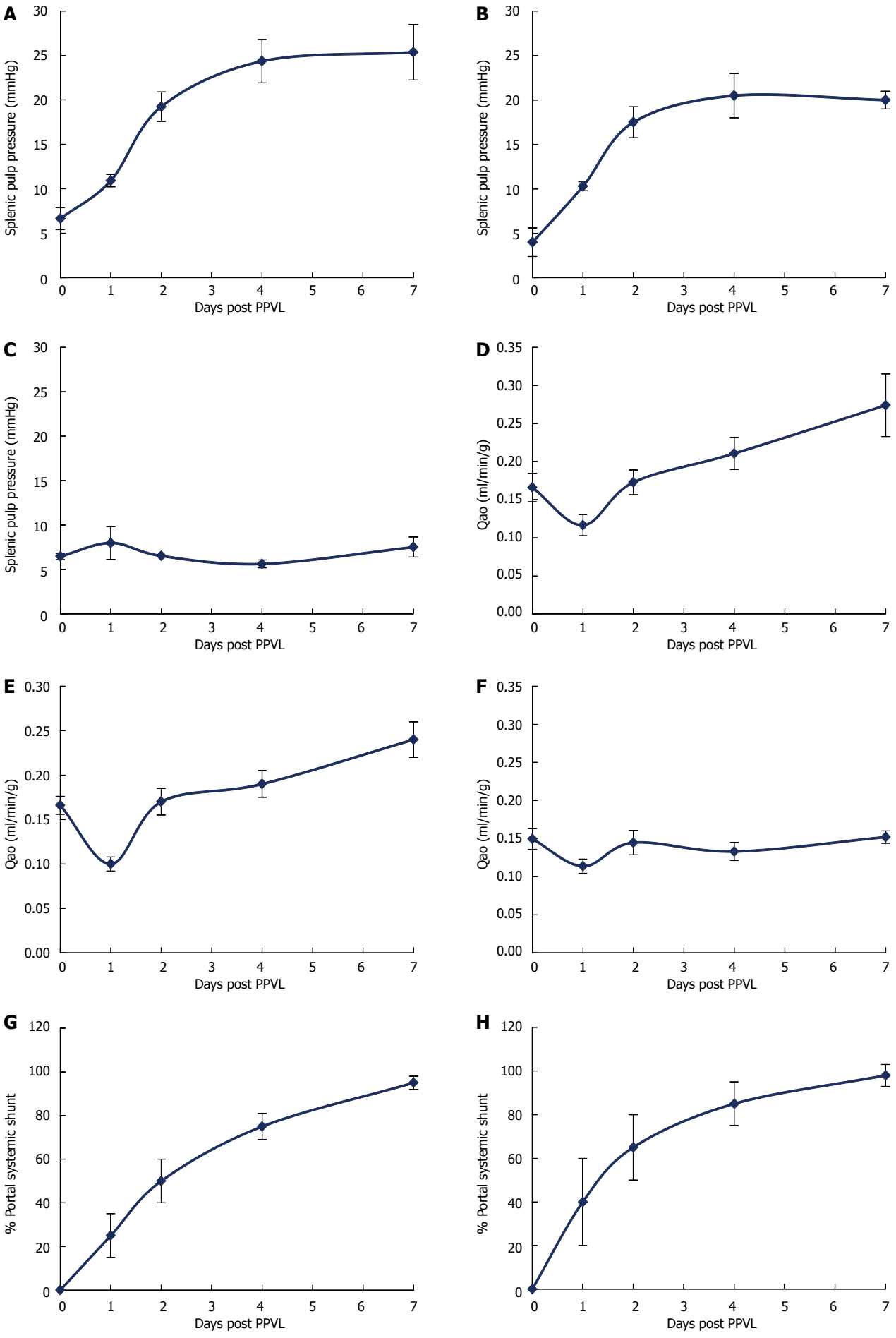
Portal systemic shunting was determined by injecting fluorescent microspheres in to the splenic vein *via* the spleen and monitoring the distribution of spheres between the lung and liver: In sham mice spheres were exclusively found in the liver, indicating normal circulation and no shunting. Following PVL sphere location changed from predominantly hepatic to predominantly pulmonary, indicative of collateral circulation. The rate of collateral circulation development was steady in wild type and iNOS$^{-/-}$ mice. (Figure 2G-H) However, in eNOS$^{-/-}$ mice the rate of collateralization was significantly slower, (Figure 2I) suggesting that acute collateralization is eNOS dependent but that alternate mechanisms are also involved.

ET1 induced aortic contractility

To better understand the interrelationship between ET1 and NO we initially performed a dose response experiment to optimize ET1 induced NO synthesis. This would confirm ETB binding and investigates vessel ET1 pressor response in the face of both constrictive and dilatory mechanisms. *In-vivo* ET1 dose was determined by monitoring plasma NOx levels following exogenous 50 μL bolus (0-20 μg/L) ET1 IV injection. Plasma NOx was increased 1hr following 2.5 (23%, P = 0.1) and 5 pmol/kg (41%, P = 0.04) ET1 IV injection. In contrast, IV injection of 10 pmol/kg ET1 reduced NOx 55% (P < 0.01). 5 pmol/kg ET1 was subsequently used for *in-vivo* experiments. Aortic response to ET1 was determined by (1) *in-vivo* monitoring of aortic blood flow; and (2) *ex-vivo* monitoring of isolated aorta segment contractility.

Aortic *in-vivo* response to ET1 was determined by monitoring abdominal aortic blood flow following IV injection of 50 μL 10 μg/L ET1 *via* the femoral vein: Bolus IV injection of 50 μL 10 μg/L (4.8 pmol/kg) ET1 to wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ mice induced a rapid reduction in abdominal aortic blood flow (50% ± 8%, 73% ± 9% and 47% ± 9% respectively). The ET1 induced reduction in flow was significantly greater in eNOS$^{-/-}$ mice compared to both wild types and iNOS$^{-/-}$ mice (P = 0.02) (Figure 3A). 7 d following PVL ET1 induced reduction in blood flow was significantly diminished in each mouse group. Abdominal aortic flow was reduced by 19% ± 9%, 32% ± 10% and 9% ± 9% in wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ mice respectively (Figure 3B). Aberrant ET1 function was significantly greater in iNOS$^{-/-}$ mice (81%) when compared to wild type (62%) or eNOS$^{-/-}$ (67%). The dose of ET1 is non-physiological and was used because it elicited a detectable increase in plasma NOx indicative of ET-B activation. Moreover, the dose given is equivalent to that used in human studies (4.8 pmol/kg)[53]. No change in heart rate was observed following ET1 injection.

Vessel *ex-vivo* response to ET1 was determined in isolated abdominal aortic segments attached


A
Splenic pulp pressure (mmHg)
Days post PPVL
B
Splenic pulp pressure (mmHg)
Days post PPVL
C
Splenic pulp pressure (mmHg)
Days post PPVL
D
Qao (ml/min/g)
Days post PPVL
E
Qao (ml/min/g)
Days post PPVL
F
Qao (ml/min/g)
Days post PPVL
G
% Portal systemic shunt
Days post PPVL
H
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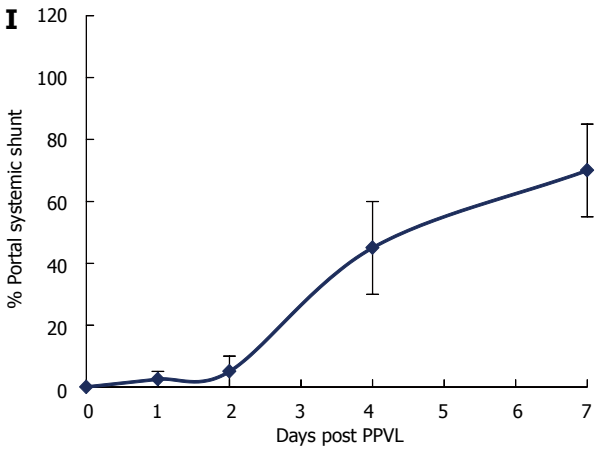

Figure 2 Partial portal vein ligation induces chronic hyperemia and persistent portal hypertension in wild type and iNOS$^{-/-}$ mice but not eNOS$^{-/-}$ mice. Wild type (A, D, G), iNOS$^{-/-}$ (B, E, H) and eNOS$^{-/-}$ (C, F, I) mice were subjected to partial portal vein ligation surgery. 0-7 d thereafter-splenic pulp pressure (A-C), aortic blood flow (D-F) and portal systemic shunting (G-I) were determined. A-C: Splenic pulp pressure was increased acutely in all mouse groups following ligation (0-1 d). After which pressure was increased further in wild type and iNOS$^{-/-}$ but not in eNOS$^{-/-}$ mice; D, E: Aortic flow was significantly reduced in wild type, iNOS$^{-/-}$ and eNOS$^{-/-}$ mice (0-1 d). In wild type and iNOS$^{-/-}$ mice this low blood flow converted to hyperemia and increased steadily. In eNOS mice flow returned to pre-surgical baseline and was not increased; G-I: Portal systemic shunting increased steadily in wild type and iNOS$^{-/-}$ mice (G, H). There was a significant delay in the development of collateral circulation in eNOS$^{-/-}$ mice (I).

to a force transducer within an isolated tissue bath: Four segments per mouse and 5 mice per group were assayed. Vessels were pre- tensioned to 100 mmHg and equilibrated for 20 min. Vessels were exposed to logarithmic increases in ET1 (10^{-8}-10^{-4} mol/L). Maximal vessel contractility was recorded. Wild type, eNOS$^{-/-}$ and iNOS$^{-/-}$ mouse aortic segments increased tension steadily in response to ET1 (Figure 3C). There was no significant difference between the ET1 aortic contractile response of wild type, eNOS and iNOS mice at doses between 10^{-8} and 10^{-5} mol/L. However, at increased doses aortic vessels from unadulterated eNOS$^{-/-}$ mice contracted significantly greater (43%, P = 0.03) then wild-type controls. 7 d following PVL contraction to 10^{-4} mol/L ET1 was reduced from 4.2 ± 0.32 to 1.7 ± 0.7 mmol/L in wild type mice, 6 ± 0.6 to 1.8 ± 0.1 mmol/L in eNOS$^{-/-}$ mice and 4.9 ± 0.7 to 1.5 ± 0.2 mmol/L in iNOS mice$^{-/-}$ (Figure 3D).

DISCUSSION

The study described in this manuscript focuses on the role of eNOS in ET-1 hypo response associated with PHT. Previous reports introduced the hypothesis that ET1 hypo-response was linked to NO and/or hyperemia[40,49,57]. This study tests this hypothesis and the potential of targeting NO biosynthesis to reduce portal pressure, variceal formation and hemorrhage. This is important because eNOS and NO are known to be important to PHT and are the basis for intervention in numerous studies[11,58,59]. To investigate the role of eNOS in PHT we hypothesized that eNOS is important to the development of ET1 hypo response and that eNOS gene deletion would prevent aberrant ET1 function in murine models. The hypothesis was challenged using the well-established pre-hepatic partial PVL of PHT and targeted eNOS and iNOS gene deleted mice. The PVL model was used because reduced extra-hepatic arterial ET1 contractile response is known to develop rapidly in this model in the absence of the milieu of inflammatory and cytokine changes associated with the carbon tetrachloride or bile duct ligation models of intra-hepatic PHT[60]. This allows us to focus on the ET1 vasculopathy in isolation from hepatic pathology. iNOS$^{-/-}$ mice were included as an isoform and gene deletion control. In both the PVL and CCl_4 models of PHT iNOS$^{-/-}$ mice develop PHT similar to wild type controls, including increased plasma NOx, hyperemia, and increased splenic pulp pressure. In contrast, eNOS$^{-/-}$ mice didn't develop hyperemia or PHT 7-14 d following PVL.

In this study we found that eNOS gene deletion increased ET1 contractility. This increase in ET1 contraction in sham eNOS$^{-/-}$ mice is probably due to absent eNOS mediated NO biosynthesis and dilation to counteract ET1 contraction *via* ETA receptor activation. However, contrary to our hypothesis we observed ET1 hypo response in eNOS$^{-/-}$ mice following PVL, suggesting that ET1 hypo-response in murine models of PHT it is a parallel occurrence rather than a pivotal component of PHT and has no distinguishable role in hyper-dynamic associated hyperemia. Therefore, our hypothesis that *eNOS* gene deletion would prevent aberrant ET1 function was false. At this point we suggest that alternative explanations for the development of ET1 hypo-response in eNOS$^{-/-}$ mice include: (1) reduced blood flow, observed immediately following PVL (0-1 d), may increase ET1 expression and modify ET1 response. Previous studies have shown increased ET1 expression following occlusion of portosystemic shunts in cirrhotic patient[61]; (2)

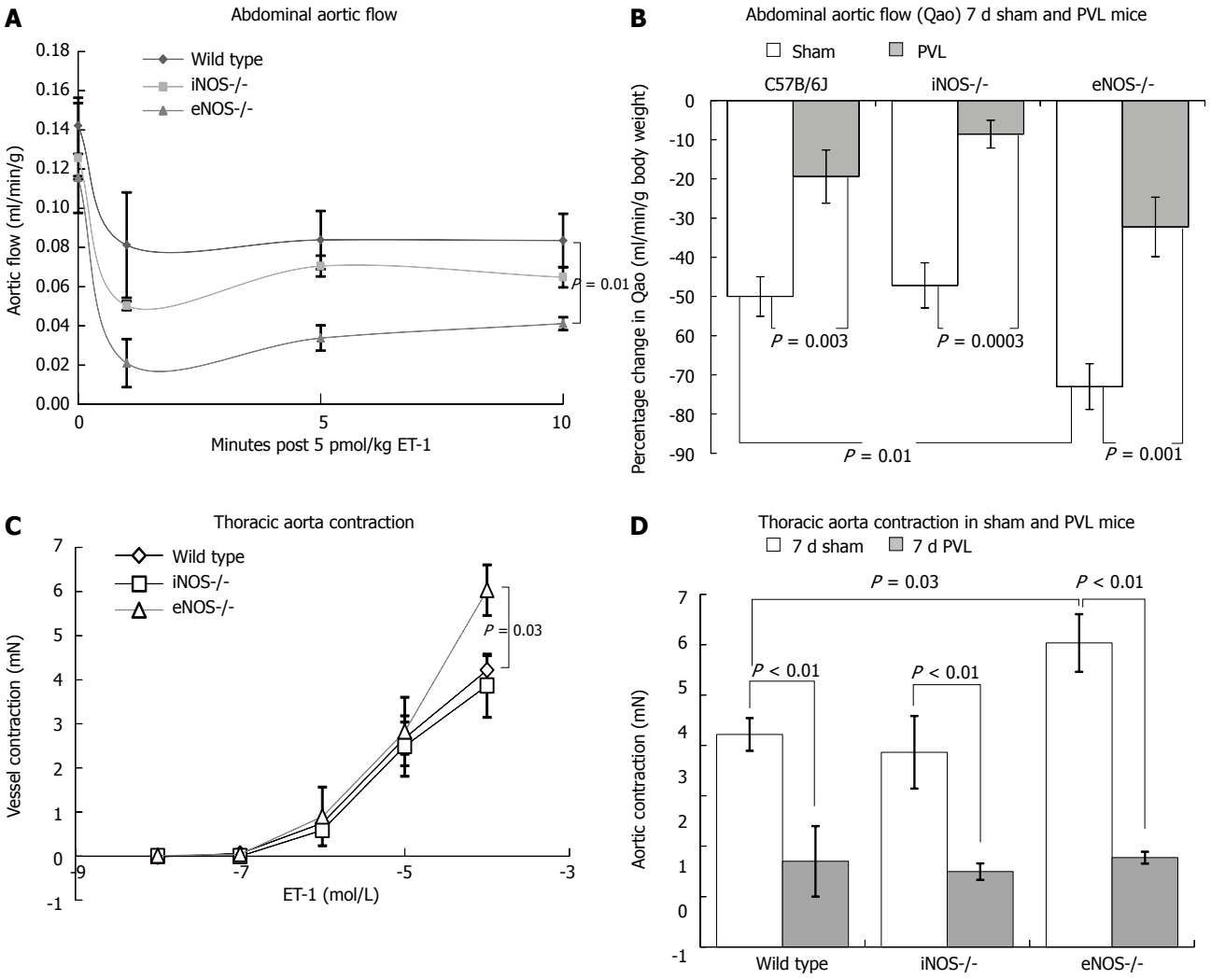

Figure 3 Endothelin-1 hypo-response develops in wild type, iNOS$^{-/-}$ and eNOS$^{-/-}$ mice following portal vein ligation. A: Aortic blood flow was monitored in unadulterated wild type (squares), iNOS$^{-/-}$ (diamonds) and eNOS$^{-/-}$ (triangle) mice prior to and following IV administration of 5 pmol/kg endothelin-1 (ET1). ET1 induced a rapid vessel contraction and subsequent reduction in flow. Response to ET1 was significantly greater in eNOS$^{-/-}$ when compared to wild type controls; B: Wild type, iNOS$^{-/-}$ and eNOS$^{-/-}$ mice were subjected to sham (open bars) or portal vein ligation surgery (PVL) (shaded bars). After 7 d changes in aortic flow was recorded following IV administration of 5 pmol/kg ET1. In all mouse groups the response to ET1 was markedly reduced following PVL (iNOS > wild type > eNOS$^{-/-}$); C: ET1 induced contraction of isolated aortic segments from unadulterated wild type (triangle) iNOS$^{-/-}$ (square) and eNOS$^{-/-}$ (triangle) mice were determined using an ADI 610M small animal myograph. Aortic vessel segments contracted to exogenous ET1. At high ET1 dose (10^{-4} mol/L) aortic vessel segments from eNOS$^{-/-}$ mice contracted significantly greater then segments from wild type controls; D: Wild type, iNOS$^{-/-}$ and eNOS$^{-/-}$ mice were subjected to sham (open bars) or portal vein ligation surgery (shaded bars). After 7 d the aorta was carefully dissected and ET1 contractility was measured. *Ex-vivo* aorta ET1 (10^{-4} mol/L) contractility was significantly decreased in vessels from 7 d wild type, iNOS$^{-/-}$ and eNOS$^{-/-}$ PVL mice when compared to shams (PVL *vs* sham, $P < 0.01$).

rapid engorgement of the portal vein by PVL activates stretch receptors in endothelial cells leading to signaling and phenotypic changes[62]; (3) formation of free radical and oxidative damage. Cell stretching, low blood flow and ET are all linked to the formation of oxygen free radicals[63-65]. Free radicals are linked to up-regulation of NO, which acts as a superoxide radical scavenger, and increases of splanchnic blood flow[66-68]. This would explain why ET1 hypo response was greater in iNOS$^{-/-}$ mice following PVL as iNOS has frequently been linked with NADPH oxidase[69]; (4) alternate NOS isoforms may play a key role. Although, normally associated with neurons NOS1 or neural NOS is increased in the mesenteric artery of PVL rats[70]. Addition studies are required to better understand the etiology of aberrant ET1 response in PHT; and (5) finally, as with all experimental models, especially using gene-modified mice, data should be used with caution. Firstly, murine models are not 100% comparable to human disease. This is especially true of the PVL model, which does not mimic the underlying causation of the majority of patients with PHT. PVL is a model of pre-hepatic PHT, which is usually caused by thrombus, or malignancies encroaching upon the portal vein. However, the PVL is a "clean" model in that it doesn't include hepatic pathology and allows for investigation of PHT vasculopathy in the absence of a pro-inflammatory background. Secondly, alternate mechanisms can compensate for gene deletion. eNOS and iNOS null mice may manifest adaptive effects

such that they may not produce outcomes as a direct consequence of a lack of eNOS or iNOS function. An alternative to using eNOS$^{-/-}$ mice would be to use the reported selective eNOS inhibitor cavtratin, a caveolin-1 derived peptide, developed within the Sessa laboratory[71,72]. Although, cavtratin is reported to inhibit eNOS with little effect on iNOS a question has been raised regarding its solubility and applicability because of its size (3 kDa)[73]. Finally, we measured ET1 response in abdominal aortic vessels rather than in mesenteric vessels used in other studies[30]. This was because of size restrictions. We were unable to isolate responsive mesenteric vessels from either sham of PVL mice. However, are able to demonstrate ET1 hypo-responsive in the abdominal aorta of PVL mice when compared to shams. Demonstrating that in PVL mice the abdominal aorta reaction to ET1 is reduced in a similar manner to mesenteric vessels of PVL rats in contrast to increased reaction to ET1 observed in the thoracic aorta of PVL rats[30,74].

Ultimately, the data presented in this manuscript suggests that targeting eNOS would not abrogate ET1 hypo-response even though previous studies have suggested a link between ET1 and eNOS *via* ETB. This does not refute the positive results seen with ET1 receptor antagonists. Correcting ET1 function has a significant role in the treatment of PHT and prevention of variceal formation and hemorrhage. Selective and non-selective ET-A and ET-B antagonists have significant potential in the treatment of various pathophysiological components of PHT[53]. However questions remain whether such antagonists should be used clinically to treat PHT because of differences between hepatic and extra hepatic outcomes. ET1 response is increased in the liver but is decreased in the systemic vasculature. Moreover, ET-B receptors on vascular smooth muscle can contribute to vasoconstriction in some circumstances and/or locations. Consequently, alternate targets are required that focus on the etiology of ET1 hypo response and receptor downstream changes. However, correcting vascular dysfunction following prolonged inflammatory liver disease might be more complicated than removing the etiological trigger. Wang *et al*[75] (2004) argue that in patients with chronic portal vein hypertension the vascular wall changes, due to the long-term dilation, and recovery will be hard even if the effect of vasodilatation is completely eliminated. More recently, Resch *et al*[76] have described mesenteric arterial remodeling, leading to decreased vessel stiffness, in the CCl_4 model of PHT. In contrast, they found no evidence of vascular remodeling in the rat PVL model of PHT, suggesting that irreversible changes are more likely a response to an inflammatory milieu and not as a consequence of mechanical changes (PVL) or increased NO biosynthesis. Because ET1 hypo response developed in the absence of an inflammatory response and was distant from mechanical/hemodynamic (stretch and low flow in the portal vein) changes it is probably in response to paracrine signaling. Further studies are required to better understand this paracrine signaling.

In conclusion, ET1 dysfunction occurs in the absence of increased NO, chronic liver disease, hyperemia or vascular remodeling and is eNOS and iNOS independent. Moreover, in the PVL model ET1 hypo-response is not sufficient, on its own, to induce a hyper-dynamic circulation or an increase in portal pressure. However, improved ET1 contractility may improve clinical options and thus decrease mortality and morbidity. Additional studies are required to determine the etiology, role and correction of ET1 hypo-response in PHT.

COMMENTS

Background

Portal hypertension is a significant complication of liver disease and can increase morbidity and mortality. Increased hepatic resistance in portal venous flow in combination with elevated portal venous flow raises portal venous pressure and promotes the vascular aberrancies and hemorrhage. Attempts to reduce portal pressure by increasing vascular resistance using vasoconstrictors are hindered by the development of a vascular hypo-response to vasoconstrictors, such as endothelin-1 (ET1). This hypo-response has been linked to increased levels of the vasodilator nitric oxide.

Research frontiers

Current beta-blocker treatment of patients with portal hypertension is problematic. Some patients do not respond and systemic blood pressure is not lowered. Others develop complications and have to terminate treatment. Consequently, alternative approaches are being sort. Amelioration of vascular response to vasoconstrictors in patients with portal hypertension would significantly improve treatment, morbidity and mortality.

Innovations and breakthroughs

By using targeted gene deletion mice this study advances our cognizable knowledge of portal hypertension. Previous studies have suggested that hypo-response to vasoconstrictors is related to an increase in the biosynthesis of the vasodilator nitric oxide. Arguing that inhibition of nitric oxide synthase will ameliorate vascular response to vasoconstrictors. In contrary to this hypothesis we demonstrate that the development of a hypo-response to vasoconstrictors is not due to over production of the vasodilator nitric oxide. Targeted gene deletion of the two main nitric oxide synthase enzymes did not ameliorate vasoconstrictor hypo-response. This information directs future study to look at alternate pathways and mechanisms other than nitric oxide.

Applications

This study guides future investigations aimed towards the development of new treatment options for patients with portal hypertension and are at risk of variceal formation/hemorrhage. By demonstrating that ET1 hypo-response is independent of NOS isoforms alternate approaches can be researched.

Terminology

Vasoconstrictor hypo-response is a condition where vascular tissues have a reduced or absent constrictive response to vasoconstrictors. The use of vasoconstrictors to increase vascular resistance and reduce flow is impaired in patients with portal hypertension. Inter alia, because of ET1 hypo-response.

Peer-review

In this study, the authors examined the importance and etiology of impaired ET1 vasoconstriction in portal hypertension. They used portal vein ligation a prehepatic model of portal hypertension that lacks the milieu of inflammatory and cytokine changes associated with the CCl_4 or bile duct ligation models of intra-hepatic portal hypertension. Although this prehepatic model of portal hypertension somewhat simplifies the complexity of involved pathways, however, it is radically different from the more clinically relevant model of CCl_4 model.

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ORIGINAL ARTICLE

Basic Study

Toxoplasma gondii causes death and plastic alteration in the jejunal myenteric plexus

Eduardo José de Almeida Araújo, Larissa Marchi Zaniolo, Suellen Laís Vicentino, Marcelo Biondaro Góis, Jacqueline Nelisis Zanoni, Aristeu Vieira da Silva, Débora de Mello Gonçales Sant'Ana

Eduardo José de Almeida Araújo, Departamento de Histologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, 86057-970 Paraná, Brazil
Larissa Marchi Zaniolo, Programa de Pós-graduação em Ciência Animal, Universidade Paranaense, 87502-210 Paraná, Brazil
Suellen Laís Vicentino, Marcelo Biondaro Góis, Jaqueline Nelisis Zanoni, Débora de Mello Gonçales Sant'Ana, Departamento de Ciências Morfológicas. Universidade Estadual de Maringa, 87020-900 Paraná, Brazil
Aristeu Vieira da Silva, Departamento de Biologia, Universidade Estadual de Feira de Santana, 44036-900 Bahia, Brazil
Author contributions: Araújo EJA, Zaniolo LM, Vicentino SL, Góis MB, Zanoni JN, da Silva AV and Sant'Ana DMG contributed equally to this work; Araújo EJA, Zanoni JN, da Silva AV and Sant'Ana DMG designed the research; Zaniolo LM, Vicentino SL and Góis MB performed the research and analyzed the data; Araújo EJA, Sant'Ana DMG and Góis MB wrote and reviewed the paper.
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Conflict-of-interest: Eduardo José de Almeida Araújo is an employee of State University of Londrina, Brazil. Jacqueline Nelisis Zanoni and Débora de Mello Gonçales Sant'Ana are an employee of State University of Maringá, Brazil. Aristeu Vieira da Silva is an employee of State University of Feira de Santana, Brazil. Eduardo José de Almeida Araújo has received research funding from Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil; Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Estado do Paraná, Brazil. Jacqueline Nelisis Zanoni has received research funding from Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil; Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Estado do Paraná, Brazil. Aristeu Vieira da Silva has received research funding from Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil; Fundação de Amparo à Pesquisa do Estado da Bahia. Débora de Mello Gonçales Sant'Ana has received research funding from Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil; Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Estado do Paraná, Brazil.
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Correspondence to: Dr. Eduardo José de Almeida Araújo, Departamento de Histologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, PO 10.011, 86057-970 Paraná, Brazil. eduardoaraujo@uel.br
Telephone: +55-433-3714327
Fax: +55-433-3715828
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Abstract

AIM: To assess the effects of ME-49 *Toxoplasma gondii* (*T. gondii*) strain infection on the myenteric plexus and external muscle of the jejunum in rats.

METHODS: Thirty rats were distributed into two groups: the control group (CG) (n = 15) received 1 mL of saline solution orally, and the infected group (IG) (n

= 15) inoculated with 1 mL of saline solution containing 500 oocysts of M-49 *T. gondii* strain orally. After 36 d of infection, the rats were euthanized. Infection with *T. gondii* was confirmed by blood samples collected from all rats at the beginning and end of the experiment. The jejunum of five animals was removed and submitted to routine histological processing (paraffin) for analysis of external muscle thickness. The remaining jejunum from the others animals was used to analyze the general population and the NADH-diaphorase, VIPergic and nitrergic subpopulations of myenteric neurons; and the enteric glial cells (S100-IR).

RESULTS: Serological analysis showed that animals from the IG were infected with the parasite. Hypertrophy affecting jejunal muscle thickness was observed in the IG rats (77.02 ± 42.71) in relation to the CG (51.40 ± 12.34), $P < 0.05$. In addition, 31.2% of the total number of myenteric neurons died (CG: 39839.3 ± 5362.3; IG: 26766.6 ± 2177.6; $P < 0.05$); hyperplasia of nitrergic myenteric neurons was observed (CG: 7959.0 ± 1290.4; IG: 10893.0 ± 1156.3; $P < 0.05$); general hypertrophy of the cell body in the remaining myenteric neurons was noted [CG: 232.5 (187.2-286.0); IG: 248.2 (204.4-293.0); $P < 0.05$]; hypertrophy of the smallest varicosities containing VIP neurotransmitter was seen (CG: 0.46 ± 0.10; IG: 0.80 ± 0.16; $P < 0.05$) and a reduction of 25.3% in enteric glia cells (CG: 12.64 ± 1.27; IG: 10.09 ± 2.10; $P < 0.05$) was observed in the infected rats.

CONCLUSION: It was concluded that infection with oocysts of ME-49 *T. gondii* strain caused quantitative and plastic alterations in the myenteric plexus of the jejunum in rats.

Key words: Enteric nervous system; Infectious diseases; Glial cells; Nitric oxide; Neuronal plasticity; Small intestine; Toxoplasmosis; Vasoactive intestinal peptide

Core tip: The authors assessed the effects of ME-49 *Toxoplasma gondii* (*T. gondii*) strain infection on the myenteric plexus and external muscle of the jejunum in rats. They found an uncommon result when *T. gondii* infection was evaluated in rats: death of myenteric neurons and enteric glial cells. In addition, the remaining neurons showed hypertrophy and the number of nitrergic neurons increased. These alterations were possibly responsible for hypertrophy of the external muscle observed in the jejunal wall. The strain (ME-49) and the life form (oocysts) of *T. gondii* used here were the determinants of all these findings.

Araújo EJA, Zaniolo LM, Vicentino SL, Góis MB, Zanoni JN, da Silva AV, Sant'Ana DMG. *Toxoplasma gondii* causes death and plastic alteration in the jejunal myenteric plexus. *World J Gastroenterol* 2015; 21(16): 4829-4839 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4829.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4829

INTRODUCTION

Several pathogens invade animals *via* the digestive tract. Some of these pathogens (viruses, bacteria, protozoans and helminths) are able to survive in the hostile environment of the intestinal lumen. However, others, such as the protozoan *Toxoplasma gondii* (*T. gondii*), break the barrier of the intestinal epithelium, invade the lamina propria (causing an inflammatory reaction) and migrate to the bloodstream to spread in the host organism, searching for sites to evade the immune system[1-4].

The consequences of oral infection with *T. gondii* may vary, depending on parasite genotype and host species, from asymptomatic infection to the development of several alterations that may lead to death of the host[1-3].

In rats, it is known that the intestinal mucosa still shows signs of injury, detected by histopathological analysis, even after *T. gondii* had crossed the intestinal barrier, spreading through the host organism, forming tissue cysts (chronic phase)[5]. In addition, components of the nervous system intrinsic to the digestive tract, the enteric nervous system (ENS), reveal signs of plasticity due to alterations induced by toxoplasmic infections in the intestinal wall. Therefore, available experimental studies carried out in rats[5-15] have shown that these plastic alterations depend on several factors such as strain; infectious stage (tachyzoites, bradyzoites, sporozoites) and inoculation route (oral or intraperitoneal) of the parasite; infection phase (acute or chronic) assessed; digestive tract region and group of nervous cells assessed. For instance, while chronic infection caused by tachyzoites from a genotype I strain (for the SAG2 gene) causes atrophy of cell bodies in ileal myenteric neurons[7], this same infection causes hypertrophy of cell bodies in colonic myenteric neurons[8].

It is also possible that other alterations can be mediated by enteric glial cells. These cells form a vast network throughout the gastrointestinal wall, especially where there are myenteric and submucosal plexi[16]. Enteric glial cells are small and star-like[17] and can be identified by the presence of specific proteins such as the glial fibrillary acidic protein, vimentin, glutamine synthetase and S100. They contain neurotransmitter precursors such as GABA and NO and express receptors for determined cytokines such as interleukin (IL)-1β, IL-6, TNFα, and neuropeptides such as neurokinin A and substance P after activation[17-19]. Due to these characteristics, they act together in the neuro-immune axis established in the intestinal wall, and are therefore able to modulate some motility functions and gastrointestinal secretions. However, just one

study has assessed enteric glial cells during *T. gondii* infection[5].

Considering the lack of studies on the impact of the infection caused by genotype Ⅱ strains on the jejunal myenteric plexus, this study was carried out to assess the possible alterations caused by oral infection with *T. gondii* oocysts (ME-49 strain, genotype Ⅱ) in the jejunum of rats. Specifically, we evaluated the thickness of intestinal wall; quantitative and morphometric of the total population of myenteric neurons as well as three subpopulations: NADH-diaphorase positive - composed of mitochondria-rich neurons; Nitrergic - produce nitric oxide; VIPergic - produce vasoactive intestinal peptide; and the total population of enteric glial cells that express the cytoplasm structural cytoplasmic protein: S-100.

MATERIALS AND METHODS

The experimental protocol of this study was previously approved by the Ethics Committee in Research Involving Animal Experimentation from Paranaense University, Brazil (Protocol 12361/2008).

Animal care and use statement

The animal protocol was designed to minimize pain or discomfort to the animals. During the experiments, the rats was maintained in an air-conditioned room (approximately 25 ℃), 12 h/12 h light/dark, with food and water *ad libitum*. Half of the animals (infected group) received, by intragastric gavage, 500 *T. gondii* sporulated oocysts in 1 mL of sterile saline solution. The rats in the control group received only sterile saline solution. Thirty-six days after infection, the rats were euthanized in a chamber saturated with halothane for tissue collection.

Experimental design

The study included thirty 60-day-old male Wistar rats (*Rattus norvegicus*), weight 258.5 ± 13.6 g, which were equally and randomly assigned into the control [control group (CG); n = 15] and infected [infected group (IG); n = 15] groups.

In order to obtain the oocysts, cats (*Felis catus*) were inoculated orally with tissue cysts of *T. gondii* (ME-49 strain, genotype Ⅱ), isolated from infected mice (*Mus musculus*). Stools were collected for seven days. Oocysts were concentrated by the Sheather method and sporulated in sulfuric acid at 2%[20].

Each rat in the IG received, 500 *T. gondii* sporulated oocysts re-suspended in 1 mL of sterile saline solution orally, while rats in the CG received only saline solution. Both groups were maintained in an air-conditioned room (approximately 25 ℃), and received commercial feed for rodents and water *ad libitum*.

Infection by *T. gondii* was confirmed by blood samples collected from all rats at the beginning and end of the experiment. The serum was submitted to the direct agglutination method[21] in order to verify the presence of serum anti-*T. gondii* antibodies. Sera were considered positive when titers were greater than 25.

Thirty-six days after infection, the rats were euthanized in a chamber saturated with halothane[22]. Necropsy was performed immediately and the jejunum was removed, using the following anatomic limits as reference: duodenojejunal flexure and ileocecal fold. Each intestinal segment was then measured, washed and underwent intestinal wall analysis techniques.

Histological analysis

A two-centimeter ring from the proximal jejunum of five animals was submitted to routine histological processing (paraffin). From each jejunum, four transversal semi-serial 4 μm-sections were stained with hematoxylin and eosin (HE). Images of the histological sections were captured by a high resolution digital camera coupled to a trinocular photomicroscope (× 20 objective). These images were analyzed by the Motic Image Plus version 2.0 in order to carry out 80 measurements of the external muscle thickness, distributed uniformly around the whole intestinal circumference.

Histochemical technique

Wholemount preparations containing the jejunal myenteric plexus of five animals from each group were submitted to the Giemsa technique to highlight the total neuronal population[23]. From these same animals, 5 cm jejunal segments were submitted to a modified NADH-diaphorase histochemistry technique. These segments were washed in Krebs solution and then immersed for 5 min in Krebs solution + 0.3% Triton X-100 and washed (2 × 10 min each) with Krebs solution and immersed for 45 min in an incubation medium containing in each 100 mL: 25 mL Nitro Blue Tetrazolium (Sigma, St. Louis, MO, United States); 25 mL phosphate buffer 0.1 mol/L, pH 7.3; 50 mL distilled water and 5 mg β-NADH (Sigma, St. Louis, MO, United States)[24]. The intestinal segments were then dissected using a stereo microscope with transillumination to remove the mucosa and submucosa in order to analyze the jejunal myenteric neuronal subpopulation rich in mitochondria (NADHd-p).

Immunohistochemical technique

Intestinal segments were washed with PBS 0.1 mol/L pH 7.4 and filled with Zamboni fixative solution[25] for 18 h at 4 ℃. After fixation, segments were opened along the mesenteric edge and washed in 80% ethanol solution to remove the fixation agent, followed by dehydration in ethanol solutions with ascending concentrations (95%-100%), deorphanization in xylol and rehydration in ethanol solutions with descending concentrations (100%, 90%, 80%, 50%) and stored in PBS + sodium azide 0.04% at 4 ℃. After microdissection, wholemount preparations with the

Table 1 Initial and final weight; length, width and area of the jejunum and thickness of the external muscle layer of the jejunum in rats from the control group and infected group

Parameters	CG	IG
Initial body weight (g)	254.8 ± 15.90	262.4 ± 11.29
Final body weight (g)	394.1 ± 14.94[1]	419.8 ± 31.53[1]
Length (cm)	108.4 ± 5.07	110.4 ± 6.83
Width (cm)	0.96 ± 0.15	0.96 ± 0.15
Area (cm^2)	104.35 ± 19.15	106.82 ± 23.53
Muscular fold thickness (μm)	51.40 ± 12.34[1]	77.02 ± 42.71[1]

[1]Significantly different ($P < 0.05$). IG: Infected group; CG: Control group.

mesenteric plexus of each animal from both groups were obtained, washed with PBS 0.1 mol/L + Triton 0.3% for 5 min, and incubated in protein blocking solution for 2 h. The wholemount preparations were then incubated separately in different solutions containing the rabbit primary antibodies: anti-VIP, anti-NOS1[26] and anti-S 100[27,28] in order to label VIPergic and nitrergic myenteric neurons and enteric glial cells, respectively. Wholemount preparations remained in a cold room (4 ℃) for 48 h and then washed three times in PBS 0.1 mol/L for 5 min. Then they were incubated in solution containing donkey anti-rabbit secondary antibody conjugated with fluorescein (1:500) at room temperature and protected from light for 2 h. The preparations were then washed three times in PBS 0.1 mol/L for 5 min, mounted using PBS/glycerol (9:1) and stored in the fridge.

Quantitative analysis

We counted the total number of myenteric neurons from each rat in 120 400X-magnified fields under the microscope (for the GIEMSA and NADH-diaphorase techniques) or in 32 images captured by a high-definition digital camera coupled to a fluorescence microscope (for the immunohistochemistry NOS^+). The result of this count was projected to one square centimeters and to the total area of the jejunum. Neurons positioned on the limits of each microscope field/image were counted in alternate fields/images. A similar procedure was adopted for counting the enteric glial cells. In this case, we counted all enteric glial cells ($S\text{-}100^+$) present in twenty × 200 magnified images captured by a high-resolution camera coupled to a fluorescent microscope. The number found in the sample area was projected to 1 mm^2.

Morphometric analysis

The area of the cellular body, cytoplasm and nucleus of 300 jejunal myenteric neurons from each animal was measured using the captured images.

Measurement of the VIP-IR varicosities of the myenteric plexus and of the cell body area of NOS-IR myenteric neurons was carried out using images captured by the AxioCam (Zeiss, Jena, Germany), a light microscope resolution camera coupled to a light microscope5 equipped with filters for immunofluorescence in a × 40 objective, then transferred to a microcomputer by the AxioVision 4.1 program. Image-Pro Plus 4.5.0.29 (Media Cybernetics, Silver Spring, MD, United States®). software was adopted during the morphometric analysis of varicosities. Areas with 400 varicosities were measured for each animal, totaling 2000 per group. Only the nerve fibers which were not inside the ganglia were analyzed[29]. Photomicrographs were obtained through images provided by a confocal microscope (LSM 5 Pascal, Zeiss®).

Statistical analysis

Data from neuronal counting were initially submitted to the Shapiro-Wilk test and those from the morphometric analyses were submitted to the D'Agostino Pearson test to verify distribution type. Data with normal distribution were expressed by mean ± SD, and those with free distribution were expressed by the median and percentiles (P25; P75). The Student *t* test was adopted to compare data between the control and experimental groups regarding independent samples (for data with normal distribution) and the Mann-Whitney for data with free distribution, and *P* values less than 0.05 were considered significant. Correlation analysis was verified with the Spearman nonparametric test. Analyses were carried out with statistics software[30]. The statistical methods in this study were reviewed by Professor Aristeu Vieira da Silva from State University of Feira de Santana (Universidade Estadual de Feira de Santana), Brazil, who is biomedical statistician and co-author of this paper.

RESULTS

The results of the serological test performed before infection showed that all rats from the CG and IG were IgM and IgG negative for *T. gondii*. At the end of the experiment, the serological test confirmed that animals from the IG were infected with *T. gondii,* while animals from the CG remained susceptible to infection. In addition, animals from the IG had loose stools when compared to animals from the CG.

At the end of the experiment, body mass of the animals in the IG was greater than that in the CG ($P < 0.05$). With regard to the size of the jejunum (length, width and area), infection did not cause any alterations ($P > 0.05$). However, the morphometric analysis of the intestinal wall revealed hypertrophy affecting jejunal muscle thickness ($P < 0.05$) in the IG compared with the CG (Table 1).

Infected animals showed death of neurons according to quantitative analyses of the myenteric population stained using the Giemsa technique. However, the number of NADHd-p neurons was unaltered. In

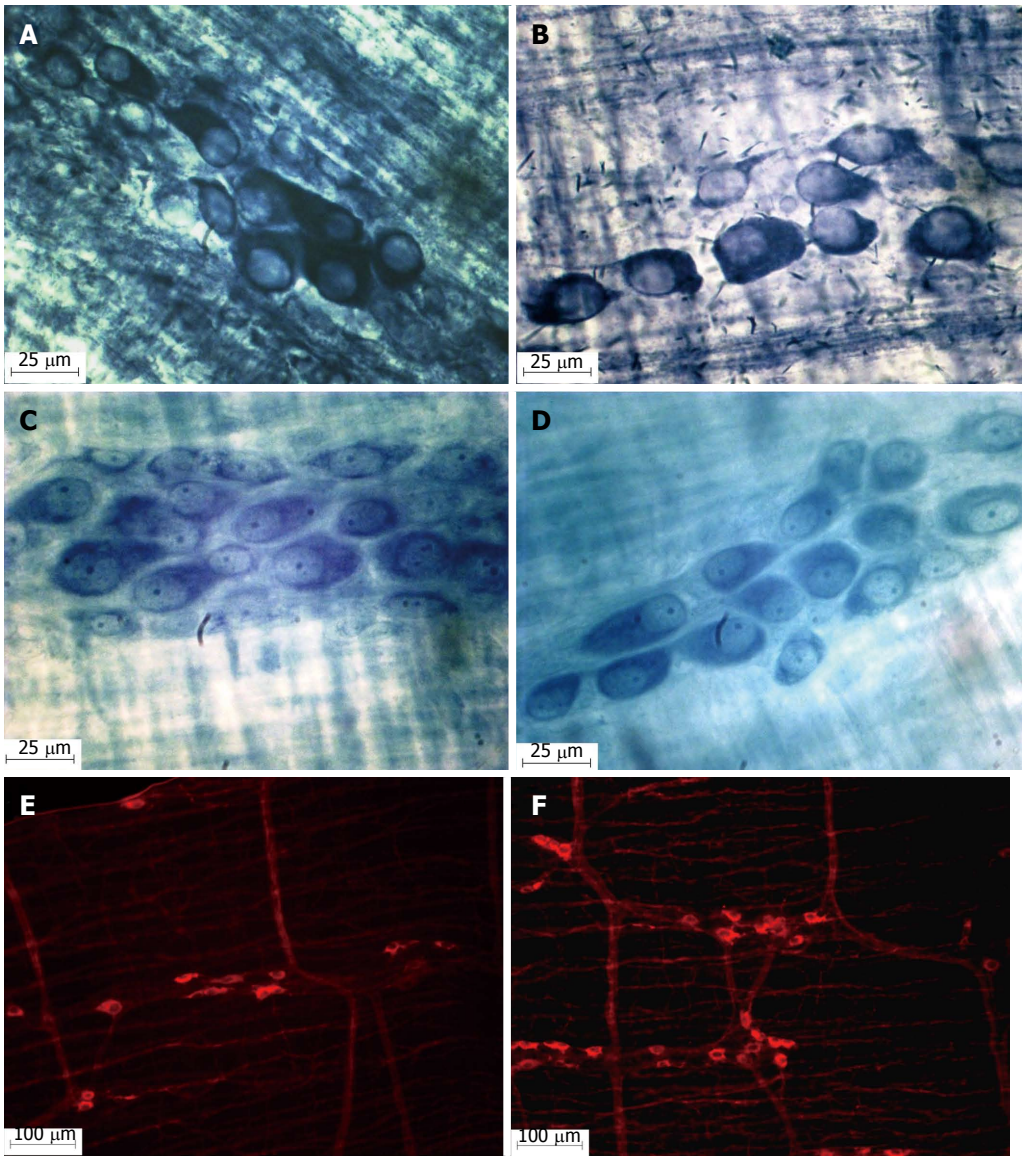

Figure 1 Photomicrograph of the myenteric ganglia in the jejunum of healthy (A, C and E) and infected rats (B, D and F); NADH-diaphorase (A and B); Giemsa (C and D). NOS-IR (E and F) showing the increase in the nitrergic myenteric neuron population in rats infected with oocysts of the ME-49 genotype Ⅱ (F) strain of *Toxoplasma gondii*.

Table 2 Population density and morphometric analysis of the cell body of myenteric neurons labeled with Giemsa, NADHd-p and NOS immunohistochemistry in healthy and infected rats with the ME-49 strain of *Toxoplasma gondii* during 36 d

Measures	GIEMSA		NADHd-p		NOS	
	CG	IG	CG	IG	CG	IG
Number of neurons/cm^2	39839.3 ± 5362.3[1]	26766.6 ± 2177.6[1]	11484.5 ± 2211.8	13155.9 ± 1319.3	7959.0 ± 1290.4[1]	10893 ± 1156.3[1]
Projection of number of neurons for the organ	4157229.4 ± 559555.1[1]	2859215.3 ± 232611.3[1]	1198410.1 ± 230807.1	1405318.8 ± 157570.2	735729.9 ± 125754.7[1]	939630.1 ± 156871.2[1]
Cell body (μm^2)	232.5 (187.2-286.0)[1]	248.2 (204.4-293.0)[1]	146.7 (96.8-202.0)[1]	155.8 (108.0-14.8)[1]	328.1 ± 2.43[1]	344.1 ± 2.2[1]
Nucleus (μm^2)	99.9 (82.5-115.3)[1]	102.6 (88.5-119.2)[1]	64.9 (43.7-88.5)[1]	76.9 (54.1-99.2)[1]	97.5 ± 0.70[1]	100.9 ± 0.67[1]
Cytoplasm (μm^2)	130.8 (99.4-175.1)[1]	141.6 (112.1-182.4)[1]	79.2 (48.0-115.9)[1]	74.2 (48.2-117.4)[1]	230.6 ± 2.1[1]	243.8 ± 1.9[1]
Ratio	0.42 (0.37-0.48)	0.41 (0.37-0.47)	0.44 (0.37-0.51)[1]	0.48 (0.40-0.57)[1]	0.30 ± 0.002	0.31 ± 0.003

[1]For the same neuronal marker, are significantly different (t, $P < 0.05$). IG: Infected group; CG: Control group.

Table 3 Smaller, medium and larger areas (μm²) of VIPergic varicosities and number of enteric glial cells/mm² from the jejunal myenteric plexus of rats from the Control Group and from the Group infected with oocysts of *Toxoplasma gondii* ME-49 strain

Parameters	CG	IG
Smaller areas (μm²)	0.46 ± 0.10[1]	0.80 ± 0.16[1]
Larger areas (μm²)	21.68 ± 5.13	24.50 ± 0.90
Medium areas (μm²)	9.28 ± 5.84	9.51 ± 5.99
Enteric glial cells/mm²	1011.18 ± 25.48[1]	807.24 ± 39.98[1]

[1]Significantly different (*t*, $P < 0.05$). IG: Infected group; CG: Control group.

addition, an increase in nitrergic myenteric neurons in the IG was observed (Table 2 and Figure 1).

The morphometric analysis of the myenteric neurons showed that cellular bodies demonstrated slight hypertrophy in the total population as well as in the nitrergic and NADHd-p subpopulations ($P < 0.05$). For the total population, hypertrophy was due to an increase in the area of cytoplasm and nucleus; however, the proportion occupied by the nucleus inside the cell body was not compromised ($P < 0.05$). This same phenomenon was observed in nitrergic neurons ($P < 0.05$). Conversely, neurons from the subpopulation with more mitochondria inside the cell body (NADHd-p) showed an increase in the nucleus area and a reduction in the cytoplasm area, causing discrete atrophy of the cell body as well as an increase in the proportion occupied by the nucleus inside the cell body ($P < 0.05$) (Table 2).

The smallest areas of the VIPergic varicosities in the IG increased by 73.9% compared to the CG ($P < 0.05$); however, the larger and medium varicosities remained unaltered in animals from the IG ($P < 0.05$). In this study, enteric glial cell S-100 IR showed a quantitative alteration ($P < 0.05$) after infection. The total number of enteric glial cells was 1537 ± 38.7 in the CG and 1227 ± 60.8 in the IG in 1.52 mm² ($P < 0.05$). Thus, there was a 25.3% reduction in the animals from the IG ($P < 0.05$). The area of the VIP-IR varicosities found in the myenteric plexus and the enteric glial cell distribution are shown in Table 3.

DISCUSSION

Experimental infection induced by *T. gondii* (ME-49 strain, genotype Ⅱ) oocysts was the cause of the alterations observed in the jejunal wall structure assessed in this study, mainly within the myenteric plexus. This finding is supported by the fact that animals from the CG remained healthy throughout the experiment, while animals from the IG showed anti-*T. gondii* serum IgG antibodies, indicating true infection by the parasite.

In general, animals are infected by ingesting tissue cysts of *T. gondii* present in raw or rare meat or oocysts found in contaminated food or water. When *T. gondii* crosses the gastrointestinal tract wall it can cause multifunctional alterations[31-33]. These alterations seem to be related to several factors such as genotype strain, life form and inoculation route (oral or intraperitoneal) of the parasite in addition to the infection phase (acute or chronic), digestive tract region and the type of cells assessed[5-15].

For the organ and host species assessed in this study, rat jejunum previously studied by our research group using oocysts of another strain (M7741 - genotype Ⅲ), showed that *T. gondii* was capable of promoting plastic alterations in the enteric neurons without leading to neuronal death during the acute[10] or chronic[12] phases. This finding was different from that observed in the present study which demonstrated that strain ME-49 caused the death of 31.2% of total myenteric neurons. Myenteric neuronal death caused by toxoplasmic infection is not a common finding. Until now this phenomenon had only been observed in the duodenum of poultry[33] and the stomach of rats[11] infected by the *T. gondii* genotype Ⅲ strain.

In addition, the proportion of nitrergic myenteric neurons increased from 17.7% in the CG to 32.8% in the IG. The number of nitrergic enteric neurons found in CG animals corresponds to 18% of the total myenteric neurons described in the literature[34]. An increase in the proportion of nitrergic myenteric neurons was also observed in the jejunum of pigs infected with the M7741 (genotype Ⅲ) strain of *T. gondii*[35], but not in the jejunum of rats infected with the same strain[12]. This shows that the ME-49 strain of *T. gondii* used in the present study was a determinant of the death of myenteric neurons in rat jejunum.

The NADHd-p jejunal myenteric neurons analyzed in this study showed no alterations due to infection; however, there was a reduction of 50% in these cells in the jejunum of pigs infected with the M7741 (genotype Ⅲ) strain of *T. gondii*[35]. Rats infected with the M7741 (genotype Ⅲ) strain of *T. gondii* also showed no alteration in the population density of NADHd-p myenteric neurons[10]. These results reinforce the fact that observable alterations in the myenteric plexus of animals infected with *T. gondii* depend on genotype strain, life form and inoculation route (oral or intraperitoneal) of the parasite in addition to the infection phase (acute or chronic), digestive tract region and the type of neurons assessed. This explains the diarrhea seen in some species when infected with *T. gondii,* while in others this clinical sign is not observed. However, some infected animals which do not exhibit diarrhea may develop constipation. In addition, some of the cases considered "asymptomatic" could represent a misunderstanding caused by lack of attention given to possible intestinal constipation. Further studies are necessary to assess the intestinal motility of animals infected by *T. gondii*.

The modifications in the myenteric neuronal density observed in rats from the IG may be related

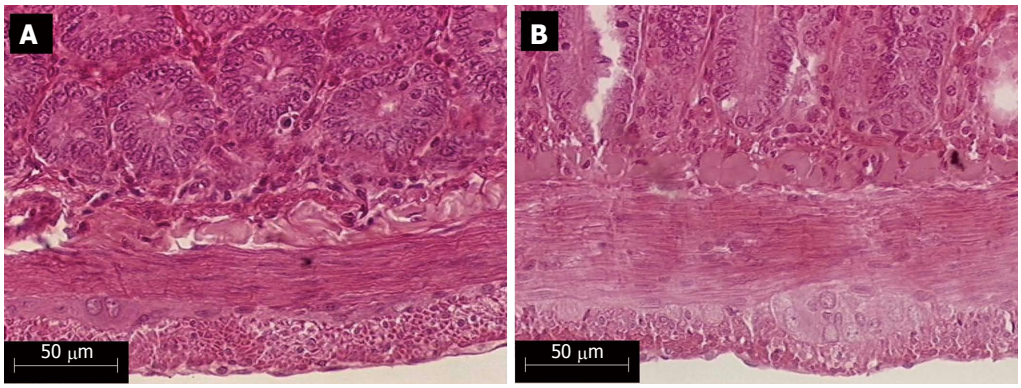

Figure 2 External muscle layer thickness of the jejunal wall of healthy (A) and infected rats (B) with the ME-49 genotype Ⅱ strain of *Toxoplasma gondii*, colored by the HE histological technique.

to the hypertrophy observed in 49.80% of the external muscle layer (Figure 2), as these neurons innervate this muscle. The death of myenteric neurons may trigger several functional disorders directly or indirectly[36-39]. Our data suggest that hypertrophy of the external muscle layer was a compensation mechanism due to neuronal loss in order to maintain the jejunum of the infected animals in an adequate condition for the digestion and absorption of nutrients. It is interesting to observe that despite morphological alterations in the intestine after 36 d of infection, our results show that the rats continued developing including gaining body mass. In the jejunum of pigs, M7741 (genotype Ⅲ) of the *T. gondii* caused atrophy of the external muscle layer after 30 d of infection and then hypertrophy 60 d after infection[40]. Hypertrophy of the external muscle layer was also found in the jejunum and ileum of chicken infected with ME-49 tissue cysts of *T. gondii* and in the jejunum of these same animals when infected with oocysts of the M7741 strain[32,41]. There are no previous studies in the literature assessing the effects of *T. gondii* infection on the external muscle layer of the intestine in rats.

The morphometric analysis of myenteric neurons revealed that, even after 36 d of infection, these cells showed hypertrophied cell bodies in both the total population as well as in the subpopulations assessed in this study. It should be emphasized that the hypertrophy observed in the cell body of the total population and nitrergic subpopulation was the result of an increase both in the nucleus area as well as in the area of the cytoplasm. On the other hand, an increase in the nuclear area (18.5%) and a reduction in the cytoplasmic area (5.3%) were observed in the NADHd-p population, leading to slight hypertrophy of their cell body.

Considering that the results from the present study showed different plasticity between NADHd-p and nitrergic myenteric neurons and that the myenteric plexus carries subpopulations of inhibitory (nitrergic/VIPergic) and excitatory (cholinergic) motor neurons[42], it is possible to infer that a considerable portion of NADHd-p neurons assessed in this study belongs to the subpopulation of cholinergic myenteric neurons. This same rationale can be applied to the population density assessment, as the increase in the proportion of nitrergic myenteric neurons (from 17.7% to 32.8% of the total) observed in animals infected by *T. gondii* suggests a reduction in the number of cholinergic neurons from 82.3% to 62.3% of the total. In this context, it is possible that the increase (18.5%) in the nuclear area of the NADHd-p myenteric neurons (possibly cholinergic, as discussed above) may be related to an increase in metabolic activity of cholinergic myenteric neurons seen as a compensatory effect in relation to the reduction in population density of these cells.

The increase in population density of nitrergic myenteric neurons along with their hypertrophy shows that infection caused by ME-49 strain *T. gondii* provoked a plastic alteration in a large part of the jejunal myenteric plexus of infected rats. Within this context we can also include the increase observed in the smaller varicosities with VIP of nerve fibers of the myenteric plexus of infected animals. As these fibers with VIP belong, mainly, to inhibitory motor neurons which also produce nitric oxide (NO)[43,44], it is suggested that the increase in the varicosities is related to an increase in NO production, thus potentializing the effects of the inhibitory motor route to recover the homeostasis of intestinal motility when looser stools were detected in infected animals. On the other hand, a parallel study with the same animals in this study showed a reduction of 28.4% in VIP-IR submucous neurons in the submucous plexus and atrophy of their cell bodies[5].

Another approach adopted by this study included the quantification of enteric glial cells which showed a reduction in these cells ($P < 0.05$) in the myenteric plexus of the jejunum of rats infected with *T. gondii.* It can be inferred that the loss of the neuronal population by 32% occurred due to a reduction in enteric glial cells. These cells play an important neurotrophic, anti-apoptotic[45-47] and neuromuscular transmitter

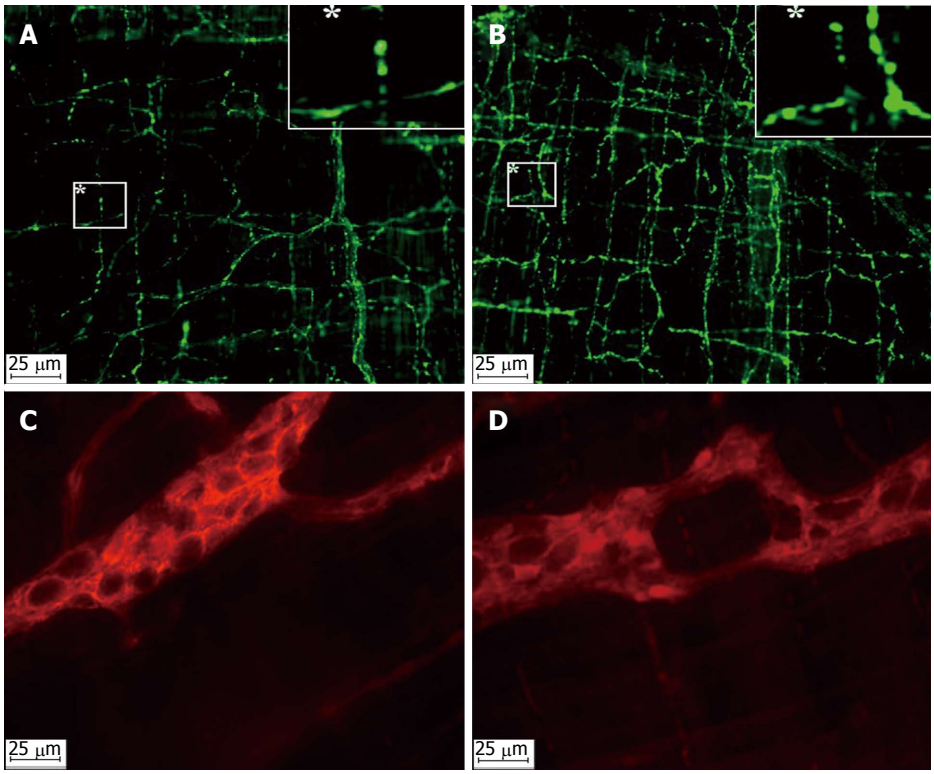

Figure 3 Photomicrographs of the VIPergic fibers (A and B) and enteric glial cells (C and D) in the jejunal myenteric plexus of healthy (A and C) and infected (B and D) rats with the ME-49 genotype II strain of *Toxoplasma gondii*.

role[42,43,36]. As a reduction of a little more than 25% of glial S-100 IR cells in the myenteric plexus of the jejunum in rats was demonstrated in this study, it is suggested that the alterations observed occurred due to the death of glial cells.

Therefore, we believe that the death of enteric glial cells was a determining factor in the changes in the metabolic profile and chemical code of the remaining neurons. This finding is supported by the fact that there were changes in the phenotypic profile in the subpopulations that predominate in the myenteric plexus and that these changes in expression would have caused an increase in the proportion of inhibitory motor neurons (nitrergic) (from 17.7% to 32.8% of the total). However, with the increase in the number of nitrergic neurons, there was an increment in NO production which may induce the release of NO by the neurons[48]. This study describes an increase of 73% in the smallest areas of the VIPergic varicosities in the myenteric plexus of animals from the IG and we question whether the increase in the smallest varicosities of the VIPergic fibers and consequently their greater expression in the myenteric plexus occurred through this mechanism (Figure 3).

VIP is an anti-inflammatory neuropeptide[49,50], therefore its increase may be related to modulation of the inflammatory reaction and recruitment of cells that will act upon tissue repair. It is believed that through this mechanism the enteric glial cells play a central role as mediators of multidirectional interactions among neurons and the immune system[18,51], which in a dynamic way contributes to the homeostasis of the whole gastrointestinal tract[52]. However, as there are no other studies assessing enteric glial cells during infection by *T. gondii*[5], we are unable to compare our results.

It is possible that all these modifications in the myenteric plexus were established after the death of 32% of the total neurons and 25% of the total enteric glial cells in this region of the digestive tract. It may be that there was effectivity in all the observed plastic alterations, as the infected animals continued developing with body mass gains, indicating that digestion and absorption of nutrients were not affected.

However, it is important to highlight that atrophy of the cell body in nitrergic myenteric neurons in the jejunum has been observed in rats infected with oocysts of the *T. gondii*: strain M7741[12]. A comparison between these findings and the results from our present study suggest that the ME-49 strain is more virulent to the myenteric plexus neurons of rat jejunum. For the population of nitrergic myenteric neurons, the *T. gondii* M7741 strain was capable of causing, in the jejunum of pigs[35], reactions similar to those observed in our study with the ME-49 strain, in rats. However, with regard to the NADHd population, pigs infected by strain M7741 showed the opposite result when compared to rats infected by the ME-49 strain.

This is the first study to assess enteric glial cell reaction to this protozoal infection. New studies are necessary to compare results and understand the questions raised here. Future studies on the assessment of the intestinal transit of animals infected by *T. gondii* strains need to be carried out to understand the impact of plastic alterations and population density in the myenteric plexus on the motility of different regions of the digestive tract.

COMMENTS

Background

Toxoplasmosis is one of the most prevalent infectious diseases worldwide. It is estimated that 40% to 70% of the world population is infected with the causative agent of the disease: *Toxoplasma gondii* (*T. gondii*). The oral route is the natural entry for this protozoan. In recent years, studies have shown that this parasite causes changes in the enteric nervous system (ENS) of different host species. The ENS is the intrinsic nervous system of the gastrointestinal tract. These changes are very heterogeneous and depend on the characteristics of the parasite and the host. The changes range from histopathological and plastic neural adaptations to the death of enteric neurons in the host. These changes are due to a number of influences linked to the parasite (genotype, inoculum size, inoculated life form, time of infection, *etc.*) and the host (species, region of the gastrointestinal tract and morphofunctional type of neuron). This study reports ENS changes caused by oocysts of the genotype Ⅱ strain of *T. gondii* on the myenteric neurons of the jejunum in rats.

Research frontiers

The comprehension of intestinal tissue response to aggressive agents such as *T. gondii* is important in understanding functional and structural alterations in the gut. Inflammatory bowel diseases affect many people and models of infection with *T. gondii* are one of the ways to study these diseases. This research group is the first to investigate the relationship between the enteric nervous system and *T. gondii*. Previous studies by our group showed that genotype Ⅲ strains promote morphological changes in enteric neurons of the jejunum, ileum and colon in rats and the jejunum in pigs. Previous studies performed using the ME-49 strain (genotype Ⅱ) showed changes in the submucosal plexus of rats, especially in VIPergic neurons.

Innovations and breakthroughs

This study is the first to describe the effects of chronic infection with the genotype Ⅱ strain of *T gondii* on different neuronal subpopulations of the myenteric plexus and related structures in the jejunum of rats. It contributes to the consolidation of an experimental model of this infectious disease and inflammatory bowel disease.

Applications

This study demonstrates that the ME-49 strain of *T. gondii* is extremely aggressive in the myenteric neurons of the jejunum in rats, as it was capable of causing death of some of these cells. Death of enteric neurons in rats infected with *T. gondii* is a uncommon finding.

Terminology

Toxoplasmosis is a disease characterized by clinical manifestations of infection caused by *T. gondii*. *T. gondii* is a protozoan that parasitizes warm-blooded animal cells. *T. gondii* has three life forms: tachyzoites (fast asexual reproduction, present in the acute phase of infection), bradyzoites (slow asexual reproduction, present in the chronic phase of infection) and sporozoites (present in oocysts which are the result of sexual reproduction of the parasite which only occurs in the intestine of felids). The oocysts are shed in the feces of felids and can contaminate food and water, which can be ingested by man and other animals leading to infection. The enteric nervous system is present in the wall of the digestive tract (esophagus to the anus). The cell bodies of these neurons are organized in two plexi. The submucosal plexus is localized in the submucosa and regulates the flow of blood and secretion of substances into the tube lumen. The myenteric plexus is present in the external muscle layer and regulates the food traffic in contact with the mucosa.

Peer-review

This paper assesses the inflammatory process in the digestive tract caused by *T. gondii* ME-49 strain infection. The objective of this study was to assess the possible effects of this parasite on the myenteric plexus and external muscle of the jejunum in rats. The findings suggest that infection by oocysts of ME-49 *T. gondii strain* caused quantitative and plastic alterations in the myenteric plexus of the jejunum in rats to maintain the homeostasis of the animals. This is a well-written paper containing interesting results.

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ORIGINAL ARTICLE

Basic Study

Hydrogen sulfide-induced enhancement of gastric fundus smooth muscle tone is mediated by voltage-dependent potassium and calcium channels in mice

Xiang-Min Meng, Xu Huang, Chun-Mei Zhang, Dong-Hai Liu, Hong-Li Lu, Young-chul Kim, Wen-Xie Xu

Xiang-Min Meng, Xu Huang, Chun-Mei Zhang, Dong-Hai Liu, Hong-Li Lu, Wen-Xie Xu, Department of Physiology, Shanghai Jiao Tong University School of Medicine, Shanghai 200240, China

Young-chul Kim, Department of Physiology, Chungbuk National University College of Medicine, Chungbuk 361-763, South Korea

Author contributions: Meng XM, Huang X and Zhang CM performed the majority of experiments; Liu DH and Lu HL performed the immunohistochemistry; Kim Y was involved in editing the manuscript; and Xu WX designed the study and wrote the manuscript.

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Correspondence to: Wen-Xie Xu, Professor, Department of Physiology, Shanghai Jiaotong University School of Medicine, 800 Dongchuan Road, 328 Wenxuan Medical Building, Shanghai 200240, China. wenxiexu@sjtu.edu.cn
Telephone: +86-21-34205639

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Abstract

AIM: To investigate the effect of hydrogen sulfide (H_2S) on smooth muscle motility in the gastric fundus.

METHODS: The expression of cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) in cultured smooth muscle cells from the gastric fundus was examined by the immunocytochemistry technique. The tension of the gastric fundus smooth muscle was recorded by an isometric force transducer under the condition of isometric contraction with each end of the smooth muscle strip tied with a silk thread. Intracellular recording was used to identify whether hydrogen sulfide affects the resting membrane potential of the gastric fundus *in vitro*. Cells were freshly separated from the gastric fundus of mice using a variety of enzyme digestion methods and whole-cell patch-clamp technique was used to find the effects of hydrogen sulfide on voltage-dependent potassium channel and calcium channel. Calcium imaging with fura-3AM loading was used to investigate the mechanism by which hydrogen sulfide regulates gastric fundus motility in cultured smooth muscle cells.

RESULTS: We found that both CBS and CSE were expressed in the cultured smooth muscle cells from the gastric fundus and that H_2S increased the smooth muscle tension of the gastric fundus in mice at low concentrations. In addition, nicardipine and aminooxyacetic acid (AOAA), a CBS inhibitor, reduced the tension, whereas Nω-nitro-L-arginine methyl ester, a nonspecific nitric oxide synthase, increased the tension. The AOAA-induced relaxation was significantly recovered by H_2S, and the NaHS-induced increase in tonic contraction was blocked by 5 mmol/L 4-aminopyridine and 1 μmol/L nicardipine. NaHS significantly depolarized the membrane potential and inhibited the voltage-dependent potassium currents. Moreover, NaHS increased L-type Ca^{2+} currents and caused an elevation in intracellular calcium ($[Ca^{2+}]_i$).

CONCLUSION: These findings suggest that H_2S may be an excitatory modulator in the gastric fundus in mice. The excitatory effect is mediated by voltage-dependent potassium and L-type calcium channels.

Key words: Gastric fundus smooth muscle; Hydrogen sulfide; Tension; Voltage-dependent potassium channel; L-type calcium channel

Core tip: The results demonstrated that the cystathionine β-synthase and cystathionine γ-lyase were both expressed in cultured smooth muscle of the gastric fundus. Hydrogen sulfide (H_2S) increased the tension of the gastric fundus and depolarized the resting membrane potential. H_2S decreased the current of voltage dependent potassium channel and calcium channel and then increased the intracellular calcium.

Meng XM, Huang X, Zhang CM, Liu DH, Lu HL, Kim Y, Xu WX. Hydrogen sulfide-induced enhancement of gastric fundus smooth muscle tone is mediated by voltage-dependent potassium and calcium channels in mice. *World J Gastroenterol* 2015; 21(16): 4840-4851 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4840.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4840

INTRODUCTION

Hydrogen sulfide (H_2S) has been proved to be a novel gasotransmitter in addition to nitric oxide (NO) and carbon monoxide (CO) in recent years[1,2]. The endogenous production of H_2S in the gastrointestinal tract has been demonstrated in tissue homogenates[3,4]. Two pyridoxal-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine g-lyase (CSE), are mainly responsible for H_2S synthesis. CBS and CSE have been found throughout the entire gastrointestinal tract[4] and are detected in several cell types, including smooth muscle cells, enteric neurons, interstitial cells of Cajal (ICC) and epithelial cells, varying between species and regions of the gastrointestinal tract[4-8].

Recently, many reports have demonstrated the role of endogenous and exogenous H_2S in gastrointestinal motility. The first work on the role of H_2S in gastrointestinal smooth muscle involved the guinea pig ileum smooth muscle, in which cyanide and nitroprusside augmented its relaxation but H_2S reversed the relaxation caused by nitric oxide[9]. The ATP-sensitive potassium (K_{ATP}) channel has been demonstrated to contribute to intestinal smooth muscle relaxation in the rat jejunum[10], the human, rat and mouse jejunum and colon[11]. Nevertheless, in the urinary bladder, H_2S increased the bladder contraction mediated by capsaicin-sensitive nerves[12].

The gastric fundus is mainly responsible for gastric receptive relaxation; for example, after swallowing, gastric accommodation of the meal involves fundic relaxation *via* activation of the vagal inhibitory pathway. Then, the stored fundic contents are gradually delivered to the caudad stomach *via* peristaltic contractions, modified by the vagal excitatory pathway[13]. This basic theory suggests that the basic tone of the gastric fundus smooth muscle is very important to gastric receptive relaxation. However, few studies have investigated the effect of H_2S on gastric fundus motility. The present study aimed to investigate the effect of H_2S on gastric fundus motility and its ion channel-based mechanism.

MATERIALS AND METHODS

Ethics

This study was carried out in strict accordance to the recommendation in the Guide for the Care and Use of Laboratory Animals of the Science and Technology Commission of P.R.C. (STCC Publication No. 2, revised 1988). The protocol was approved by the Committee on the Ethics of Animal Experiments of Shanghai Jiaotong University School of Medicine (Permit Number: Hu 686-2009).

Animals

Adult male ICR mice aged 5 wk (20-35 g) were provided by the Experimental Animal Center of the Chinese Academy of Sciences, Shanghai, China. The mice were housed at a constant temperature (20-25 ℃) under a 12 h light/dark cycle with free access to water and food.

Tissue preparation and isometric measurements

The mice were killed by cervical dislocation, and the stomach was removed quickly, usually in 2 min, and placed in aerated (95% O_2 and 5% CO_2) Krebs solution containing the following (in mmol/L): NaCl 121.9, $NaHCO_3$ 15.5, KCl 5.9, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.5, and $CaCl_2$ 2.5. The stomach was cut along the lesser curvature, washed with iced Krebs, pinned to the base of a Sylgard dish with the mucosa facing upward, and the mucosa and submucosa were removed. Full-thickness muscle strips (2 mm × 8 mm) of the fundus were obtained along the circular axis. A silk thread (USP 5/0) was attached to both ends of the strips, and the strips were hung along the circular axis in 8-mL organ baths perfused with warm (37 ℃) oxygenated Krebs solution. Mechanical activity was recorded by an isometric force transducer (RM6240C, Chengdu Instrument Factory, China) connected to an amplifier. The strip was equilibrated for 30 min with 0.3-0.5 g of the basal tension before addition of the experimental drugs.

Preparation of cultured smooth muscle cells and immunocytochemistry

Mouse gastric smooth muscle was isolated as described

above, with a few modifications. After washing three times in phosphate-buffered saline (PBS) with 1% antibiotic/antimycotic (Gibco, Grand Island, NY, United States), the muscle was planted in six-well plates immersed in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, United States), supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic (Gibco, Grand, NY, United States). The culture medium was changed every 48 h, and the cells were subcultured for 4-6 d.

A double-labeling immunocytochemical study was used to examine the expression of CBS and CSE. Cells grown on polylysine-coated sterile glass coverslips were washed three times with 0.1 mol/L PBS and fixed with 4% paraformaldehyde for 20 min at 4 ℃. The cells were washed in PBS for 10 min and incubated in PBS containing 10% normal goat serum for 30 min on ice, followed closely by being incubated with either rabbit anti-CBS (1:100, Abcam Ltd., Hong Kong) or rabbit anti-CSE polyclonal antibody (1:100, Proteintech Group, Ltd., United States) mixed with mouse monoclonal anti-smooth muscle α-actin (1:100, Santa Cruz Ltd., United States) at 4 ℃ overnight. After washing, the cells were incubated at room temperature with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:100, Jackson Immuno Research, West Grove, PA, United States) mixed with Dylight 594-conjugated goat anti-rabbit IgG (1:100, ImmunoReagents Inc, Raleigh, NC, United States) for 30 min. Nuclei were stained with 4',6-diamidino-2-phenylindole for 5 min. The controls used the same procedure but omitted the primary antibodies. The cells were observed under a fluorescence microscope (BX3, Olympus, Tokyo, Japan).

Intracellular microelectrode recordings

The strips (8 mm × 10 mm) were pinned in a chamber with a piece of Sylgard in the bottom with the circular muscle side up and perfused with Krebs solution. A 2-h equilibration is necessary before performing the recording. Nicardipine is present to lessen the movement of the strips. We used a glass microelectrode filled with 3 mol/L KCl (30-60 MΩ of resistance) to impale the cells. Membrane potentials were recorded with a standard electrometer (Duo 773, WPI Inc., Sarasota, FL, United States). The 3% KCl-agar bridge between the bath solution and the Ag-AgCl reference electrode was used to stabilize the electrode potentials.

Cell preparation and voltage patch-clamp experiment

Smooth muscle cells were prepared from the fundus as described above. The strip was incubated in a Ca^{2+}-free solution containing the following (in mmol/L): NaCl 135, KCl 5, glucose 10, Hepes 10, and $MgCl_2$ 1.2, adjusted to pH 7.4 with Tris. The strip was cut into pieces and incubated in 1 mL of digestive medium (Ca^{2+}-free solution) containing 2 mg of collagenase I (Sigma-Aldrich, St. Louis, MO, United States), 500 μg of papain (Sigma-Aldrich, St. Louis, MO, United States), 2 mg of bovine serum albumin (Sigma-Aldrich, St. Louis, MO, United States) and 1.5 mg of DTT at 37 ℃ for 10 min. After digestion, the tissue fragment was reserved and washed with modified Kraft-Bruhe (KB) solution containing the following (in mmol/L): glutamic acid 50, taurine 20, EGTA 0.5, Hepes 10, $MgCl_2$ 3, KCl 50, KH_2PO_4 20, and glucose 10, adjusted to pH 7.4 with KOH. Then, the solution was triturated with a glass pipette and kept in modified Kraft-Bruhe (KB) solution. The suspension was transferred to a perfusion chamber on the stage of an inverted microscope, and the cells were recorded after being allowed to settle for 30 min. The cells were perfused in physiologic saline solution (PSS) containing the following (in mmol/L): NaCl 135, KCl 5, $CaCl_2$ 2.5, glucose 10, Hepes 10, and $MgCl_2$ 1.2, adjusted to pH 7.4 with Tris. A single 4-channel perfusion system (BPS-4, ALA Inc., Westbury, NY, United States) was used to change the perfusate. The whole-cell patch-clamp technique was used to record the transient outward potassium current and L-type Ca^{2+} current with an EPC-10 amplifier (HEKA Elektronik, Lambrecht, Germany). The pipette resistance was 2-4 MΩ. For recording the transient outward potassium current, the pipette was filled with a solution comprising the following (in mmol/L): KCl 20, potassium-aspartic acid 110, di-tris-creatine phosphate 2.5, Mg-ATP 5, Hepes 5, $MgCl_2$ 1.0 and EGTA 10, adjusted to pH 7.3 with Tris. For recording the L-type Ca^{2+} channel current, the pipette was filled was a solution comprising the following (in mmol/L): CsCl 130, $MgCl_2$ 1, Na_2ATP 5, Na_2GTP 0.5, EGTA 11 and HEPES 10, adjusted to pH 7.3 with CsOH.

$[Ca^{2+}]_i$ measurement

The cells were obtained as previously described and placed on polylysine-coated slides. They were cultivated in a carbon dioxide incubator at 37 ℃. $[Ca^{2+}]_i$ was measured in cells loaded with 1 μmol/L fura-3 acetoxymethyl ester (fura-3AM) (Sigma-Aldrich, St. Louis, MO, United States) dissolved in PSS containing 1 μmol/L F127 in a carbon dioxide incubator for 1 h. After fura-3AM loading, the cells were washed three times in PSS and placed under a fluorescence microscope (BX3, Olympus, Tokyo, Japan). The cells were perfused in a flowing PSS perfusion solution at room temperature.

Drugs

Sodium hydrogen sulfide (NaHS), 4-aminopyridine (4-AP), nicardipine, aminooxyacetic acid (AOAA), DL-propargylglycine (PAG), Nω-nitro-L-arginine methyl ester (L-NAME) were all purchased from Sigma (Sigma-Aldrich, St. Louis, MO, United States). All were dissolved in distilled water except nicardipine, which was distilled in DMSO (dimethyl sulfoxide).

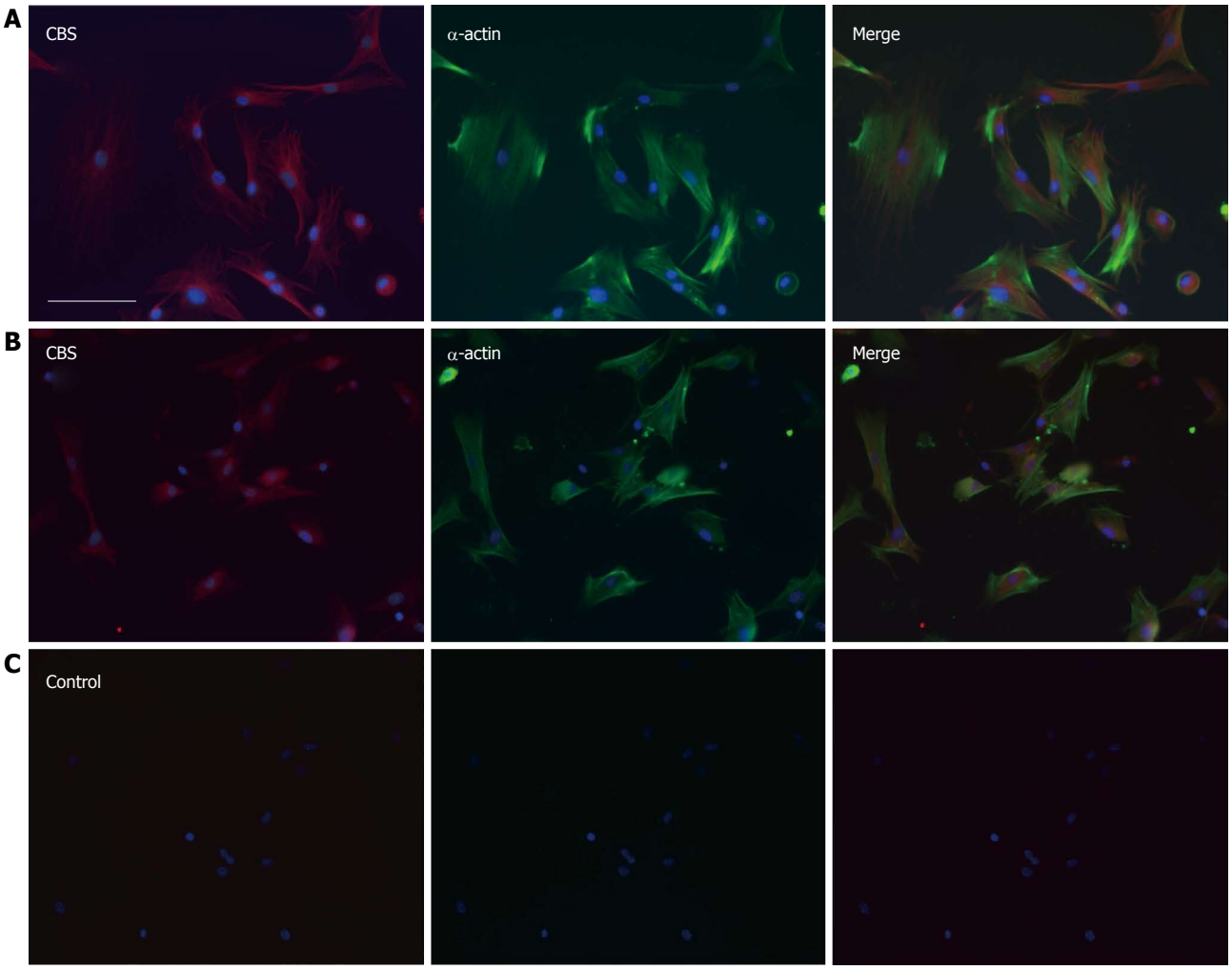

Figure 1 Double immunofluorescence labeling of cystathionine β-synthase and cystathionine γ-lyase proteins in cultured mouse gastric smooth muscle cells. A: CBS-IR is present in α-actin IR smooth muscle cells; B: CSE-IR is present in α-actin IR smooth muscle cells; C: Negative control without primary antibodies (scale bar =100 μm). CBS: Cystathionine β-synthase; CSE: Cystathionine γ-lyase; IR: Immunoreactivity.

Statistical analysis

The data were analyzed using Origin 7.5 software and are expressed as mean ± SE. Data from multiple groups were evaluated using one-way analysis of variance followed by a post-hoc Bonferroni test, whereas Student's paired *t*-test was used to evaluate paired data sets. A P value < 0.05 was considered statistically significant.

RESULTS

Expression of CBS and CSE in gastric fundus smooth muscles

To determine whether H_2S can be generated from the gastric fundus smooth muscle, CBS and CSE protein immunoreactivity (IR) was examined by double immunofluorescence labeling of cultured fundus smooth muscle cells. We found that both CBS and CSE were expressed in α-actin-positive cells (Figure 1), which suggests that H_2S can be endogenously generated in gastric fundus smooth muscle cells.

Effect of exogenous and endogenous H_2S on gastric fundus smooth muscle contraction

We observed the effect of H_2S on gastric fundus smooth muscle tonic contraction. We observed that NaHS, an H_2S donor, significantly enhanced the tension of the fundus smooth muscle at lower concentrations. The basal tension was increased from 0 mg in the control to 6.71 ± 2.11, 16.86 ± 5.67, 33.57 ± 10.32, 52.86 ± 13.06, 72.00 ± 9.62, 84.43 ± 6.56, and 52.57 ± 5.99 mg in force in cells treated with NaHS at 60, 90, 120, 150, 180, 210, and 240 μmol/L, respectively (P < 0.05, n = 10; Figure 2Aa, Ab). L-NAME (100 μmol/L), a non-specific inhibitor of NOS, increased the tension of fundus smooth muscle strips from 0 mg in the control to 52.40 ± 16.47 mg in force (P < 0.05, n = 10), whereas the CBS inhibitor AOAA (1 mmol/L) decreased the tension from 0 mg in force in the control to -241.30 ± 28.57 mg in force (P < 0.05, n = 10, Figure 2Ba, Bb). The AOAA-induced decrease in the tension was reversed by NaHS (Figure 3Aa, Ab, Ad; P < 0.05, n = 10). Interestingly, the CSE inhibitor,

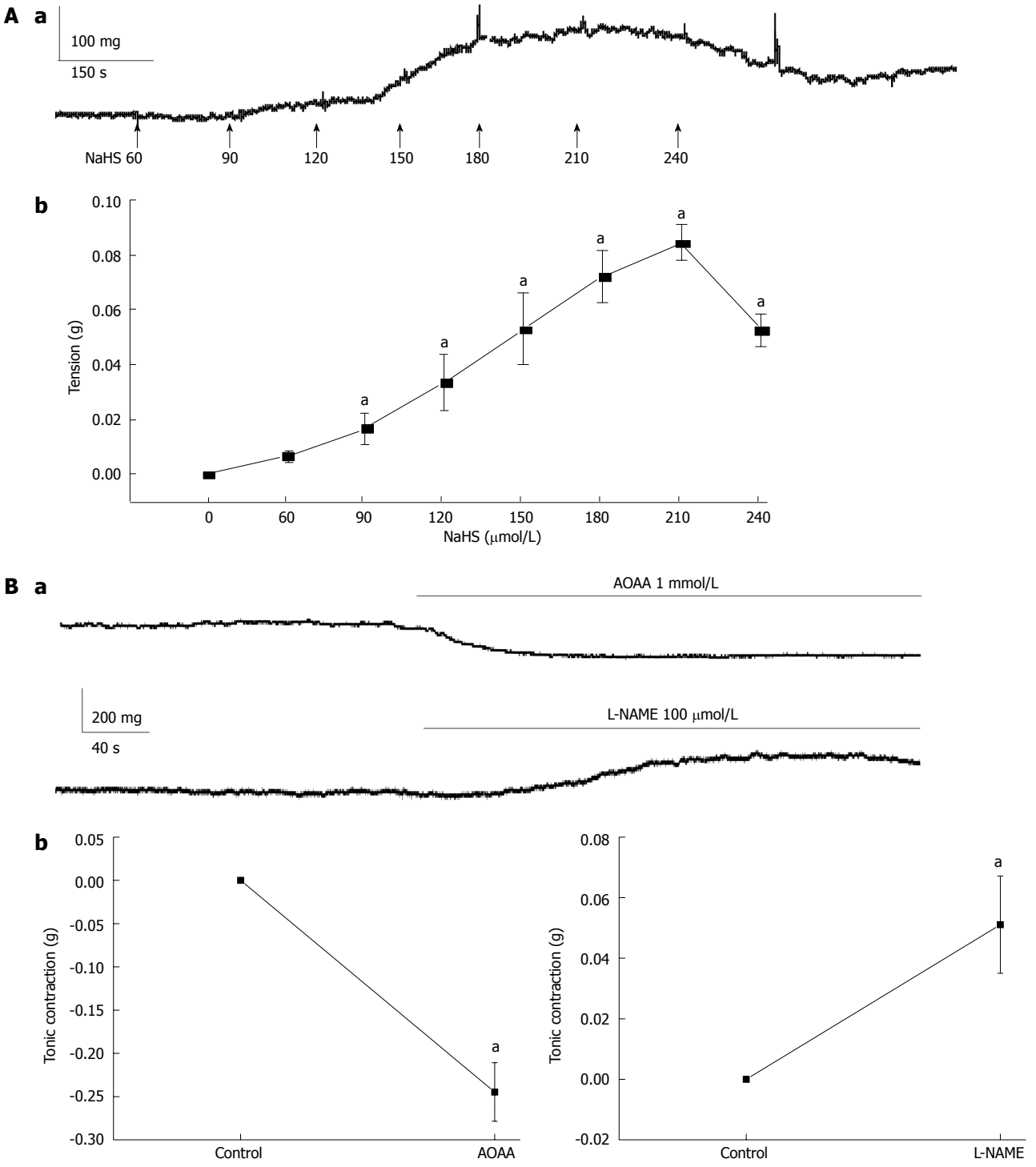

Figure 2 Effect of H_2S on the tension of gastric smooth muscle. A: Different doses of NaHS significantly enhanced the gastric fundus smooth muscle tension (a: Representative trace of changes in gastric fundus smooth muscle tension induced by different concentrations of NaHS; b: Summarized graph showing the effects of different concentrations of NaHS in inducing basal tension); B: Effects of endogenous H_2S on the gastric fundus smooth muscle tension (a: Representative traces of AOAA- and L-NAME-induced basal tension; b: Summarized graph showing the changes in AOAA and L-NAME-induced tonic contraction). Data are expressed as mean ± SE, $n = 10$, $^aP < 0.05$, *vs* the control. AOAA: Aminooxyacetic acid; L-NAME: Nω-nitro-L-arginine methyl.

PAG (1 mmol/L), did not significantly affect the tension (from 0 mg in force by the control to -4.25 ± 2.81 mg in force, Figure 3Ac, $P > 0.05$, $n = 10$), which indicates that CBS may predominate over the gastric fundus in modulating smooth muscle contraction. Consequently, we investigated whether potassium and calcium channels are involved in the NaHS-induced excitatory effect on fundus smooth muscle tonic contraction. 4-AP (5 mmol/L), a voltage-dependent potassium channel blocker, elicited strong tonic contraction and completely blocked the NaHS-induced enhancement of fundus smooth muscle tone (Figure 3Ba, Bb; $n = 8$). We then tested the effect of nicardipine, an L-type calcium channel blocker, on NaHS-induced fundus

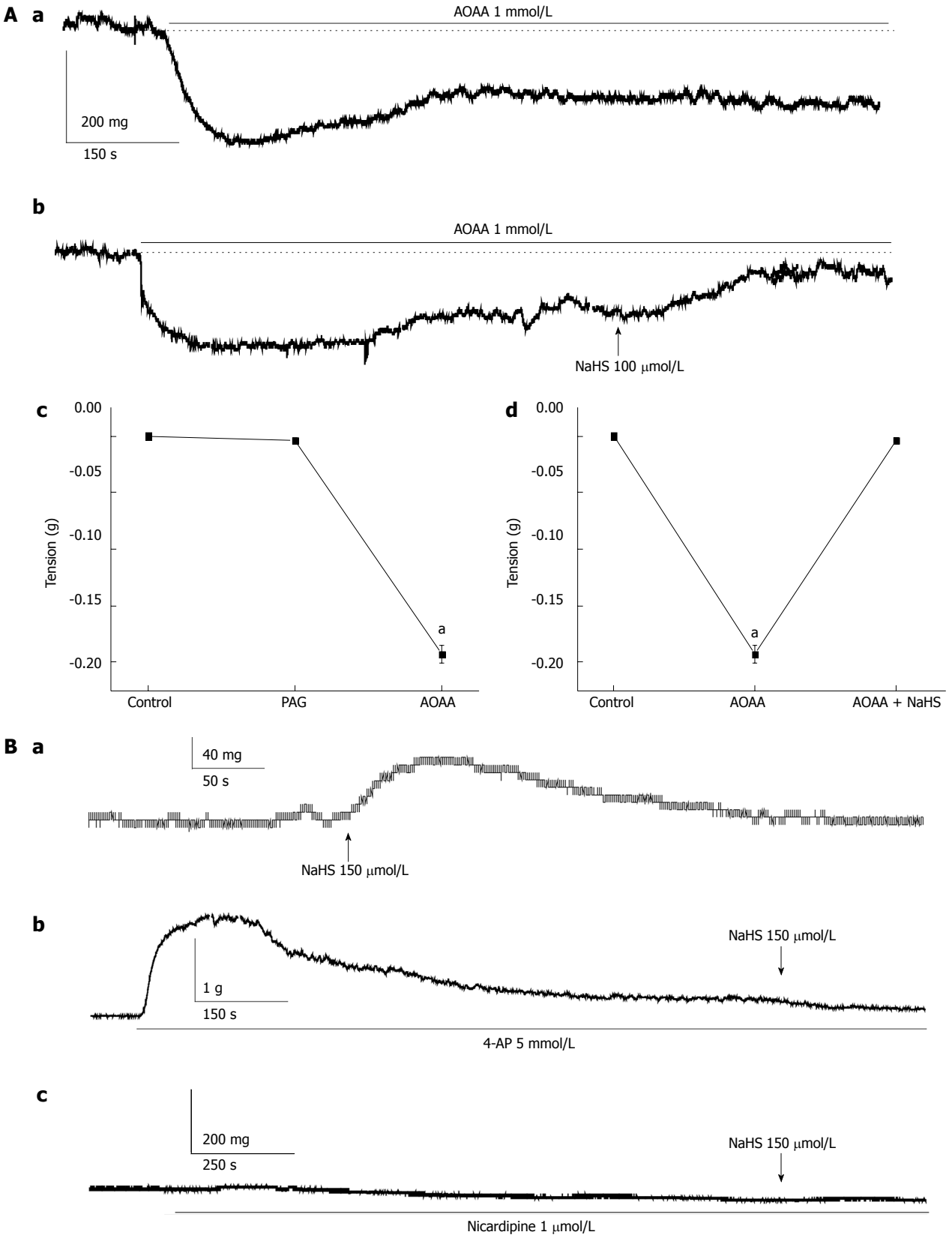

Figure 3 Effects of aminooxyacetic acid, DL-propargylglycine and ion channel blockers on NaHS-induced gastric fundus smooth muscle tonic contraction. A: Effects of AOAA and PAG on gastric fundus smooth muscle basal tension (a, b: H_2S significantly recovered the AOAA-induced decrease in basal tension; c: Summarized graph showing the changes in AOAA- and PAG-induced tonic contractions; d: The recovery effect of NaHS on the AOAA-induced decrease in gastric fundus smooth muscle tension); B: Effects of a potassium channel blocker and L-type calcium channel blocker on NaHS-induced tonic contraction (a: Representative traces of NaHS-induced tonic contraction; b,c: Effect of 4-AP (5 mmol/L) on NaHS-induced tonic contraction, and effect of nicardipine (1 μmol/L) on NaHS-induced tonic contraction. Data are expressed as mean ± SE, $n = 10$, $^aP < 0.05$, *vs* the control. AOAA: Aminooxyacetic acid; PAG: DL-propargylglycine.

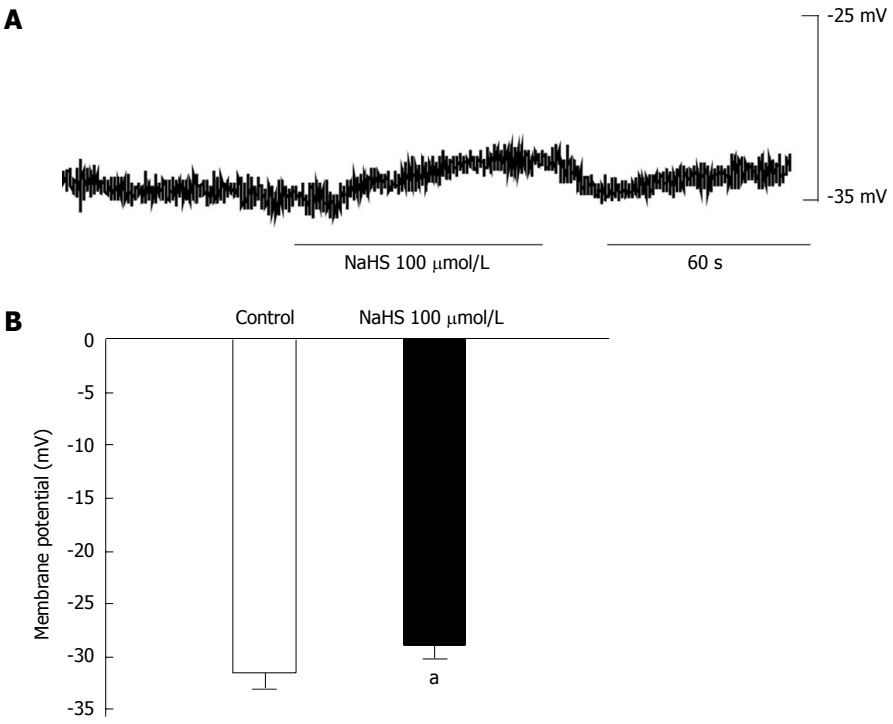

Figure 4 Effect of NaHS on the membrane potential of the gastric fundus smooth muscle. A: Raw trace of the NaHS-induced change in membrane potential; B: Summarized graph showing the change in the NaHS-induced increase in the membrane potential. Data are expressed as mean ± SE, $n = 6$, $^{a}P < 0.05$ *vs* the control.

smooth muscle tonic contraction. As shown in Figure 3Ba and Bc ($n = 8$), nicardipine (1 μmol/L) completely blocked the excitatory effect of NaHS (150 μmol/L) on fundus smooth muscle tonic contraction. These results suggest that the excitatory effect of NaHS may be mediated *via* the voltage-dependent potassium channels and L-type calcium channels, resulting in the depolarization of membrane potential and Ca^{2+} influx.

Effect of NaHS on the membrane potential

To further understand the above results, we observed the effect of NaHS (100 μmol/L) on membrane potential by intracellular recording. We found that NaHS depolarized the membrane potential from -31.82 ± 1.36 mV in the control to -25.44 ± 1.13 mV (Figure 4A, B; $P < 0.05$, $n = 6$). The result indicates that the NaHS-induced excitatory effect may be related to depolarization of the membrane potential.

Effect of NaHS on voltage-dependent potassium current and L-type calcium current

To determine the mechanism of NaHS-induced membrane potential depolarization, we further examined the effect of NaHS on the voltage-dependent potassium current (IKv). Initially, IKv was elicited by a single depolarizing step pulse (in which the membrane potential was held at -60 mV and depolarized to +60 mV in 10-s intervals) for 440 ms using the whole-cell patch-clamp technique in freshly dispersed fundus smooth muscle cells. The mean peak current was increased from 777.26 ± 59.78 pA in the control to 753.89 ± 89.70 pA, 659.86 ± 48.04 pA, and 559.06 ± 36.02 pA by 50 μmol/L, 100 μmol/L, and 200 μmol/L NaHS, respectively (Figure 5Aa, Ab, Ac; $P < 0.05$, $n = 6$). To further determine the effect of NaHS on the current-voltage (I-V) relationship of IKv, IKv was elicited by a step voltage command pulse from -40 mV to +100 mV for 400 ms with a 20-mV increment in 10-s intervals. NaHS significantly decreased IKv at every membrane potential level from +20 mV to +100 mV in the I-V curve (Figure 5Ba). The IKv at +60 mV was decreased by 16.18% ± 4.96% (Figure 5Bb, $P < 0.05$, $n = 6$) with the application of 200 μmol/L NaHS. These results suggest that IKv may contribute to NaHS-induced membrane potential depolarization in mouse gastric fundus smooth muscle.

The L-type calcium current (ICa) was activated by a single depolarizing step pulse (in which the membrane potential was held at -80 mV and depolarized to 0 mV in 10-s intervals) for 440 ms first by using the whole-cell patch-clamp technique. The inward calcium current was increased with the application of a succession of NaHS. The peak relative currents were increased from 1 in the control to 1.13 ± 0.13, 1.26 ± 0.05, and 1.34 ± 0.08, by 50 μmol/L, 100 μmol/L, and 200 μmol/L NaHS, respectively (Figure 6Aa, Ab, Ac; $P < 0.05$, $n = 6$). The effect of NaHS on the I-V relationship of ICa is shown in Figure 6. The bath application of 200 μmol/L NaHS showed augmentation of the peak current on the I-V curve. NaHS significantly increased ICa at membrane potentials from -10 mV to +10 mV in the I-V curve (Figure 6Bb; $P < 0.05$, $n = 6$). The ICa at 0 mV was increased by 22.10% ± 3.90% (Figure 6Bb; $P < 0.05$, $n = 6$) with the application of 200 μmol/L NaHS.

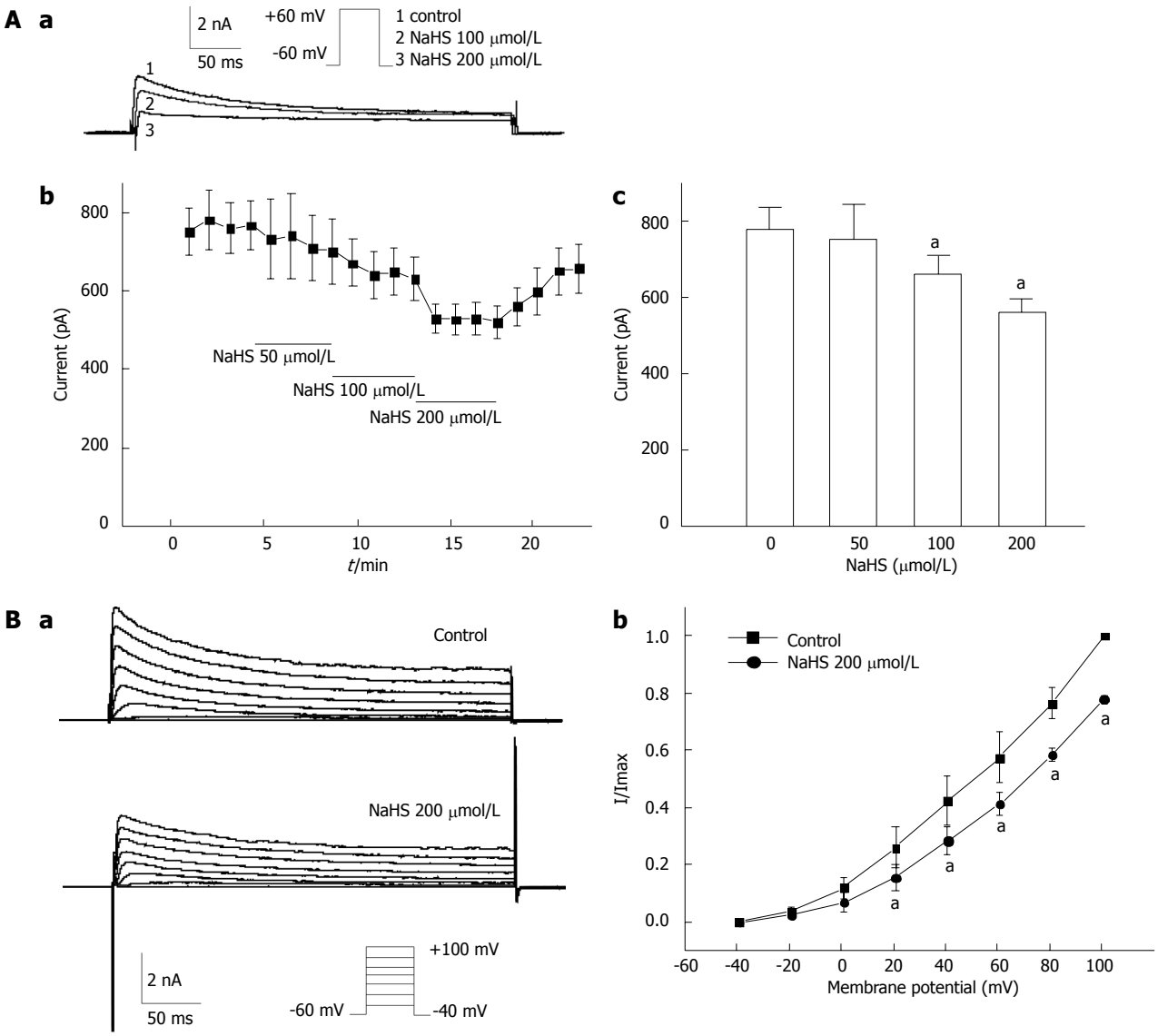

Figure 5 Effects of NaHS on IKv in gastric fundus smooth muscle cells. Aa: Representative traces elicited by a single depolarized step pulse; Ab: The time-dependent effect of different concentrations of NaHS on IKv; Ac: Summarized graph showing the changes in the NaHS-induced inhibition of IKv elicited by a single depolarized step pulse; Ba: Representative traces of the NaHS-induced decrease in IKv in gastric smooth muscle cells; Bb: The I-V relation curve of the NaHS-induced change in IKv. Data are expressed as mean ± SE, $n = 6$, $^aP < 0.05$ *vs* the control.

These results suggest that the L-type calcium channel is involved in the excitatory effect of NaHS on fundus smooth muscle contraction.

Effect of NaHS on $[Ca^{2+}]_i$

The above results suggest that NaHS-induced depolarization activates the L-type calcium channel *via* the inhibition of voltage-dependent potassium currents. Thus, we directly observed the effect of NaHS on changes in intracellular calcium levels. NaHS has been shown to elicit an increase in intracellular Ca^{2+} in cultured fundus smooth muscles cells (Figure 7Aa and Ab). The F/Fo response to NaHS varied in magnitude from 1.02 ± 0.004 in the control to 1.06 ± 0.015, 1.18 ± 0.037, 1.33 ± 0.047, and 1.27 ± 0.023 when treated with 50 μmol/L, 100 μmol/L, 200 μmol/L, and 250 μmol/L NaHS, respectively, with the maximum at 200 μmol/L (Figure 7Ab; $P < 0.05$, $n = 6$).

Because extracellular Ca^{2+} entry can result from the opening of L-type calcium channels, we observed whether the change in intracellular Ca^{2+} is associated with L-type calcium channels. When perfused with PSS containing 200 μmol/L NaHS, the F/Fo was markedly increased (Figure 7Ba), but pretreatment with 1 μmol/L nicardipine caused the NaHS-induced increase in F/Fo to be almost completely abolished (Figure 7Ba). Then, we perfused the sample with PSS containing 1 μmol/L nicardipine and 200 μmol/L NaHS, which attenuated the increase in intracellular Ca^{2+} induced by 200 μmol/L NaHS (Figure 7Bb). These results indicate that NaHS increases the intracellular Ca^{2+} levels *via* L-type calcium channels.

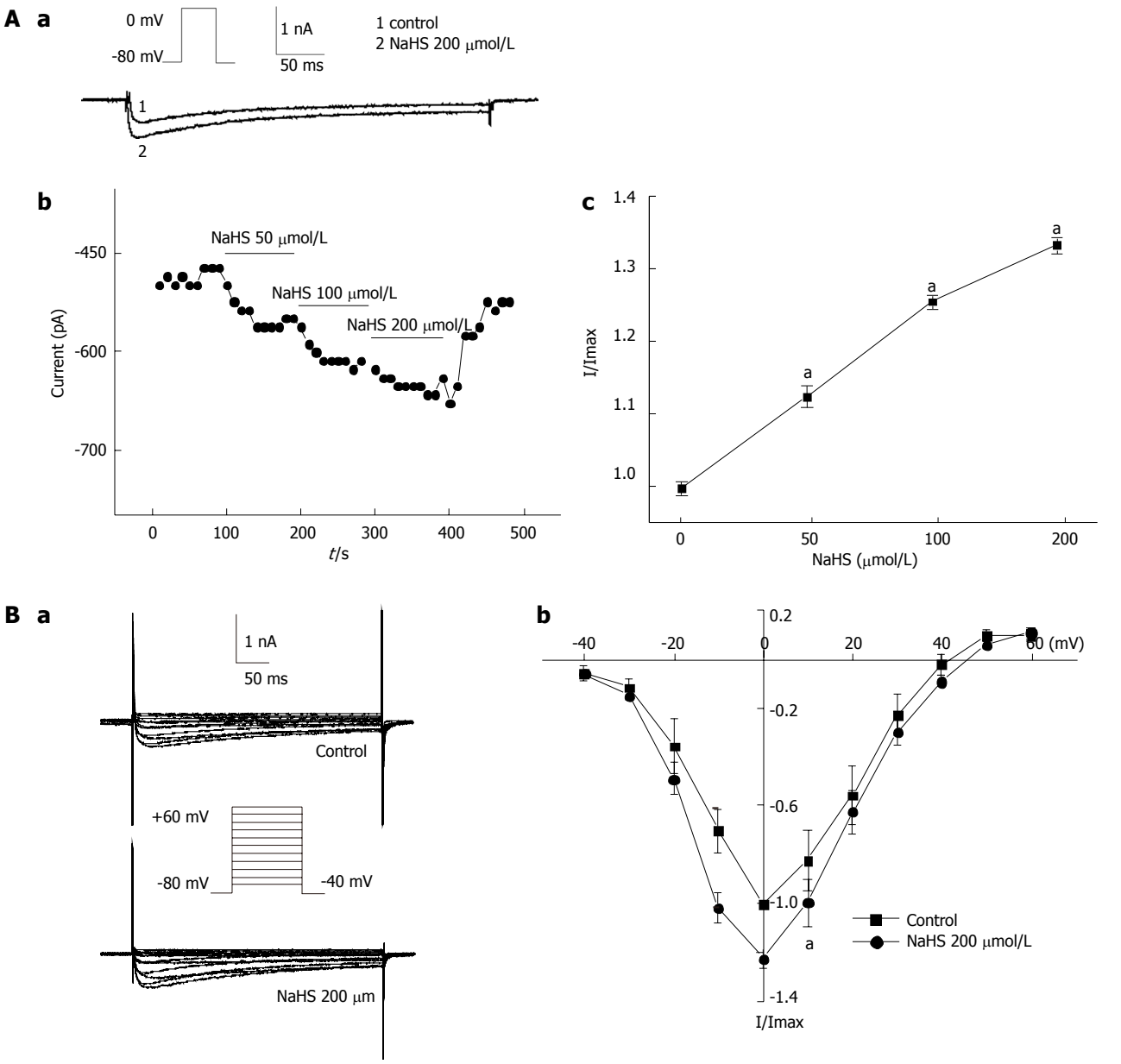

Figure 6 Effects of NaHS on ICa in gastric fundus smooth muscle cells. Aa: Representative traces elicited by a single depolarized step pulse; Ab: The time-dependent effect of different concentrations of NaHS on ICa; Ac: Summarized graph showing the change in the NaHS-induced inhibition of ICa elicited by a single depolarized step pulse; Ba: Representative traces of the NaHS-induced increase in ICa in gastric smooth muscle cells; Bb: The I-V relation curve of the NaHS-induced change in ICa. Data are expressed as mean ± SE, $n = 6$, $^aP < 0.05$ *vs* the control.

DISCUSSION

Gasotransmitters are gas molecules endogenously synthesized in a regulated manner, causing well-defined physiological and/or pathophysiological effects, acting at specific cellular and molecular targets and employing specific mechanisms of inactivation[2,14,15]. H_2S is of particular interest in the gastrointestinal tract as it is both produced both by gastrointestinal tissues and generated in large quantities by the bacterial flora in the lumen of the gut[3,4,15-18]. In the gastrointestinal tract, both excitatory and inhibitory effects on smooth muscle have been reported. For example, NaHS concentration-dependently relaxed prostaglandin F2a-contracted circular muscle strips of mouse fundus and distal colon[19,20]. NaHS also exerted relaxant effects on guinea pig, rabbit and rat ileum and jejunum preparations[3,10,19,21,22]. Furthermore, NaHS inhibits peristaltic activity in the mouse small intestine and colon[11]. Our previous studies indicated dual effects of H_2S on the spontaneous contraction of gastric antral smooth muscle; for example, a low concentration of NaHS increased tonic contraction, whereas a high concentration reduced the amplitude and tone of gastric smooth muscle spontaneous contraction in guinea pigs[23]. We have explored that NO and H_2S play opposite roles in regulating the tension of gastric antrum before, and they share no common pathways[24]. These studies suggest that the role of H_2S in the regulation of gastrointestinal motility

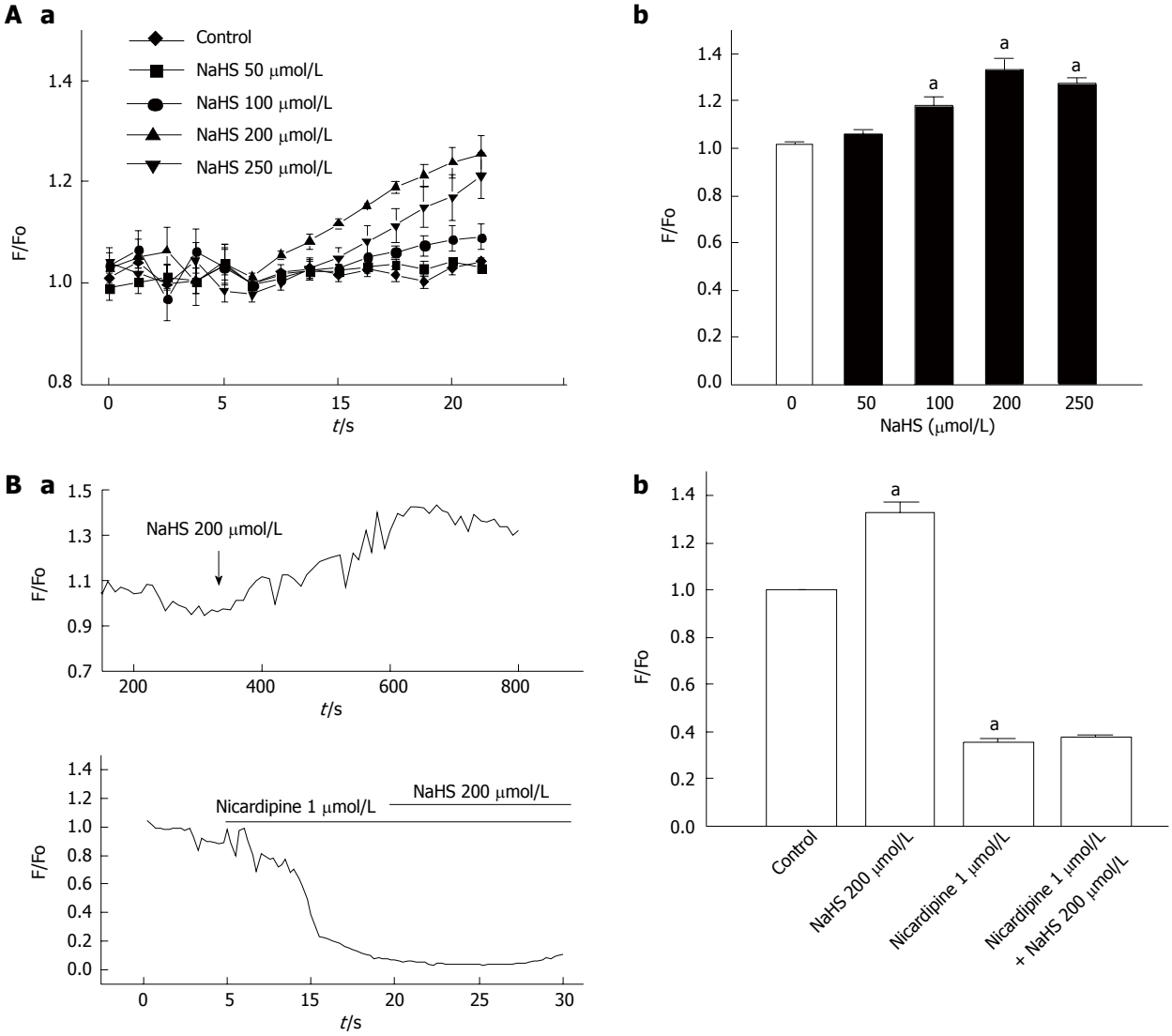

Figure 7 Effect of NaHS on $[Ca2+]_i$ in cultured gastric fundus smooth muscles. Aa: The time-dependent effects of different concentrations of NaHS on $[Ca2+]_i$. Ab: Summary graph showing the effects of the NaHS-induced increase in intracellular calcium; Ba: Raw traces of the NaHS-induced increase in intracellular calcium. Representative traces showing the effect of nicardipine on the H2S-induced increase in intracellular calcium; Bb: Summary graph showing the effects of nicardipine on the H2S-induced increase in intracellular calcium in the presence of nicardipine. Data are expressed as mean ± SE, $n = 6$, $^aP < 0.05$ *vs* the control.

displays a regional variation. The stomach gastric fundus is involved in receptive contraction concerned with gastric accommodation, and the gastric antrum is involved in gastric emptying concerned with the pyloric pump; in contrast, the jejunum and colon are involved in migrating the motility complex aimed at absorption. Therefore, the effects of H_2S in the stomach might differ from those in the jejunum and colon.

In the present study, we found that both CBS and CSE, which catalyze the generation of H_2S, were expressed in primary cultured smooth muscle cells (Figure 1). We deduce that H_2S can be generated endogenously and continuously in gastric smooth muscle cells and influences physiological processes. Meanwhile, we observed that NaHS at lower concentrations increased the basal tension of smooth muscles in the gastric fundus (Figure 2). AOAA, an inhibitor of CBS, decreased the basal tension, and PAG, an inhibitor of CSE, did not affect the tension significantly (Figure 3). All these results demonstrate that CBS may be the predominant enzyme in the gastric fundus. Although AOAA is widely used as an inhibitor of several pyridoxal phosphate-dependent enzymes, including aspartate transaminase, 4-aminobutyrate and dopa-decarboxylase[25], it may also inhibit NADH shuttles[26]. In contrast, Martin *et al*[4] have demonstrated that AOAA reduces H_2S generation. In our study, we also observed that the decrease in the tension induced by AOAA was significantly reversed by NaHS (Figure 3), suggesting that the AOAA-induced inhibitory effect on gastric fundus smooth muscles was partially mediated by inhibition of CBS to generate endogenous H_2S. NO is a well-known relaxation agent for smooth muscle as a contrary experiment control, L-NAME, a nonspecific inhibitor of NOS, significantly enhanced the tension of fundus smooth muscle. As

shown in our results, the effect of AOAA on gastric fundus smooth muscle is the opposite to that of L-NAME, which indicates that H_2S may be an excitatory gaseous transmitter in the gastric fundus under physiological conditions.

It is undeniable that membrane potential is important for electric-contraction coupling. Furthermore, distinct from other parts of the gastrointestinal tract, gastric fundus smooth muscle cells are electrically quiescent or, in some occasions, generate the discharge of membrane noises[27]. We observed the membrane potential of gastric fundus smooth muscles using the intracellular recording technique and found that 100 μmol/L NaHS significantly depolarized the membrane potential (Figure 4). Because voltage-dependent potassium channels are the most important regulators of maintaining the resting membrane potential, we observed the effect of 4-AP, an inhibitor of IK_V, on the NaHS-induced excitatory effect in succession and found that the NaHS-induced tonic contraction of fundus smooth muscle was completely blocked by 4-AP. Gastrointestinal smooth muscle cells express voltage-dependent calcium channels[28,29], and these channels are the backbone of electric-contraction coupling in the gut[30]. Therefore, we also used nicardipine to block the L-type calcium channels and found that the NaHS-induced tonic contraction was completely abolished (Figure 3). These results suggest that H_2S may be activated by L-type calcium channels through the inhibition of IK_V and depolarization of the membrane potential.

However, H_2S inhibited L-type calcium channels, resulting in the inhibition of intracellular calcium concentrations in rat cardiomyocytes[31]. Moreover, H_2S raised the intracellular calcium concentrations in endothelial cells *via* K_{ATP} channels and the Na+-Ca^{2+} exchanger[32]. These studies indicated that the function of H_2S may be complicated in different tissues. In the present study, to further explore the ion channel mechanism involved in the NaHS-induced excitatory effect on fundus smooth muscles, we observed the effect of H_2S on IK_V, L-type calcium current and intracellular calcium concentration using the whole-cell patch-clamp and calcium imaging techniques. We found that H_2S inhibited IK_V (Figure 5) but increased the L-type calcium current (Figure 6) and intracellular calcium levels (Figure 7). The H_2S-induced increase in intracellular calcium was significantly blocked by nicardipine (Figure 7). We conclude that the excitatory effect of NaHS on the fundus smooth muscle was mediated by intracellular calcium due to the activation of L-type calcium channels *via* inhibition of the IK_V-induced depolarization of the membrane potential. The present study demonstrates that H_2S is an excitatory gaseous molecule in the gastric fundus in mice, but its exact mechanism still needs further investigation.

In summary, we showed that the H_2S-producing enzymes CBS and CSE are expressed in the gastric fundus in mice. H_2S at physiological concentrations may excite the fundus smooth muscles and induce tonic contraction. CBS may be more important for the excitatory effect of endogenous H_2S on gastric motility. Endogenous H_2S induces the depolarization of membrane potential *via* the inhibition of voltage-dependent potassium channels. In succession, NaHS-induced depolarization activates L-type calcium channels, raises the intracellular calcium level and finally induces fundus smooth muscle tonic contraction. Under physiological conditions, endogenous H_2S and NO levels might maintain a relative balance to ensure the basic physiological tone of fundus smooth muscle.

COMMENTS

Background

Hydrogen sulfide is considered a gaseous signal molecular for its wide effects in pathophysiology process. Numerous studies have shown that hydrogen sulfide serves as a vasodilator. The gastric fundus is responsible for receptive relaxation and little is known on the effect of hydrogen sulfide on the fundus.

Research frontiers

Hydrogen sulfide is mainly catalyzed by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). The authors previous studies have shown that hydrogen sulfide enhanced the tension of gastric antrum *via* voltage dependent potassium channels and ATP sensitive potassium channels. In this study, the authors demonstrate that hydrogen sulfide-induced enhancement of gastric fundus smooth muscle tone is mediated by voltage-dependent potassium and calcium channels in mice.

Innovations and breakthroughs

Recent reports have highlighted the effects of hydrogen sulfide on gastrointestinal muscle and enteric nervous system. This is the first study to report that hydrogen sulfide enhances the tension of gastric fundus smooth muscle *via* raising intracellular calcium.

Applications

This study may represent a future strategy for therapeutic intervention in disorders of gastrointestinal motility by understanding the mechanism of action of hydrogen sulfide on gastric fundus tension.

Terminology

Hydrogen sulfide is mainly catalyzed by CBS and CSE. In the gastrointestinal tract, hydrogen sulfide is involved in gastrointestinal motility, absorption and secretion.

Peer-review

The authors of the manuscript entitled "Hydrogen sulfide-induced enhancement of gastric fundus smooth muscle tone mediated by voltage-dependent potassium and calcium channels in mice" present a very nice work of basic science physiology on gastric smith muscles.

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ORIGINAL ARTICLE

Basic Study

Alterations in serotonin, transient receptor potential channels and protease-activated receptors in rats with irritable bowel syndrome attenuated by Shugan decoction

Hai-Lian Shi, Chu-Hsuan Liu, Li-Li Ding, Yu Zheng, Xiao-Yan Fei, Lu Lu, Xue-Ming Zhou, Jian-Ye Yuan, Jian-Qun Xie

Hai-Lian Shi, Chu-Hsuan Liu, Yu Zheng, Xiao-Yan Fei, Lu Lu, Jian-Ye Yuan, Jian-Qun Xie, Institute of Digestive Diseases, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China
Hai-Lian Shi, Li-Li Ding, Institute of Chinese Materia Medica, Shanghai Key Laboratory of Complex Prescription, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China
Xue-Ming Zhou, Experimental Animal Center, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China
Author contributions: Shi HL and Liu CH carried out all the experiments, analyzed the data and wrote the paper, and contributed equally to this study; Ding LL performed the HPLC analysis; Yuan JY and Xie JQ designed the experiments, analyzed the data, revised the paper, and contributed equally to this study; Zheng Y, Fei XY, Lu L and Zhou XM performed parts of the experiments and provided valuable suggestions for this study; all authors have read and approved the final manuscript.
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Correspondence to: Jian-Ye Yuan, Associate Professor, Institute of Digestive Diseases, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, 530 Lingling Road, Xuhui District, Shanghai 200032, China. yuanjianye@hotmail.com
Telephone: +86-21-64385700
Fax: +86-21-64398310
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Abstract

AIM: To determine the molecular mechanisms of Shugan decoction (SGD) in the regulation of colonic motility and visceral hyperalgesia (VHL) in irritable bowel syndrome (IBS).

METHODS: The chemical compounds contained in SGD were measured by high-performance liquid chromatography. A rat model of IBS was induced by chronic water avoidance stress (WAS). The number of fecal pellets was counted after WAS and the pain pressure threshold was measured by colorectal distension. Morphological changes in colonic mucosa were detected by hematoxylin-eosin staining. The contents of tumor necrosis factor (TNF)-α in colonic tissue and calcitonin-gene-related peptide (CGRP) in serum were measured by ELISA. The protein expression of serotonin [5-hydroxytryptamide (5-HT)], serotonin transporter (SERT), chromogranin A (CgA) and CGRP in

colon tissue was measured by immunohistochemistry.

RESULTS: SGD inhibited colonic motility dysfunction and VHL in rats with IBS. Blockers of transient receptor potential (TRP) vanilloid 1 (TRPV1) (Ruthenium Red) and TRP ankyrin-1 (TRPA1) (HC-030031) and activator of protease-activated receptor (PAR)4 increased the pain pressure threshold, whereas activators of PAR2 and TRPV4 decreased the pain pressure threshold in rats with IBS. The effect of SGD on pain pressure threshold in these rats was abolished by activators of TRPV1 (capsaicin), TRPV4 (RN1747), TRPA1 (Polygodial) and PAR2 (AC55541). In addition, CGRP levels in serum and colonic tissue were both increased in these rats. TNF-α level in colonic tissue was also significantly upregulated. However, the levels of 5-HT, SERT and CgA in colonic tissue were decreased. All these pathological changes in rats with IBS were attenuated by SGD.

CONCLUSION: SGD alleviated VHL and attenuated colon motility in IBS, partly by regulating TRPV1, TRPV4, TRPA1, PAR2, 5-HT, CgA and SERT, and reducing CGRP and TNF-α level.

Key words: Shugan decoction; Visceral hyperalgesia; Serotonin; Transient receptor potential; Protease-activated receptor; Serotonin transporter; Calcitonin-gene-related peptide; Tumor necrosis factor-α

Core tip: The present study demonstrated that Shugan decoction alleviated visceral hyperalgesia and attenuated colon motility in a rat model of irritable bowel syndrome, partly by regulating the expression of transient receptor potential (TRP) vanilloid 1, TRP vanilloid 4, TRP ankyrin-1 and protease-activated receptor 2, serotonin, chromogranin A and serotonin transporter, and reducing the levels of calcitonin-gene-related peptide and tumor necrosis factor-α.

Shi HL, Liu CH, Ding LL, Zheng Y, Fei XY, Lu L, Zhou XM, Yuan JY, Xie JQ. Alterations in serotonin, transient receptor potential channels and protease-activated receptors in rats with irritable bowel syndrome attenuated by Shugan decoction. *World J Gastroenterol* 2015; 21(16): 4852-4863 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4852.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4852

INTRODUCTION

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder characterized by altered bowel evacuation, bloating and visceral pain, without anatomical or biochemical abnormalities[1,2]. The pathological mechanism of IBS is not well understood. In recent years, laxatives or antidiarrheal agents, with or without spasmolytics, as well as serotonergic agents [*e.g.*, tegaserod, a serotonin 5-hydroxytryptamine (5-HT_4) agonist, and alosetron, a 5-HT_3 antagonist], have achieved some therapeutic effects, but have not obtained satisfactory results due to their adverse effects[3]. Thus, to date, there are no standard therapeutic agents to treat IBS. Acupuncture and traditional Chinese medicine (TCM) have attracted increased attention due to their potential in the treatment of IBS. The efficacy of acupuncture on visceral hypersensitivity and related motility dysfunction in IBS has been demonstrated[4]. In China, there are many clinical formulas of TCM used in the treatment of IBS[2,5], for example, TongXieYaoFang (TXYF), is a classic TCM prescription[2] consisting of white attractylodes rhizome (Baizhu), white peony root (Baishao), dried old orange peel (Chenpi) and Ledebouriella root (Fangfeng). Shugan decoction (SGD) is another formula used to treat patients with diarrhea-predominant IBS, which is composed of TXYF and *Radix bupleuri* (Chaihu), and can alleviate visceral pain and improve bowel habits[6,7]. SGD shows an excellent therapeutic effect on patients who lack coordination between the liver and the spleen (TCM syndrome type)[6]. However, the functional mechanism of SGD is poorly understood.

Visceral hypersensitivity and gastrointestinal dysmotility are two primary characteristics of IBS. Transient receptor potential vanilloid type 1 (TRPV1) and TRP ankyrin-1 (TRPA1) have been shown to play an important role in visceral hypersensitivity, and are correlated with severity of abdominal pain in patients with IBS and inflammatory bowel disease (IBD), as well as IBS-like symptoms in rats with water avoidance stress (WAS)[8-12]. Alterations in protease-activated receptor (PAR)2 and PAR4 have also been indicated in visceral pain in IBS patients and stressed mice[13-16]. Serotonin (5-HT) in both the central and peripheral nervous systems, is involved in visceral hypersensitivity and gut motility disorders which can be attenuated by acupuncture and TCM[2,4,5,17]. Although their mechanisms have not been fully elucidated, regulation of the 5-HT signaling pathway has been shown to play an important role in relieving the symptoms of IBS. In recent studies, it was demonstrated that many types of endocrine cells are affected in IBS patients[18-20]. Lower densities of cells positive for 5-HT and peptide YY are found in the colon of patients with IBS, compared with healthy individuals. In addition, the density of chromogranin A (CgA) is reduced in the colon of patients with IBS[18-20].

A suitable animal model is important for elucidating the pathophysiological process of IBS. Previous studies have shown that repeated exposure to WAS induces sustained visceral hypersensitivity and gastrointestinal motility disorders in rats[21]. Therefore, WAS-exposed rats could reflect the typical characteristics of IBS. In the present study, the efficacy of SGD and its underlying mechanisms of action on visceral hypersensitivity and

Table 1 Formulation of Shugan decoction and TongXieYaoFang

Chinese name	Latin name	Common name	Drug (dry, g)	Formula names	
Baizhu	*Rhizoma Atractylodis Macrocephalae*	White attractylodes rhizome	9.0	TongXieYaoFang	Shugan decoction
Baishao	*Radix Paeoniae Alba*	white peony root	6.0		
Chenpi	*Pericarpium Citri Reticulatae*	dried old orange peel	6.0		
Fangfeng	*Radix Saposhnikoviae*	Ledebouriella root	4.5		
Chaihu	*Radix bupleuri*	Radix bupleuri	6.0		

colonic dysmotility were evaluated using WAS in a rat model of IBS.

MATERIALS AND METHODS

Drug and materials

White attractylodes rhizome (Baizhu), white peony root (Baishao), dried old orange peel (Chenpi), ledebouriella root (Fangfeng), and *Radix bupleuri* (Chaihu) were purchased as crude herbs from the Yanghetang Pharmacy (Shanghai, China). Rabbit anti-CgA polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States); the S_{ABC} kit was purchased from Boston Biochem (Wuhan, China). Methanol was obtained from Merck (Darmstadt, Germany). Triple deionized water was from Millipore (Bedford, MA, United States) and was prepared for all aqueous solutions. Ruthenium Red, capsaicin, HC-030031, polygodial, AY-NH2, RN1747 and AC55541 were obtained from Tocris Bioscience (MI, United Kingdom). Saikosaponin d, penoniflorin, poncirin, synephrine, atractylenolide Ⅲ, isofraxidin, hesperidin and neohesperidin were purchased from Shanghai Research and Development Centre for Standardization of Chinese Medicines (Shanghai, China). Calcitonin-gene-related peptide (CGRP) ELISA kit was purchased from Cusabio Biotech (Wuhan, China). Tumor necrosis factor (TNF)-α ELISA kit was obtained from ExCell (Shanghai, China). Rabbit anti-5-HT antibody was obtained from ShangHai Xiangsheng Biotechnology Co. Ltd. (Shanghai, China).

Preparation of SGD and TXYF extracts

SGD is comprised of five types of herbs with different mass ratios, which are listed in Table 1, and the total mass used was 31.5 g (common dose for adult humans). TXYF contained four types of herbs with a total mass of 25.5 g dry herbs, which are also listed in Table 1. The dry powder of an aqueous extract of SGD or TXYF was prepared by the Herbal Chemistry Laboratory at Shanghai University of Traditional Chinese Medicine. SGD and TXYF extracts were prepared as follows. Briefly, 315 g crude SGD and 255 g crude TXYF were powdered and soaked in 8 volumes of water for 12 h, boiled for 2 h, filtered, and the liquid collected. The procedure was then repeated. The liquid from the two procedures was mixed together and evaporated under vacuum until the aqueous phase was extracted completely. After drying in a vacuum drying oven, 125 g SGD powder and 100 g TXYF powder were obtained separately.

Chromatography analysis

The samples were determined using the Agilent 1100 chromatographic system, consisting of a solvent degasser, a quaternary gradient pump, a diode array detector, and a data station with analytical software (Chemstation 8.03; Agilent, SF, CA, United States). The separation was performed on a Kromasil C18 analytical column (250 mm × 4.6 mm, 5 μm) and maintained at 25 ℃. The mobile phase was a mixture of methanol/0.1% phosphoric acid aqueous solution (85:15, v:v) with a flow rate of 1 mL/min. The injection volume was 10 μL and the detection wavelength was set at 210 nm. The standard solutions of saikosaponin d, penoniflorin, poncirin and atractylenolide Ⅲ were prepared with methanol.

Animals and treatments

Male Sprague-Dawley rats (150-200 g body weight) were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China), and fed in the Experimental Animal Center of Shanghai University of Traditional Chinese Medicine. All animals were maintained at 22 ℃ with a 12-h light/dark cycle and had free access to rodent chow and water. All animal experiments were performed according to the protocols approved by the Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine. The rats were randomly divided into the control and WAS groups. After 3 d, the WAS rats were randomly divided into five subgroups: (1) IBS model group; (2) SGD-H group (1.97 g/kg); (3) SGD-M group (0.98 g/kg); (4) SGD-L group (0.49 g/kg); and (5) TXYF group (0.68 g/kg). SGD or TXYF extract was dissolved in distilled water and rats were treated by intragastric administration 1 h before WAS on day 4 and maintained for 7 d. The rats in the control and model groups were treated with distilled water.

WAS

WAS was induced as described previously[21], with minor modifications. The rats were placed on an island-like plastic platform (10 cm × 8 cm × 8 cm), which was fixed in the center of a plexiglass pool (45 cm × 25 cm × 25 cm) filled with fresh tap water (25 ℃) up to 7 cm (1 cm below the top of the platform), for 1 h once daily and repeated for 10 consecutive days.

Stress procedures were performed between 09:00 and 11:30 am to minimize diurnal variations in response.

Measurement of fecal pellet output

Fecal pellet output was used to estimate distal colonic motility as a validated index[22]. Fecal pellets found in the pool were counted at the end of 1 h WAS. Control animals were left in the home cage for 1 h to count the fecal pellets.

Colorectal distension and pain pressure threshold

Colorectal distension (CRD) induces contraction of the abdominal and hind limb muscles, and is termed abdominal withdrawal reflex (AWR), which has been validated as a quantitative method to determine visceral sensitivity in rats[10,23]. Behavioral responses to CRD were assessed by measuring the pressure threshold of CRD, that is, the minimum pressure that induced the first AWR. Immediately after the end of 1 h WAS on the 10th day, the rats were lightly anesthetized with ether while a deflated latex balloon (5 mm diameter at full inflation) was inserted intra-anally into the descending colon and rectum with its end 1 cm proximal to the anal sphincter. The balloon catheter (2 mm diameter) was fixed at the root of the tail. The rats were then placed into a small Lucite cubicle (20 cm × 8 cm × 8 cm) and allowed to recover from anesthesia for 30 min before testing. To measure the pressure threshold of CRD, the colorectal balloon was progressively inflated with an increment of 5 mmHg until pain behavior was observed, namely, at the first sign of obvious contraction in the abdominal wall. The measurement was repeated three times with at least 5-min intervals for recovery. During the measurement, the investigators were blinded to the treatment groups.

Hematoxylin-eosin staining

Rats were deeply anesthetized with sodium pentobarbital, and the colonic tissues were excised and fixed in 10% formaldehyde solution. After being paraffin embedded and sectioned, the paraffin slides were deparaffinized in xylene Ⅰ, Ⅱ and Ⅲ for 15, 10 and 10 min, respectively, and dehydrated in 100%, 95%, 85% and 75% ethanol for 5 min, respectively. The sections were stained with hematoxylin-eosin (HE), dehydrated in 95%, 85% and 75% ethanol, cleared in xylene, and finally mounted with Permount Mounting Medium (Shanghai, China). Morphological changes in the colonic mucosa were observed and photographed under a light microscope (200 × objective) equipped with a Nikon color digital camera system.

ELISA

Blood samples were centrifuged at 3000 rpm, at 4 ℃ for 10 min. Serum was collected and immediately frozen in liquid nitrogen and stored at -80 ℃ for further study. The frozen colonic tissues were homogenized and lysed in tissue lysis buffer, and centrifuged at 12000 *g*, at 4 ℃ for 10 min. The supernatant was collected. The level of calcitonin-gene-related peptide (CGRP) in serum was measured by ELISA (Cusabio Biotech), and the level of TNF-α in colonic tissue was determined by ELISA (ExCell), according to the manufacturer's instructions.

Immunohistochemistry

The sections were deparaffinized in xylene and an ethanol gradient. The endogenous peroxidase was quenched in methanol: 30% H_2O_2 (9:1) for 10 min, and microwaved for 10 min in citrate buffer (10 mmol/L, pH 6.0) for antigen retrieval. Sections were blocked in 5% bovine serum albumin at 37 ℃ for 1 h, followed by incubation with goat anti-rabbit polyclonal CgA antibody (Santa Cruz Biotechnology) at 1:30 dilution, or rabbit anti-5-HT polyclonal antibody (Xiangsheng, Shanghai, China) at 1:100 dilution overnight at 4 ℃, then followed by incubation with secondary antibody-peroxidase conjugate at 37 ℃ for 1 h. PBS was used for washing after each step. Subsequent visualization was performed using diaminobenzidine as a chromogen (Dako, Denmark).

Positive expression was displayed by brown color, and the cell nuclei were stained blue by hematoxylin. Positive expression of CgA and 5-HT in the colon was observed and photographed under a light microscope at × 200 magnification. Five randomly selected × 200 magnification fields were evaluated using a BH2 microscope (Olympus, Tokyo, Japan) equipped with a Nikon 4500 digital camera. The area and optical density (OD) of CgA- and 5-HT-positive cells in each field were counted and processed by the computer-aided automatic image analysis system (Qiu Wei, Shanghai, China). The immunohistochemical index was calculated as the average integral OD: [(positive area × OD)/ total area].

Statistical analysis

Each value represents mean ± SD. Differences between two groups were analyzed using the Student's *t* test, and statistical comparisons among more than two groups were subjected to One-way analysis of variance (ANOVA) followed by Dunnett's test using GraphPad Prism 5.0. $P < 0.05$ was considered statistically significant.

RESULTS

Chemical compounds in water extract of SGD

The ingredients in the water extract of SGD were determined by high-performance liquid chromatography (HPLC) analysis. Although many chemical compounds were detected by HPLC-MS/MS analysis (data not shown), we could not obtain all the standard chemical compounds shown in the HPLC-MS/MS analysis, thus, we were only able to confirm the following chemical ingredients in the water extract of SGD: saikosaponin d, penoniflorin, poncirin, and atractylenolide Ⅲ (Figure 1). The other chemical ingredients should be analyzed further.

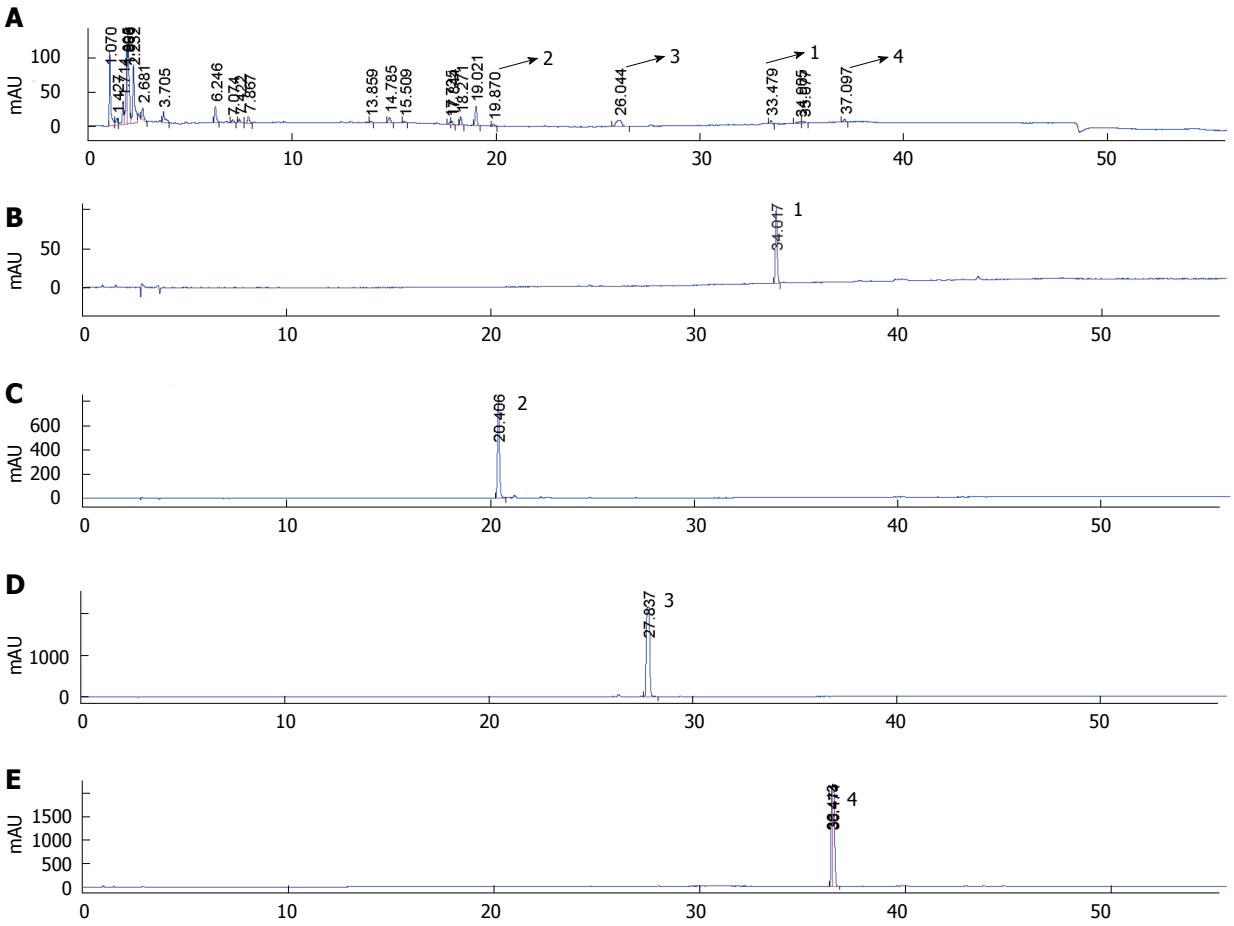


Figure 1 Analysis of the chemical constituents of Shugan decoction by high-performance liquid chromatography. HPLC chromatogram of SGD and standard reference monitored at 210 nm; Peak 1: saikosaponin d; Peak 2: penoniflorin; Peak 3: poncirin; Peak 4: atractylenolide Ⅲ. A: HPLC chromatogram of SGD; B: HPLC chromatogram of saikosaponin d; C: HPLC chromatogram of penoniflorin; D: HPLC chromatogram of poncirin; E: HPLC chromatogram of atractylenolide Ⅲ. SGD: Shugan decoction; HPLC: High-performance liquid chromatography.

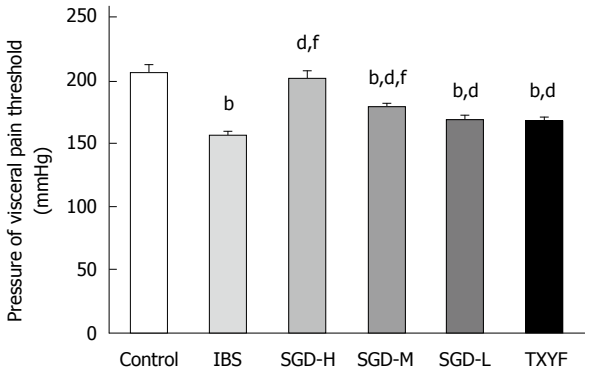


Figure 2 Abdominal withdrawal reflex pressure threshold measured in response to colorectal distension. Data are presented as mean ± SD, $^{b}P < 0.01$ *vs* control; $^{d}P < 0.01$ *vs* irritable bowel syndrome; $^{f}P < 0.01$ *vs* TongXieYaoFang. Statistical comparisons were performed using One-way ANOVA followed by Dunnett's test using GraphPad Prism 5.0. (n = 9 or 10). IBS: Irritable bowel syndrome; SGD: Shugan decoction; TXYF: TongXieYaoFang.

Effects of water extract of SGD on WAS-induced visceral hyperalgesia and colon hypermotility

At the end of 10 d of WAS stimulation, the AWR pressure threshold of CRD was measured. The AWR pressure threshold of rats in the IBS model group was significantly lower than that of rats in the control group ($P < 0.01$), which demonstrated that visceral hyperalgesia (VHL) was successfully induced by WAS (Figure 2). The AWR pressure threshold of SGD-treated rats was significantly higher than that of the IBS model rats ($P < 0.01$). TXYF was able to reverse the AWR pressure threshold in model rats ($P < 0.01$). However, SGD-H and SGD-M had a greater effect on AWR pressure threshold than TXYF ($P < 0.01$, $P < 0.01$, respectively).

As shown in Figure 3, compared with control rats, the number of fecal pellets produced by model rats was significantly increased ($P < 0.01$), then attenuated by the water extract of SGD ($P < 0.01$). TXYF also decreased the number of fecal pellets produced by model rats ($P < 0.01$). However, SGD-H had a better effect on fecal pellet output than TXYF ($P < 0.05$).

TRP channels and PARs involved in visceral hypersensitivity induced by WAS

Blockers of TRPV1 (Ruthenium Red) and TRPA1 (HC-030031), or the PAR4 agonist (AY-NH2) increased the AWR pressure threshold in IBS model rats ($P <$

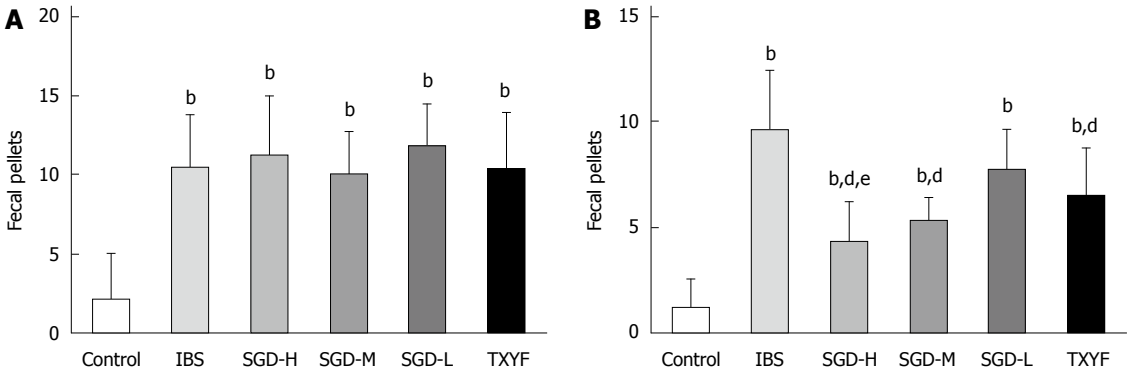


Figure 3 Colon transit measured by fecal pellet output in 1 h. A: Colon transit measured by fecal pellet output in 1 h on day 3; B: Colon transit measured by fecal pellet output in 1 h on day 10. Data are presented as mean ± SD, $^{b}P < 0.01$ *vs* control; $^{d}P < 0.01$ *vs* irritable bowel syndrome; $^{e}P < 0.05$ *vs* TongXieYaoFang. Statistical comparisons were performed using One-way ANOVA followed by Dunnett's test using GraphPad Prism 5.0. (n = 10). IBS: Irritable bowel syndrome; SGD: Shugan decoction; TXYF: TongXieYaoFang.

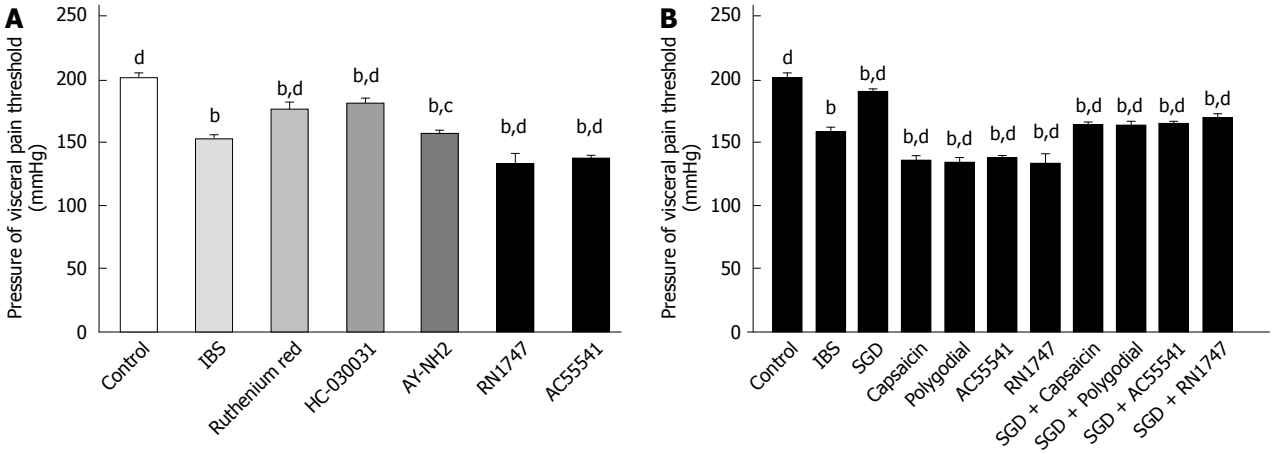


Figure 4 Transient receptor potential channels and protease-activated receptors involved in water avoidance stress-induced visceral hyperalgesia. A: TRP channels and PARs involved in WAS-induced VHL; B: TRP channels and PARs involved in the effect of SGD on VHL. The blockers or activators of TRP channels and PARs were given by intraperitoneal injection after WAS on day 10. One hour later, rats were subjected to colorectal distension and AWR pressure threshold measurement. Ruthenium Red: blocker of TRPV1, 5 mg/kg; HC-030031: blocker of TRPA1, 5 mg/kg; AY-NH2: activator of PAR4, 1 mg/kg; RN1747: activator of TRPV4, 5 mg/kg; AC55541: activator of PAR2, 0.78 mg/kg; capsaicin: activator of TRPV1, 5 mg/kg; Polygodial: activator of TRPA1, 5 mg/kg. Ruthenium Red was dissolved in distilled water. Other blockers or activators were dissolved in 10% dimethylsulfoxide/5% Tween 80/ 85% saline. Data are presented as mean ± SD, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* IBS. Statistical comparisons were performed using One-way ANOVA followed by Dunnett's test using GraphPad Prism 5.0. (n = 5-7). TRP: Transient receptor potential; PAR: protease-activated receptor; WAS: Water avoidance stress; VHL: Visceral hyperalgesia; IBS: Irritable bowel syndrome; SGD: Shugan decoction.

0.01, $P < 0.01$, $P < 0.05$, respectively) (Figure 4A). In contrast, agonists of PAR2 (AC55541), TRPV1 (capsaicin), TRPV4 (RN1747) and TRPA1 (polygodial) decreased AWR pressure threshold in model rats ($P < 0.01$, $P < 0.01$, $P < 0.01$, $P < 0.01$, respectively) (Figure 4A and B). The effect of SGD on AWR pressure threshold was weakened or abolished by agonists of PAR2 (AC55541), TRPV1 (capsaicin), TRPV4 (RN1747) and TRPA1 (polygodial) ($P < 0.01$, $P < 0.01$, $P < 0.01$, $P < 0.01$, respectively).

Effects of water extract of SGD on CGRP level in serum and colonic mucosa of WAS rats

ELISA and immunohistochemistry indicated that CGRP in serum ($P < 0.01$) or in colonic tissue of WAS rats was elevated, compared with control rats. The water extract of SGD and TXYF reduced CGRP expression in serum ($P < 0.05$, $P < 0.01$, respectively) (Figure 5A) and colonic tissue (Figure 5B) of WAS rats.

5-HT and CgA expression in colonic mucosa of WAS rats

To determine whether the expression of 5-HT and CgA in the colonic tissue of rats was affected by WAS, the expression of 5-HT and CgA was detected by immunohistochemistry. As shown in Figure 6, after repeated exposure to WAS, the expression of 5-HT or CgA in colonic mucosa of IBS model rats was lower than that in the colonic mucosa of control rats ($P < 0.05$, $P < 0.05$, respectively). Treatment with the water extract of SGD increased expression of 5-HT and CgA in the colonic mucosa of IBS model rats ($P < 0.01$, $P < 0.01$, respectively). TXYF increased the expression of CgA ($P < 0.01$), but not 5-HT ($P > 0.05$) in colonic mucosa.

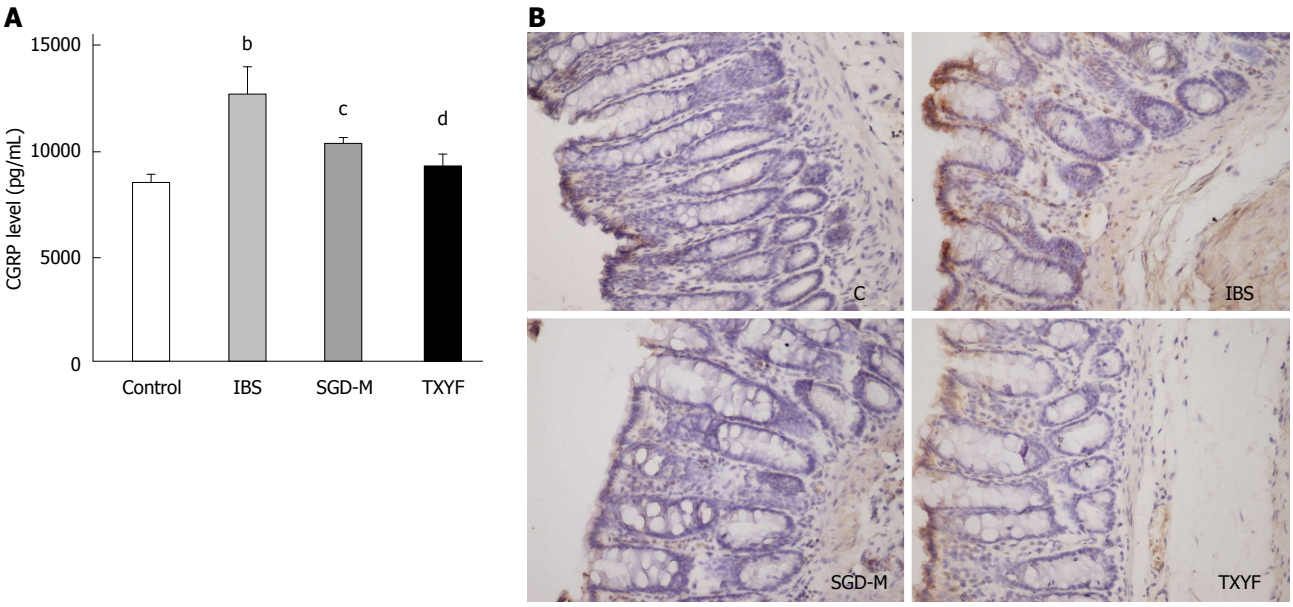
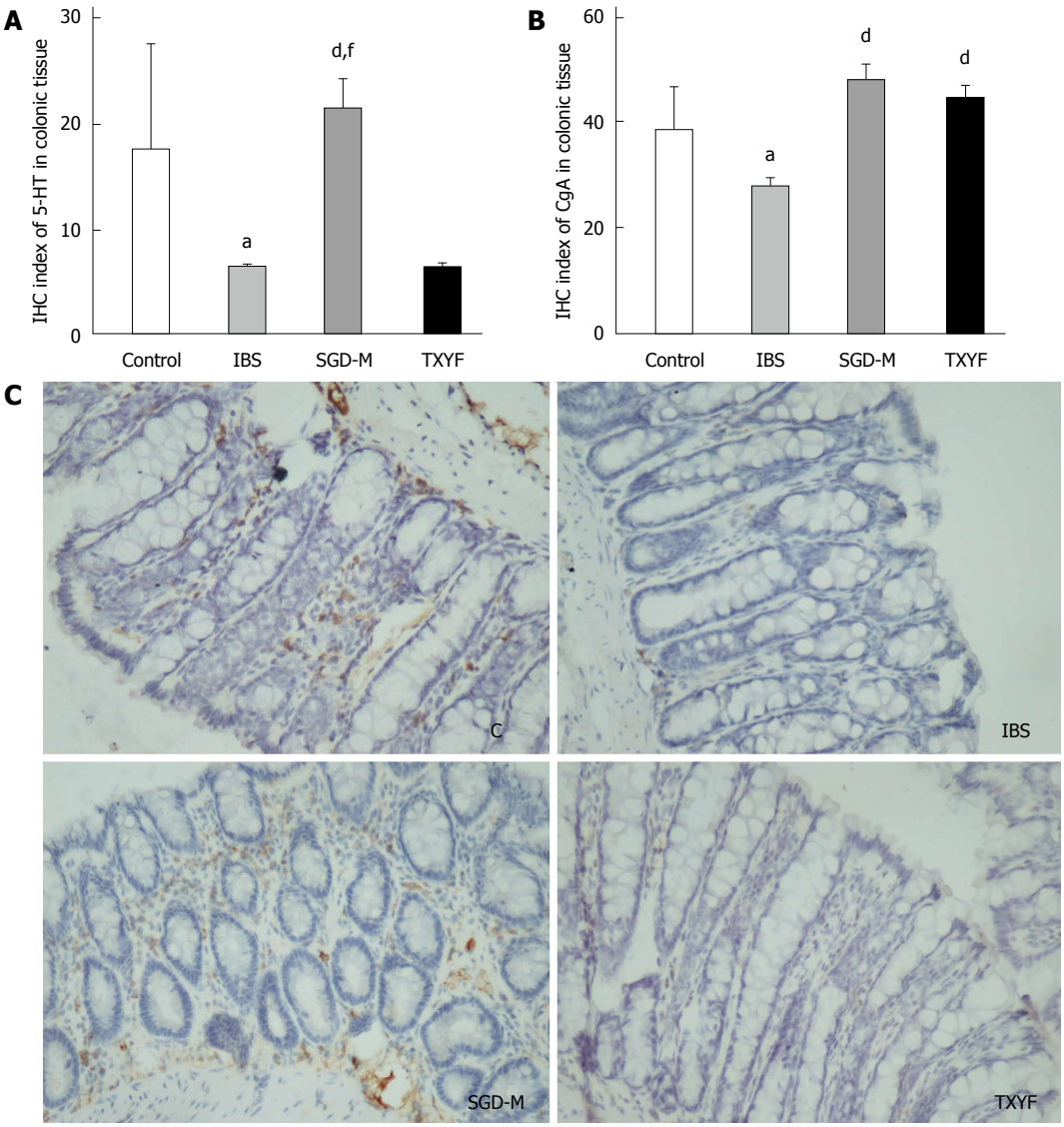


Figure 5 Calcitonin-gene-related peptide level in serum and colonic tissues. A: CGRP level in serum measured by ELISA; B: CGRP expression in colonic tissues measured by immunochemistry. Data are presented as mean ± SD, $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* IBS. Statistical comparisons were performed using One-way ANOVA followed by Dunnett's test using GraphPad Prism 5.0. (n = 3 or 4). CGRP: Calcitonin-gene-related peptide; IBS: Irritable bowel syndrome; SGD: Shugan decoction; TXYF: TongXieYaoFang.



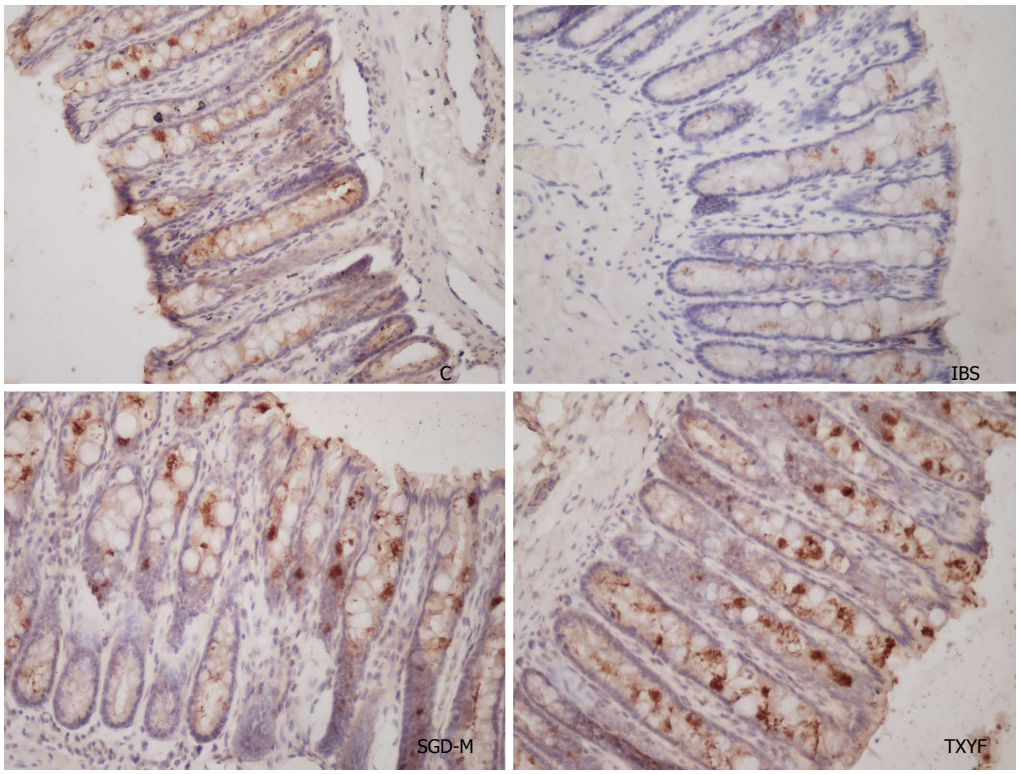


Figure 6 Endocrine cells in colonic tissues. A, C: 5-HT levels in colonic tissues measured by IHC; B, D: CgA expression in colon tissues measured by immunohistochemistry. Data are presented as mean ± SD, $^{a}P < 0.05$ *vs* control; $^{d}P < 0.01$ *vs* IBS, Statistical comparisons were performed using One-way ANOVA followed by Dunnett's test using GraphPad Prism 5.0.; $^{f}P < 0.01$ *vs* TXYF, difference between SGD-M and TXYF was analyzed by Student's *t* test using GraphPad Prism 5.0. (n = 3-5). 5-HT: 5-hydroxytryptamide; IHC: Immunohistochemistry; IBS: Irritable bowel syndrome; SGD: Shugan decoction; TXYF: TongXieYaoFang.

Protein expression of serotonin transporter in colonic mucosa of WAS rats

Figure 7 shows that the protein expression of serotonin transporter (SERT) was significantly decreased in colonic tissues of IBS model rats, compared with control rats. After treatment with the water extract of SGD or TXYF, SERT expression in colonic tissues was increased.

TNF-α level in colonic tissue or serum of WAS rats

Although HE staining showed no inflammation in the colonic tissues of rats with chronic WAS (data not shown), Figure 8 shows that the level of TNF-α in colonic tissue of IBS model rats was higher than that in control rats ($P < 0.05$). The water extract of SGD, but not TXYF significantly reduced the TNF-α level in colonic tissue of WAS rats ($P < 0.05$, $P > 0.05$, respectively).

DISCUSSION

In this study, we found that the water extract of SGD attenuated VHL by inhibiting the activation of TRPV1, TRPV4, TRPA1 and PAR2 and regulating 5-HT, SERT and CgA expression, as well as TNF-α and CGRP levels, which may contribute to the improvement in VHL and abnormal colonic motility in WAS rats.

Currently, the detailed pathological mechanism of IBS is not clear. Due to the difficulty in removing colonic tissue samples from IBS patients, animal models of IBS are pivotal in clarifying the pathogenesis of IBS. To date, repeated WAS and neonatal maternal separation have been used to create animal models of IBS to study the pathological process or to evaluate the efficacy of western medicines or TCM[21,23]. In previous studies, rats with repeated exposure to WAS developed persistent VHL, exhibited anxiety-like behaviors, and increased fecal pellet output[21,23]. In our study, the AWR pressure threshold in response to CRD in model rats was lower than that in control rats, which indicated that the WAS rats suffered from VHL. The number of fecal pellets excreted after 1 h exposure to WAS in model rats was increased compared with control rats, which showed that abnormal colonic motility was present in the model rats. The water extract of SGD significantly improved this dysfunction (visceral hypersensitivity and abnormal colonic motility).

Abnormal pain perception or visceral hypersensitivity is considered to be an important pathological basis underlying the symptoms of some IBS patients[11]. The mechanism of action of SGD on VHL was also investigated in the present study. TRPV1 is widely expressed in the colonic afferent dorsal root ganglia (DRG) neurons and throughout the gastrointestinal tract. TRPV1 is a ligand-gated ion channel that modulates the sensation of pain and thermal hyperalgesia as well as colonic hypersensitivity[24,25]. TRPA1 is

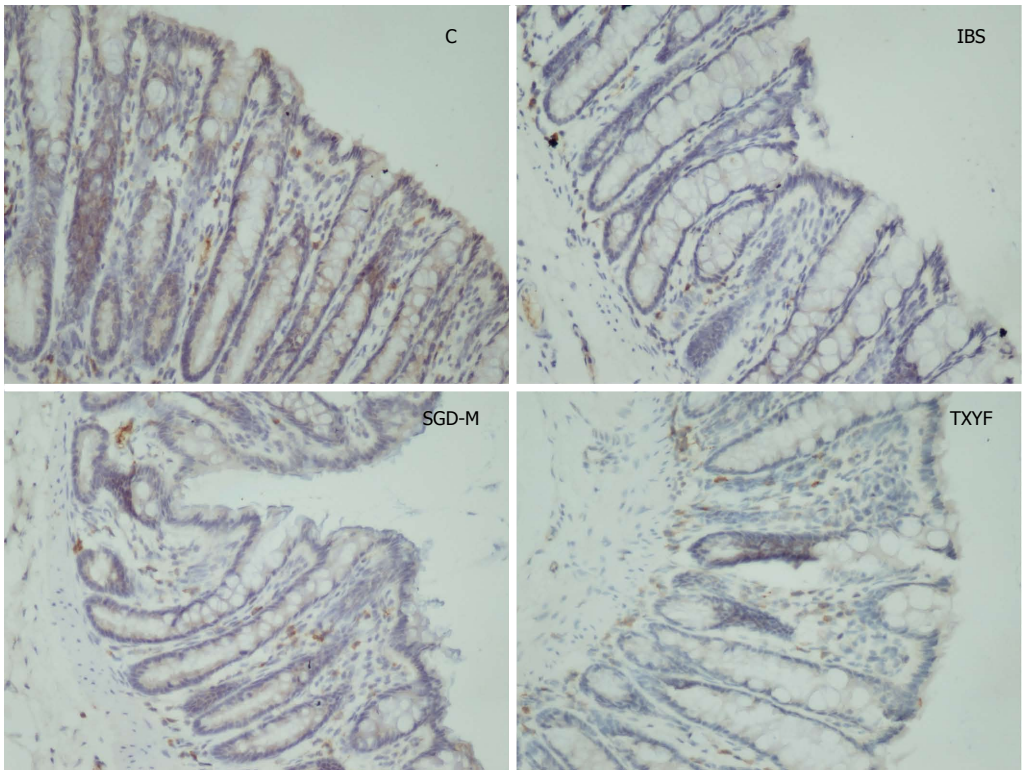


Figure 7 Serotonin transporter expression in colon tissue measured by immunochemistry. IBS: Irritable bowel syndrome; SGD: Shugan decoction; TXYF: TongXieYaoFang.

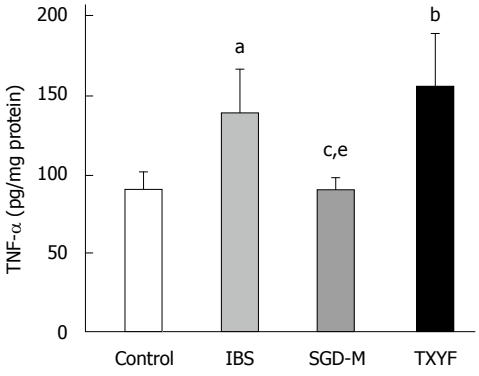


Figure 8 Tumor necrosis factor-α level in colonic tissue. Data are presented as mean ± SD, $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control, Statistical comparisons were performed using One-way ANOVA followed by Dunnett's test using GraphPad Prism 5.0.; $^{c}P < 0.05$ *vs* IBS; $^{e}P < 0.05$ *vs* TXYF, difference between SGD-M and TXYF was analyzed by the Student's t test using GraphPad Prism 5.0. ($n = 4$). TNF: Tumor necrosis factor; SGD: Shugan decoction; IBS: Irritable bowel syndrome; TXYF: TongXieYaoFang.

coexpressed with TRPV1 in primary sensory neurons[26], and has attracted the attention of researchers investigating gut sensation. TRPA1 deletion markedly reduces colitis-induced mechanical hyperalgesia in mice[27]. Moreover, activation of TRPA1 is proposed to contribute to the somatic hyperalgesia triggered by tissue damage and inflammation[28]. In WAS rats, protein expression of TRPV1 and TRPA1 in the colonic afferent DRG is significantly upregulated[10]. In our previous studies, increased expression of TRPV1 was also found in the DRG and colonic tissue of WAS rats with IBS-like symptoms[23,29]. Some studies have shown that increased TRPV1 expression is also present in the colonic tissue of IBS patients[11,30]. However, evidence of the involvement of TRPV1 in visceral perception in IBS and in WAS rats with IBS-like symptoms is still lacking. In our previous studies, SGD decreased the elevated expression of TRPV1, substance P and CGRP in the DRG and colonic tissue[23,29]. However, whether TRPV1 is involved in the therapeutic effect of SGD on VHL of WAS-induced IBS-like symptoms has not been clarified. In a neonatal maternal separation IBS murine model, expression of the TRPV4 gene was significantly increased[31]. However, the role of TRPV4 in visceral perception in IBS is not well understood. In the present study, blockers of TRPV1 and TRPA1 and an activator of TRPV4 showed that TRPV1, TRPV4 and TRPA1 are involved in WAS-induced VHL. PARs play an important role in the modulation of visceral pain[32]. PAR2 is highly expressed in the gastrointestinal tract and implicated in the genesis of hyperalgesia in a number of models of colitis[32]. Activation of PAR2 in the lumen affects visceral pain[33]. In rats, the intracolonic infusion of PAR2 agonists (SLIGRL and trypsin) induces delayed hypersensitivity to colonic distension[34]. In contrast, PAR4 may mediate antinociceptive activity. PAR4 activation significantly increases the nociceptive threshold in response to noxious stimuli[35]. Thus, blockade of PAR2 at the periphery and/or inhibition of colonic luminal protease activity may be new targets for treating gut hypersensitivity and IBS. In the present study, activators of PAR2 and PAR4 showed that

activation of PAR2, but not PAR4 was involved in WAS-induced VHL. However, PAR4 activation increased AWR pressure threshold. Moreover, activators of TRPV1, TRPV4, TRPA1 and PAR2 also demonstrated that SGD attenuated VHL in WAS rats by inhibiting TRPV1, TRPV4, TRPA1 and PAR2. Whether there is crosstalk between TRPV1, TRPV4, TRPA1 and PAR2 warrants further study.

5-HT is an important neurotransmitter which is involved in mediating psychological stress, VHL and gastrointestinal dysmotility by influencing the sympathetic, parasympathetic, and enteric nervous systems[18,36]. In healthy individuals, 5-HT is released mainly from enterochromaffin (EC) cells in the gastrointestinal tract and is inactivated *via* reuptake by SERT from the mucosa into nerve fibers[37]. Altered SERT expression and function in IBS could result in abdominal hypersensitivity and abnormal colonic motility[5,18]. Previous studies indicated that mucosal 5-HT and SERT were both decreased in ulcerative colitis, diarrhea-predominant IBS (IBS-D), and constipation-predominant IBS (IBS-C)[18]. However, Kerckhoffs *et al*[38] reported that IBS-C patients have increased mucosal 5-HT concentration and EC cell numbers, whereas IBS-D patients have reduced mucosal 5-HT concentration. Other studies have also shown that SERT expression was increased in the colon together with an increased concentration of 5-HT in neonatal maternally separated rats[5]. Thus, altered SERT expression in different states may be associated with different processes of IBS development, which should be investigated in further studies. In the present study, compared with control rats, expression of SERT and 5-HT was significantly decreased in the colonic mucosa of WAS rats. The water extract of SGD upregulated the expression of SERT and 5-HT in the colon. The above results indicated that the water extract of SGD regulated the expression of 5-HT and SERT to attenuate IBS-like symptoms. These results also suggested that the decreased level of 5-HT in the colon possibly resulted from reduced biosynthesis of 5-HT, but not a change in 5-HT reuptake by SERT.

Endocrine cells are also important in the pathological process of IBS[20]. Recent research demonstrated that colonic endocrine cells are altered in patients with IBS[18-20]. Increased numbers of mast cells in the terminal ileum are observed in patients with IBS[39]. It is reported that there are low densities of 5-HT-immunoreactive (EC) cells, peptide-YY-immunoreactive cells and CgA-immunoreactive (enteroendocrine) cells in the colon of patients with IBS[20]. In the present study, low expression of CgA and 5-HT was seen in the colonic mucosa of WAS rats, which indicated low densities of CgA-positive and 5-HT-positive endocrine cells. These changes were improved by the water extract of SGD.

Although HE staining results did not show inflammation in colonic tissue in model rats, the level of TNF-α in colonic tissue was significantly increased, but not serum TNF-α levels, and was decreased by the water extract of SGD. Foley *et al*[40] proved that TNF-α decreased SERT function and expression in Caco 2 cells. In the present study, the change in SERT and TNF-α was similar. However, whether there was a correlation between the expression of SERT and the level of TNF-α in this model requires clarification. In other research, TNF-α stimulated the expression and secretion of CGRP from rat trigeminal ganglion neurons[41]. In the present study, serum and colonic tissue CGRP levels were both increased in model rats after repeated exposure to WAS, accompanied by an increase in the level of TNF-α in colonic tissue. Following treatment with the water extract of SGD, the alterations in TNF-α and CGRP levels in model rats was attenuated.

In conclusion, the water extract of SGD enhanced the decreased AWR pressure threshold in response to CRD and attenuated abnormal colonic transit mainly by inhibiting TRPV1, TRPV4, TRPA1 and PAR2, but also by regulating 5-HT, SERT and CgA, as well as possibly reversing the imbalance in colonic 5-HT-positive and CgA-positive endocrine cells, thus decreasing the level of CGRP and TNF-α.

ACKNOWLEDGMENTS

We thank Dr. Ying Dong for help in statistical analysis.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder and not an organic disease. It is characterized by altered bowel evacuation, bloating and visceral pain, without anatomical or biochemical abnormalities. Currently, there are no standard drugs to treat IBS. Traditional Chinese medicine (TCM) has shown promising effects on IBS, however, the mechanism of action requires clarification. In this investigation, the efficacy of Shugan decoction (SGD) and its underlying mechanisms in IBS were evaluated in IBS-like rats with visceral hypersensitivity and colonic dysmotility.

Research frontiers

The pathological mechanism of IBS is not well understood. Visceral hypersensitivity and gastrointestinal dysmotility are two pivotal characteristics of IBS. Transient receptor potential vanilloid type 1 (TRPV1), TRP ankyrin-1 (TRPA1), protease-activated receptor (PAR)2 and PAR4, as well as serotonin [5-hydroxytryptamine (5-HT)] in the central and peripheral nervous systems play important roles in abdominal pain severity and gut motility disorders in IBS and inflammatory bowel disease. In the present study, SGD alleviated visceral hyperalgesia (VHL) and attenuated colon motility in model rats, partly by regulating TRPV1, TRPV4, TRPA1, PAR2, 5-HT, chromogranin A (CgA), serotonin transporter (SERT) and reducing calcitonin-gene-related peptide (CGRP) and tumor necrosis factor (TNF)-α level.

Innovations and breakthroughs

TRPV1, TRPA1, PAR2, PAR4, 5-HT and 5-HT-positive endocrine cells are involved in abdominal pain severity and gut motility disorders in IBS patients. The effect of TCM on the above molecules, as well as its functional mechanism, is poorly understood. In the present investigation, the water extract of SGD attenuated the decrease in pain pressure threshold in response to colorectal distension and attenuated abnormal colonic transit, mainly by inhibiting TRPV1, TRPV4, TRPA1 and PAR2, and regulating 5-HT, SERT and CgA, as well as possibly reversing the imbalance in colonic 5-HT-positive and CgA-positive endocrine cells, decreasing the level of CGRP and TNF-α.

Applications

Increased stress in daily life has led to an increase in IBS, and the development of therapeutic agents is urgently needed. TCM has some beneficial effects on IBS, however, its use in IBS patients is hindered by our lack of understanding

of its mechanism of action. In the present investigation, SGD relieved VHL and attenuated abnormal colonic transit, mainly by inhibiting TRPV1, TRPV4, TRPA1 and PAR2, regulating 5-HT, SERT and CgA, as well as possibly reversing the imbalance of colonic 5-HT-positive and CgA-positive endocrine cells, decreasing the level of CGRP and TNF-α. Thus, the present study shows the beneficial therapeutic effect and functional mechanism of TCM such as SGD in IBS patients.

Terminology

IBS is a functional gastrointestinal disorder, and is characterized by altered bowel evacuation, bloating and visceral pain, without anatomical or biochemical abnormalities. TongXieYaoFang (TXYF) is a classic TCM prescription and consists of white attractylodes rhizome (Baizhu), white peony root (Baishao), dried old orange peel (Chenpi) and Ledebouriella root (Fangfeng). SGD is another formula used to treat patients with diarrhea-predominant IBS, which is composed of TXYF and *Radix bupleuri* (Chaihu), and can alleviate visceral pain and improve the bowel habits of patients with a lack of coordination between the liver and the spleen (TCM syndrome type). TRPV1 is widely expressed in the colonic afferent dorsal root ganglia neurons and throughout the gastrointestinal tract. TRPV1 is a ligand-gated ion channel that modulates pain sensation and thermal hyperalgesia as well as colonic hypersensitivity. TRPA1 is coexpressed with TRPV1 in primary sensory neurons. PAR2 is highly expressed in the gastrointestinal tract and implicated in the genesis of hyperalgesia in a number of models of colitis. 5-HT is an important neurotransmitter, which alters visceral perception and motor dysfunction in IBS by influencing the sympathetic, parasympathetic, and enteric nervous systems.

Peer-review

The authors described Shugan-decoction attenuates the alteration of 5-HT, TRP channels and PAR receptors of rats exposed to water avoidance stress. The article is informative and well-presented.

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ORIGINAL ARTICLE

Basic Study

Inflammatory microenvironment and expression of chemokines in hepatocellular carcinoma

Ke-Qi Han, Xue-Qun He, Meng-Yu Ma, Xiao-Dong Guo, Xue-Min Zhang, Jie Chen, Hui Han, Wei-Wei Zhang, Quan-Gang Zhu, Hua Nian, Li-Jun Ma

Ke-Qi Han, Xue-Qun He, Meng-Yu Ma, Xiao-Dong Guo, Xue-Min Zhang, Jie Chen, Hui Han, Wei-Wei Zhang, Department of Oncology, Shanghai Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai 200336, China
Quan-Gang Zhu, Hua Nian, Department of Pharmacy, Shanghai Yueyang Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai 200336, China
Li-Jun Ma, Department of Oncology, Tongren Hospital of Shanghai Jiaotong University School of Medicine, Shanghai 200336, China
Author contributions: Han KQ and Ma LJ designed and performed the experiments; He XQ, Ma MY, Guo XD, Zhang XM, Chen J, Han H, Zhang WW, Zhu QG and Nian H provided vital reagents and analytical tools and were also involved in editing the manuscript; Han KQ and He XQ wrote the paper.
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Ethics approval: The study was reviewed and approved by the Institutional Review Board of Shanghai University of Traditional Chinese Medicine.
Institutional animal care and use committee: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (No. 20130413).
Animal care and use statement: The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for two weeks prior to experimentation. All animals were euthanized by barbiturate overdose (150 mg/kg pentobarbital sodium, iv) for tissue collection.
Conflict-of-interest: All the authors declare that they have no conflicts of interest.
Data sharing: No additional data are available.

Correspondence to: Dr. Li-Jun Ma, Department of Oncology, Tongren Hospital of Shanghai Jiaotong University School of Medicine, 1111 Xianxia Road, Shanghai 200336, China. ljma5@hotmail.com
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Abstract

AIM: To study the inflammatory microenvironment and expression of chemokines in hepatocellular carcinoma (HCC) in nude mice.

METHODS: CBRH-7919 HCC cells were injected into the subcutaneous region of nude mice. Beginning two weeks after the challenge, tumor growth was measured every week for six weeks. The stromal microenvironment and inflammatory cell infiltration was assessed by immunohistochemistry in paired tumor and adjacent peritumoral samples, and macrophage phenotype was assessed using double-stain immunohistochemistry incorporating expression of an intracellular enzyme. A chemokine PCR array, comprised of 98 genes, was used to screen differential gene expressions, which were validated by Western blotting. Additionally, expression of identified chemokines was knocked-down by RNA interference, and the effect on tumor growth was assessed.

RESULTS: Inflammatory cell infiltrates are a key feature of adjacent peritumoral tissues with increased macrophage, neutrophil, and T cell (specifically helper

and activated subsets) infiltration. Macrophages within adjacent peritumoral tissues express inducible nitric oxide synthase, suggestive of a proinflammatory phenotype. Fifty-one genes were identified in tumor tissues during the progression period, including 50 that were overexpressed (including *CXCL1*, *CXCL2* and *CXCL3*) and three that were underexpressed (*CXCR1*, *Ifg* and *Actb*). RNA interference of *CXCL1* in the CBRH-7919 cells decreased the growth of tumors in nude mice and inhibited expression of CXCL2, CXCL3 and interleukin-1β protein.

CONCLUSION: These findings suggest that *CXCL1* plays a critical role in tumor growth and may serve as a potential molecular target for use in HCC therapy.

Key words: Chemokines; Gene expression profile; Hepatocellular carcinoma; PCR array; RNA interference

Core tip: An orthotopic transplantation tumor model of hepatocellular carcinoma (HCC) with CBRH-7919 cells was established. Inflammatory cell infiltration and macrophage phenotype were assessed by immunohistochemistry. A chemokine PCR array was used to identify differentially expressed genes, and tumor growth was assessed after knockdown with RNA interference. This study describes the inflammatory microenvironment and differential expression of chemokines in hepatocellular carcinoma. The data suggest that *CXCL1* plays a critical role in tumor growth and may serve as a potential molecular target for use in HCC therapy.

Han KQ, He XQ, Ma MY, Guo XD, Zhang XM, Chen J, Han H, Zhang WW, Zhu QG, Nian H, Ma LJ. Inflammatory microenvironment and expression of chemokines in hepatocellular carcinoma. *World J Gastroenterol* 2015; 21(16): 4864-4874 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4864.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4864

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide, and the fifth leading cause of cancer-related deaths[1,2] due to its rapid growth, early metastasis, and relationships with chronic hepatitis. At the time of diagnosis, surgical resection remains the most effective treatment for early disease. However, more than 75% of patients relapse within five years, and the overall survival for HCC patients has not yet been improved[3].

Recent studies have indicated that the tumor inflammatory microenvironment plays an essential role in the progression of HCC. The tumor microenvironment plays a critical role in modulating the process of liver fibrosis, hepatocarcinogenesis, epithelial-mesenchymal transition, tumor invasion and metastasis[4-8]. The tumor microenvironment consists of: (1)hepatic stellate cells, fibroblasts, immune cells (including regulatory and cytotoxic T cells and tumor-associated macrophages), and endothelial cells; (2) growth actors (including transforming growth factor [TGF]-1 and platelet-derived growth factor); (3) proteolytic enzymes (such as matrix metalloproteinases and tissue inhibitor of metalloproteinases); and (4) extracellular matrix proteins and inflammatory cytokines. These play a critical role in HCC development, tumor control and response to treatment[9,10].

The aim of this study was to elucidate the mechanisms that underlie chronic inflammation in HCC disease progression in order to identify potential therapeutic targets. We also discuss the current understanding of each component of the tumor microenvironment and their roles in the pathogenesis of HCC. Thus, understanding the inflammatory microenvironment is critical to promote understanding of the molecular, cellular and pathophysiological mechanisms of HCC, and is essential for the development of new therapeutic strategies. Nevertheless, to date, the inflammatory microenvironment and differential expression pattern of chemokines in hepatocellular carcinoma is still not clear.

In this study, infiltration of inflammatory cells in the stromal microenvironment was assessed by immunohistochemistry in paired tumor and adjacent peritumoral samples. Macrophage phenotype was assessed using double-staining immunohistochemistry, incorporating expression of an intracellular enzyme. PCR array analysis was used to evaluate the expression profiles of chemokines and their receptors in subcutaneous CBRH-7919 cell xenograft tumors[11] and peritumoral tissues. The expression of chemokines identified by the PCR array were verified by Western blotting and immunohistochemistry. Additionally, knockdown of chemokines by RNA interference (RNAi) was used to assess the effect on tumor growth.

MATERIALS AND METHODS

Cell cultures

The human hepatocellular carcinoma cell line CBRH-7919 (Chinese Academy of Science, Shanghai, China) was used in this study. Cells were cultured at 37 ℃ in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco of Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 10% fetal bovine serum, 100 mg/mL penicillin G, and 50 μg/mL streptomycin (Life Technologies of Thermo Fisher Scientific).

Animal model

Male Balb/c nude mice were obtained from Laboratory

Figure 1 Establishment of mouse model with CBRH-7919 cells. A xenograft tumor is apparent in the subcutaneous abdominal region.

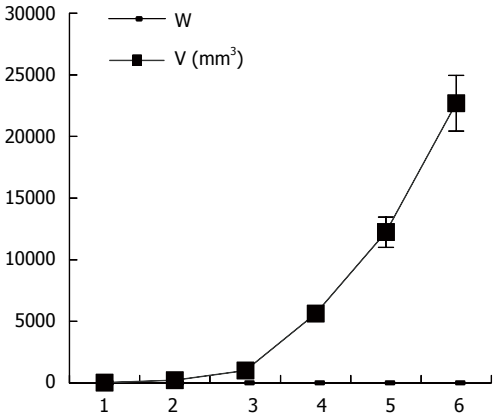

Figure 2 Tumor growth curve determined by the international veterinary information service system.

Animal Service Center of the Medical College of Shanghai. All mice were maintained under specific pathogen free conditions and had free access to sterilized food and autoclaved water. These experimental procedures were approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine.

Male Balb/c nude mice (4 wk of age, 15-18 g) were subcutaneously injected with 0.1 mL of a CBRH-7919 cell suspension (1×10^7 cells) using a 21-gauge needle. Mice were observed after two weeks, and their tumors were excised, weighed, and measured. A portion of the tumor tissue was fixed in 10% formalin for subsequent histologic examination, and the remaining tissue was snap frozen in liquid nitrogen and stored at -70 ℃ for molecular studies.

Observation of inflammatory cell infiltration and macrophage phenotype

Inflammatory cell phenotype was assessed by immunohistochemistry on three tumor tissues and adjacent normal biopsies. Tissue was stained using the Envision$^+$ biotin-free system[12] incorporating either the Envision$^+$ peroxidase-linked biotin-free system (K5007) or CSA Ⅱ biotin-free tyramide signal amplification (K1497; Dako of Agilent Technologies, Santa Clara, CA, United States) depending on antibody requirements. One microscopic high-powered field (HPF) from each sample was digitally imaged (× 640 magnification) with normal tissue as representative of tissue type, distinct from the area of most positive staining. The number of positive cells was counted to give a score of inflammatory cellular infiltrate.

Macrophage phenotype was also assessed in three pairs of tumor and adjacent tissue biopsies as described above. A double-stain immunohistochemical technique was used, incorporating detection of an intracellular enzymatic marker of macrophage function, namely [inducible nitric oxide synthase (iNOS), a proinflammatory classically activated macrophage) or arginase I (alternatively activated macrophage][13,14], identified by peroxidase-linked immunoreactivity. The area of most positive macrophage infiltration within one HPF (× 680 magnification), distinct from lymphoid aggregation, was identified under fluorescent light at 580 nm using a Texas red filter set and digitally imaged, and also captured under standard optical light. The two images were imported into Corel-Paint X3 (version 13; Corel Corp., Ottawa, CA) to assess macrophage infiltrate.

Histologic evaluation

Formalin-fixed tumors were embedded in paraffin, and 4 μm sections were cut and stained with hematoxylin and eosin (HE).

Isolation of total RNA

HCC tissue samples were dissolved with 1 mL of Trizol reagent (Invitrogen of Thermo Fisher Scientific) and homogenized; the sample volume did not exceed 10% of the volume of Trizol reagent. The homogenized samples were incubated at 15-30 ℃ for 5 min in clear polypropylene tubes to permit the complete dissociation of nucleoprotein complexes. Chloroform (0.2 mL) was added to the tube. After vigorous shaking, the mixture was incubated again at 15-30 ℃ for 2-3 min. After centrifugation at 12000 × *g* for 15 min at 4 ℃, the RNA in the aqueous phase was moved to a fresh RNA-free tube and mixed with 0.5 mL isopropyl alcohol. The samples were incubated at 15-30 ℃ for 10 min and centrifuged at 12000 × *g* for 10 min at 4 ℃. The supernatant was removed and the RNA pellet was washed once with 75% ethanol, redissolved in RNase-free water, and stored at -70 ℃. All samples were treated with MinElute (Qiagen, Vinlo, Netherlands) to remove residual DNA. The quality of the RNA was analyzed on an RNA chip by means of a bioanalyzer (model 2100; Agilent Technologies); the 260/280 ratio of array-tested RNA was 1.8-2.0.

OligoDNA microarray analysis

Files were extracted from Agilent Feature Extraction Software (version 9.5.3) and imported into the Agilent GeneSpring GX software (version 7.3 or later) for further analysis. The microarray datasets were normalized in GeneSpring GX using the Agilent FE one-color scenario (mainly median normalization). The positive effect of this median normalization is

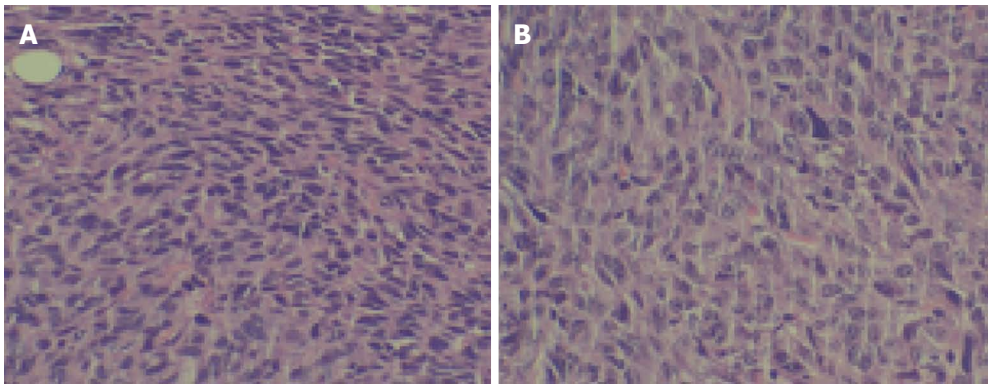

Figure 3 Tumor tissues from nude mice were stained with hematoxylin and eosin. A: Tumor tissue (× 200); B: Peritumor tissue (× 200).

Table 1 Observation of inflammatory cell phenotype in paired tumor and adjacent peritumoral samples, *n* = 3

Inflammatory cell	Tumor sample	Peritumoral samples	Mean difference	(95%CI)	*P*
T helper cell	12.09 ± 10.74	20.08 ± 13.31	7.53	(2.75-13.31)	0.005
Cytotoxic T cell	7.25 ± 8.07	6.05 ± 8.41	11.87	(21.60-2.14)	0.800
B cell	2.88 ± 6.36	4.31 ± 6.87	10.87	(19.60-4.07)	0.600
Activated T cell	2.12 ± 2.37	6.96 ± 5.17	5.10	(3.17-7.02)	0.005
NK cell	0.29 ± 1.30	2.30 ± 5.18	1.83	(0.21-3.45)	0.060
Macrophage	9.03 ± 9.97	19.76 ± 9.41	8.07	(4.00-12.13)	0.005
Mast cell	9.00 ± 5.35	10.44 ± 9.00	0.78	(21.07-3.09)	0.600
Neutrophil	1.56 ± 3.22	14.83 ± 14.76	13.28	(8.37-18.19)	0.005
Plasma cell	5.59 ± 7.11	6.50 ± 8.00	0.67	(20.16-4.14)	0.800
iNOS+ cells median (range)	0 (0-3)	10 (6-16)	-	-	0.005
Arginase I+ cells median (range)	1 (0-2)	0 (0-1)	-	-	0.005

Data are expressed as mean ± SD unless otherwise indicated. iNOS: Inducible nitric oxide synthase.

illustrated in a box-plot, and genes marked present ("All Targets Value") were chosen for data analysis. Finally, a fold-change analysis was carried out by calculating the ratio between the treatment and the control to identify differentially expressed genes. A cutoff value of twofold change was used; genes with expression levels that differed by at least twofold from the mean in at least one sample were selected for further evaluation. The gene expression profiling data complied with the Minimum Information About Microarray Experiments standard. The microarray experiment was completed by Shanghai KangChen Bio-tech Company (Shanghai, China).

Immunohistochemistry

Paraffin blocks were cut (6 μm sections), and sections were deparaffinized followed by antigen retrieval using citric acid buffer (pH 6.0, 95 ℃ for 15 min). Slides were treated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. After 20 min of blocking in 1% bovine serum albumin (BSA), the slides were incubated overnight at 4 ℃ with anti-human CXCL1 (Ab86436), CXCL2 (Ab25130), CXCL3 (Ab10064), and CXCR1 (Ab60254) antibodies (all goat polyclonal antibodies, 1:250 in 1% BSA; Abcam, Cambridge, United Kingdom). Next, the slides were incubated with 2 μg/mL of biotinylated anti-goat IgG secondary antibody (Vector Laboratories, Burlingame, CA) for 40 min at room temperature. Subsequently, the sections were stained using Standard Ultra-Sensitive ABC Peroxidase Staining kit (Pierce of Thermo Fisher Scientific) and 3,3′-diaminobenzidine (Vector Laboratories), and counterstained by hematoxylin. Mouse xenograft tumors from the hepatocellular cancer cell line CBRH-7919, known to stain strongly for CXCL1, CXCL2, CXCL3 and CXCR1[15] were used as a positive control.

Western blot analysis

HCC cell extracts from tumor and peritumoral tissues were analyzed using antibodies against CXCL1, CXCL2, CXCL3 and their receptor CXCR1. Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the intracellular amount of GAPDH was analyzed as a loading control. Finally, the immunoreactive bands were developed on X-ray film using an ECL Western Blotting Analysis System (Amersham, Buckinghamshire, United Kingdom) according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA was isolated as described above, and the concentration was measured by using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). RNA was converted to cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher

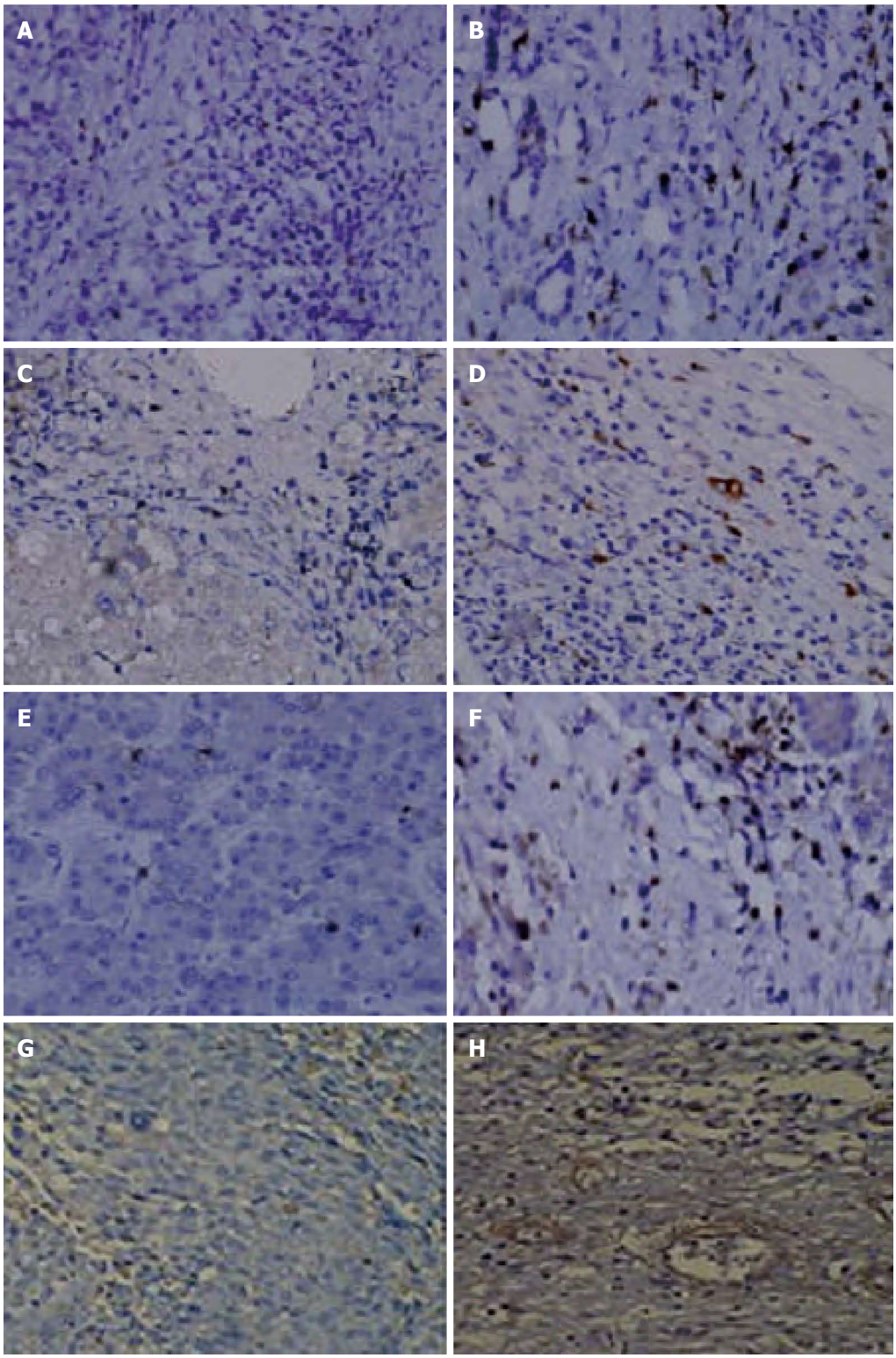

Figure 4 Inflammatory cell infiltration in tumors compared to adjacent peritumoral tissues. A, C, E: Peritumoral tissues; B: Increased macrophage infiltrate in tumor tissue; D: Increased neutrophil infiltrate in tumor tissue; F: CD25+, activated T cells were increased in tumor tissue.

Scientific). The level of CXCL1 mRNA expression was evaluated by qRT-PCR performed using DyNAmo ColorFlash SYBR Green qPCR Kit on an ABI 7300 system (Applied Biosystems of Thermo Fisher Scientific). The following primers were used: CXCL1, 5′-TAGAAGGTGTTGAGCGGGAAG-3′ (sense) and 5′-TGAGACGAGAAGGAGCATTGG-3′ (antisense); GAPDH, 5′-GTCGGTGTGAACGGATTTG-3′ (sense) and 5′-TCCCATTCTCAGCCTTGAC-3′ (antisense).

RNA interference

The siRNAs against CXCL1 were designed and ordered

Table 2 Upregulated chemokine-related genes in tumor tissues compared with peritumoral tissues

Accession No.	Gene name	Gene symbol	Fold change
A01	C5aR	*C5ar1*	8.86
A02	CCR10	*Ccbp2*	5.39
A04	Scya11	*Ccl11*	97.95
A05	Scya12	*Ccl12*	4.75
A07	CKb11	*Ccl19*	71.66
A08	HC11	*Ccl2*	25.81
A11	CKb-6	*Ccl24*	17.46
B06	MRP-1	*Ccl6*	20.28
B07	MCP-3	*Ccl7*	22.47
B08	MCP-2	*Ccl8*	16.51
B09	MRP-2	*Ccl9*	20.72
B10	Cmkbr1	*Ccr1*	5.2
B11	Cmkbr9	*Ccr10*	2.01
C01	Ccr2a	*Ccr2*	14.64
C02	CKR3	*Ccr3*	14.14
C04	CD195	*Ccr5*	9.77
C05	CCR-6	*Ccr6*	4.4
C11	mcmklrl	*Cmklr1*	8.96
C12	Cklf	*Cmtm2a*	2.25
D01	Cklfsf3	*Cmtm3*	28.58
D02	Cklfsf4	*Cmtm4*	2.94
D03	Cklfsf5	*Cmtm5*	4.14
D04	Cklfsf6	*Cmtm6*	5.27
D05	Cxc3	*Cx3cl1*	32.58
D07	KC	*Cxcl1*	23.49
D08	C7	*Cxcl10*	3.15
D09	Cxc11	*Cxcl11*	4.84
D10	Pbsf	*Cxcl12*	246.94
D12	KS1	*Cxcl14*	323.14
E02	Zmynd15	*Cxcl16*	2.3
E04	Gm1960	*Cxcl3*	12.76
E05	LIX	*Cxcl5*	458.57
E09	Cd183	*Cxxr3*	12.42
E10	CD184	*Cxcr4*	3.16
E12	B0NZ0	*Cxcr6*	9.26
F01	Rdc1	*Cxcr7*	3.6
F02	CCBP1	*Darc*	68.4
F04	AI853548	*Gpr17*	7.55
F05	MOP1	*Hifla*	12.83
F07	KIAA4048	*Il16*	3.96
F09	Il-4	*Il4*	3.73
F10	Il-6	*Il6*	87.67
F11	CR3	*Itgam*	5.51
F12	2E6	*Itgbz*	15.7
G02	Crk1	*Mapk14*	6.74
G03	Cxcl4	*Pf4*	9.21
G04	Cxcl7	*Ppbp*	14.62
G06	Tgfb	*Tgfb1*	12.46
G07	Ly105	*Tlr2*	8.67
G08	Lps	*Tlr4*	10.41
G09	Tnfa	*Tnf*	2.37
G12	Ccxcr1	*Xcr1*	17.78
H02	beta2m	*B2m*	6.14
H03	Gapd	*Gapdh*	41.27
H04	Gur	*Gusb*	3.63

from Shanghai GenePharma Co., Ltd. CBRH-7919 cells were transfected with the siRNAs using Lipofectamine RNAi Max (Invitrogen) according to manufacturer's protocol. Cells were incubated for 48 h, and knock-down efficiency was determined by both qRT-PCR and Western blot analysis. The siRNA with the sequence 5'-GTCTCAGGACAGAGAAGTT-3' showed the highest efficiency in the knockdown of CXCL1 and was used in this study. Western blot analysis was used to assess the expression of CXCL2, CXCL3 and interleukin (IL)-1β.

Table 3 Downregulated chemokine-related genes in tumor tissues compared with peritumoral tissues

Accession No.	Gene name	Gene symbol	Fold change
E07	I18ra	*Cxcr1*	-4.18
F06	Ifng	*Ifg*	-2.02
H01	β-actin	*Actb*	-2.80

Statistical analysis

Data are summarized as mean ± SE. All statistical calculations were performed using either Student's *t* or Wilcoxon's rank sum tests performed with SPSS version 18 (SPSS Inc., Chicago, IL, United States). A $P < 0.05$ was considered statistically significant. The statistical methods of this study were reviewed by Wenjun Li.

RESULTS

Establishment of an HCC mouse model in vivo

As shown in Figure 1, the tumor growth developed rapidly between weeks 1-6 after xenograft tumor transplantation. The mice were evaluated for the formation of a tumor every week. Fourteen days after challenge with the CBRH-7919 cells, a local tumor was initially observed in the subcutaneous abdominal region of the mice (Figure 2).

HE staining confirmed tumorigenesis. Tumor tissue characteristics included atypia, a large core, darker colors, various cell sizes, irregular shapes, and nuclear division. Many of these characteristics were similar to those of the original CBRH-7919 cells (Figure 3).

Observation of inflammatory cell infiltration and macrophage phenotype

Macrophage, helper T cells, activated T cells, and neutrophils were significantly increased in peritumoral tissue compared to tumor tissue (*P*s < 0.005) (Table 1). There were no iNOS+ proinflammatory macrophages in the tumor tissue, whereas an average of 10 cells (range: 6-16) with positive expression were found within peritumoral tissue. The median number of arginase I+ cells within the tumor and peritumoral tissues were 1 (range: 0-2) and 0 (range: 0-1), respectively ($P < 0.005$). The relative proportion of regulatory to proinflammatory macrophages was higher in peritumoral tissue (Figure 4).

Expressions of chemokine-related genes in HCC

The expressions of chemokine-related genes during HCC progression were evaluated in tumor and peritumoral tissues using a chemokine PCR array. A total of 50 genes were identified as upregulated, including *CXCL1*, *CXCL2*, *CXCL3* and *IL-1β*, whereas *CXCR1*, *Ifg* and *Actb* were downregulated in tumor

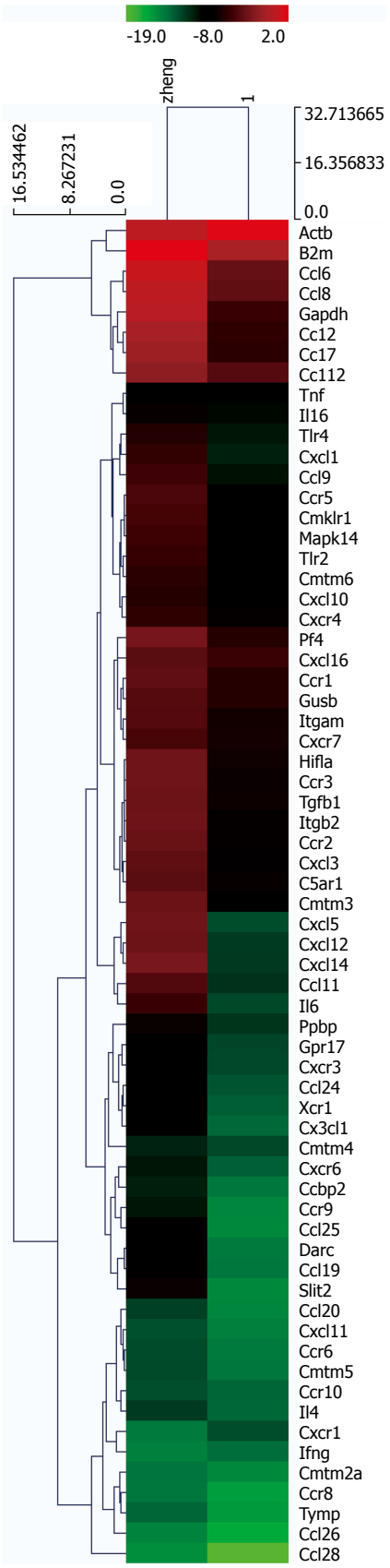

Figure 5 Hierarchical clustering analysis and line plots of genes with altered expression in tumor tissues compared with peritumoral tissues. Expression profiles of chemokine-associated genes were obtained using a PCR microarray. Two-dimensional hierarchical clusters were prepared in GeneSpring 6.1 and Gene Tree View using these gene expression profiles. Red: Upregulation; Green: Downregulation.

tissues (Tables 2 and 3; Figure 5).

Immunohistochemical and western blot analyses

The expression of selected genes was analyzed using immunohistochemistry and Western blotting (Figure 6) to confirm the changes observed by the PCR microarray. These results were consistent with those of the microarray analysis. CXCL1, CXCL2, and CXCL3 expression was higher and CXCR1 expression was lower in tumor tissues during HCC progression compared to the controls (Figure 6).

Tumor transplantation experiment

Gene silencing of CXCL1 inhibits the growth of CBRH-7919 tumors *in vivo.* Dissection of the subcutaneous tumors and evaluation in mice were carried out on the 45th day. Results showed the complete formation of tumors in all mice (Figure 7). The volume of tumors in the group with shCXCL1 were significantly smaller than those in the control group ($P < 0.05$).

CXCL1 silencing inhibits expression of CXCL2, CXCL3 and IL-1β

Western blotting analysis was performed to detect the expression of CXCL2, CXCL3 and IL-1β after knockdown of CXCL1. The results indicate that the protein expression levels of CXCL2, CXCL3 and IL-1β were significantly decreased compared with those of the control group and NC-RNAi-LV group ($P < 0.01$) (Figure 8).

DISCUSSION

Although efforts have been made to investigate the cellular and molecular pathways involved in cancer-related inflammation, as well as their potential as cancer biomarkers and therapeutic targets, the mechanisms regarding how inflammation contributes to cancer initiation, progression, metastasis, and angiogenesis remain largely unknown. In this study, we investigate the inflammatory microenvironment and expression of chemokines in HCC in nude mice. Our results show that transplanted tumors contained cells with inflammatory and macrophage phenotypes. A chemokine PCR array was used to identify differential gene expressions, which were validated by western blotting. Additionally, knockdown CXCL1 by RNAi suppressed tumor growth and expression of CXCL2, CXCL3 and IL-1β.

Chemokines and their receptors play an intricate role in HCC progression[16,17]. Chemokines are divided by their different structure and function into four families: including CC, CXC, C, and CX$_3$C. CXC chemokines are the second largest family of chemokines, and increasing evidence suggests that chemokine expression is associated with tumor angiogenesis, tumor, progression, and metastasis[18]. CXCL1 is a growth-regulated oncogene with melanoma growth-stimulating activity. Studies have

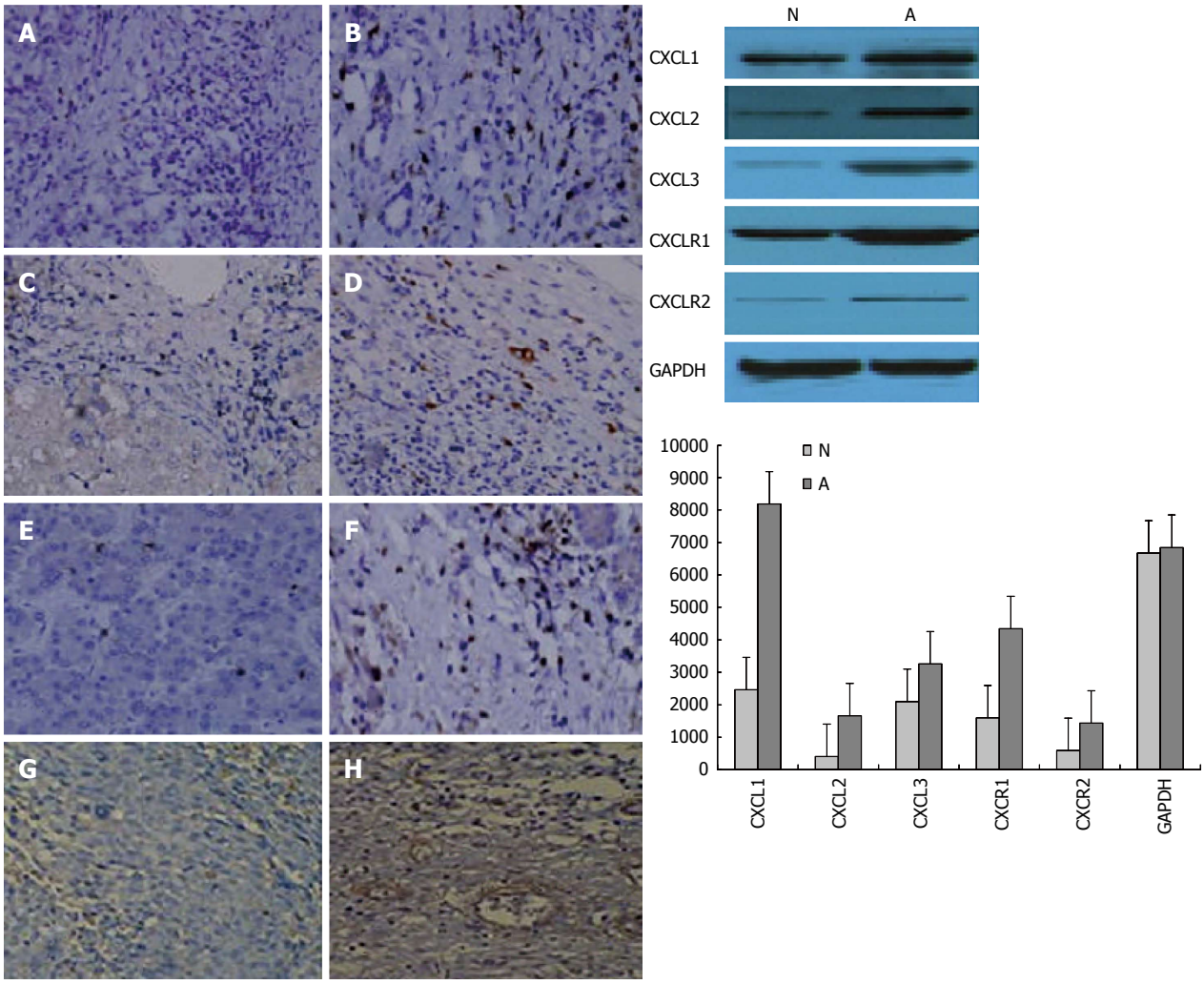

Figure 6 CXCL1, CXCL2, CXCL3, and CXCR1 expression in carcinoma cells xenografted into nude mice. Immunostaining for A, B: CXCL1; C, D: CXCL2; E, F; CXCL3; and G, H: CXCR1 in pertumoral (A, C, E and G) and tumor (B, D, F and H) tissues (× 200). Western blot analyses of the tumor and peritumoral tissues from the CBRH-7919 cell line or shown to the right in tumor (N) and peritumoral (A) tissues.

shown that CXCL1 can regulate tumor epithelial-stromal interactions that facilitate tumor growth and invasion, and CXCL1 has also been associated with angiogenesis[19-22]. CXCL1 is primarily regulated by growth factors/mediators, such as vascular endothelial growth factor (VEGF), TGF-β, c-Jun N-terminal kinase, and phosphoinositol 3-kinase. For instance, VEGF can stimulate CXCL1 release in both a time- and concentration-dependent manner, and this can be inhibited by VEGF receptor antagonists[23-26].

Some studies suggest that chemokines also mediate tumor metastasis. Both CXCL1 and CXCL2 are closely related to metastasis[23]. CXCL1 and CXCL2 expression can be suppressed by inhibiting phosphorylated IκBa and nuclear factor (NF)-κB activation. NF-κB promotes the survival of premalignant epithelial cells while also stimulating the release of proinflammatory mediators. NF-κB affects the expression of at least 400 genes with a variety functions, including inflammation, invasion and metastasis. Thus, downregulation of chemokines represents a potential treatment for cancer[4,27-29].

CXCL2 and CXCL3 are upregulated by proinflammatory cytokines. Our study shows that CXCL1, CXCL2, and CXCL3 are upregulated and CXCR1 is downregulated in the tumors of mice with HCC. These CXC chemokines and chemokine receptor are also expressed in several solid tumors. In human colon carcinoma cell lines, CXCL1 and its receptor CXCR2 have been associated with metastatic potential and are thought to modulate cell proliferation and invasion in both an autocrine and paracrine manner. CXCL1 is also overexpressed in human bladder carcinomas, and this increase in expression is associated with higher bladder carcinoma grade and stage. Studies have also reported that CXCL1 is overexpressed in colon, renal, gastric, skin, and breast cancers. However, other studies on CXCL1 expression in non-small cell lung cancer have reported the opposite results[30-32].

CXCR1 binds to only CXCL-8. Several studies have suggested that CXCR1 is an important player in tumor progression. Neutralization CXCR1 using small molecule antagonists affects cell proliferation and

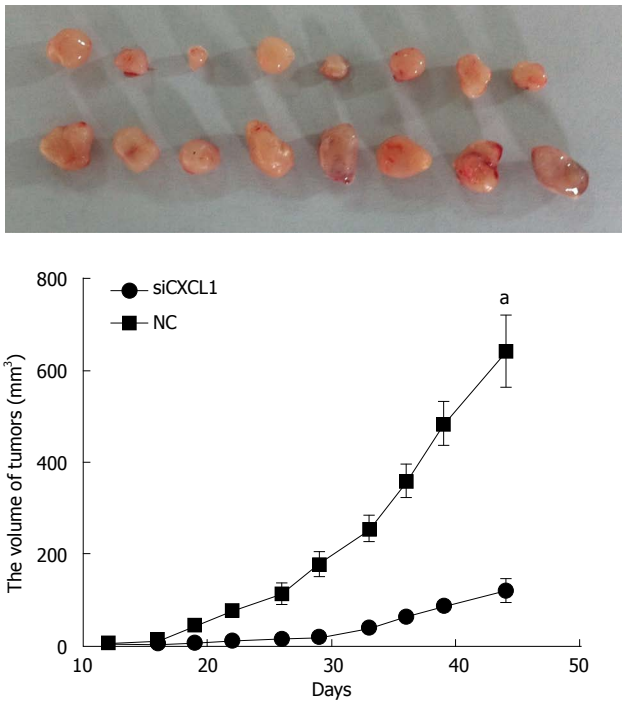

Figure 7 Growth CBRH-7919 xenografts with silencing of *CXCL1*. Tumor growth curve was determined by the International Veterinary Information Service system. All data are expressed as mean ± SD; $^{a}P < 0.05$ *vs* control (NC). shCXCL1: Short hairpin RNA targeting CXCL1.

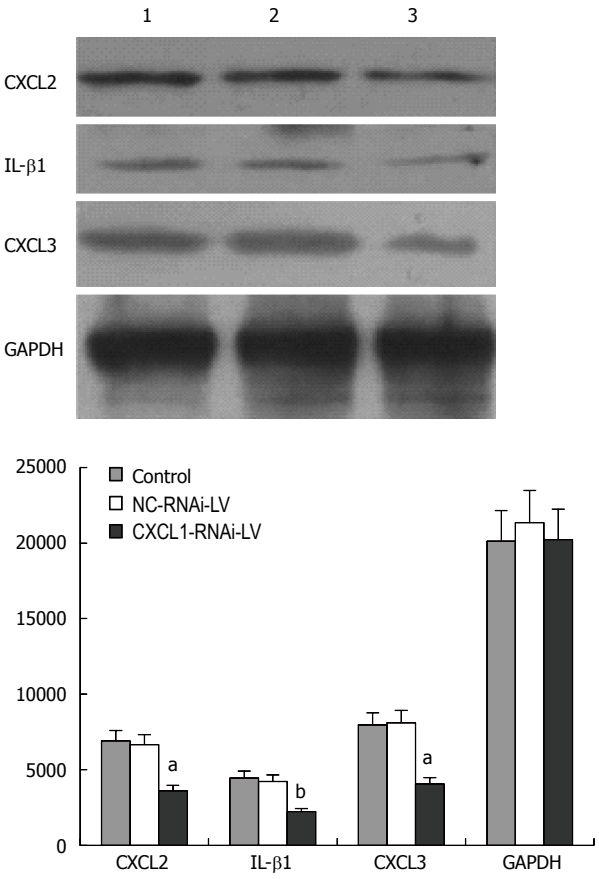

Figure 8 Expression of CXCL2, CXCL3 and IL-1β with *CXCL1* silencing. Representative Western blot showing expression in control untreated (1), control RNA interference (NC-RNAi-LV; 2), and CXCL1-targeted RNA interference (CXCL1-RNAi-LV; 3) groups; $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

migration. Recent reports have also demonstrated CXCR1 expression in all melanoma cases, irrespective of stage and grade, and modulating CXCR1 expression and/or activity has been shown to regulate malignant melanoma growth, angiogenesis, and metastasis[33-35]. In addition, activating both CXCR1 and CXCR2 increased the rate of cell proliferation in prostate cancer[36,37].

RNAi is one of the post-transcriptional gene silencing mechanisms, which has emerged as a powerful tool to induce loss-of-function phenotypes and is now widely used in the research of gene analysis and therapy. A lentivirus is a retrovirus, and lentiviral vectors can efficiently deliver si/shRNA-expression cassettes into various cells with sustained expression and potent function of the encoded siRNAs[38].

Our study shows that 50 chemokine-related genes were upregulated in HCC tissues and three additional genes were downregulated. Western blotting confirmed the changes in CXCL1, CXCL2, CXCL3, and CXCR1 expression in the CBRH-7919 HCC mouse model. Furthermore, we used a chemically modified siRNA, which is thought to be more stable and effective than a non-modified siRNA. The introduction of siRNA targeting CXCL1 into CBRH-7919 resulted in efficient and specific inhibition of CXCL1 expression, as demonstrated by Western blotting. The results show that gene silencing of CXCL1 inhibits the growth of CBRH-7919 tumors *in vivo* and significantly decreases protein expression levels of CXCL2, CXCL3 and IL-1β. Our study provides the first evidence that the targeted silencing of CXCL1 expression results in inhibited growth in HCC. These findings support the hypothesis that CXCL1 plays an important role in protecting CBRH-7919 cells from cell death, thus indicating CXCL1 is a target for future therapeutic interventions.

COMMENTS

Background

Recent reports show that inflammation is a crucial factor in the tumor microenvironment, and has become a hot topic in the past few years. The tumor microenvironment is an indispensable participant in the neoplastic process, promoting proliferation, survival, and migration of such tumors, and consists of cancer cells, tumor-associated fibroblasts, and chemokines. Chemokines play a paramount role in tumor progression, angiogenesis, invasion and metastasis. Hepatocellular carcinoma (HCC) tumors express a number of chemokines/receptors, including CXCL12-CXCR4, CX3CL1-CX3CR1, and CCL20-CCR6. However, the inflammatory microenvironment and differential expression pattern of chemokines in HCC is still not clear.

Research frontiers

In this study, inflammatory cell infiltration in the stromal microenvironment was assessed by immunohistochemistry in paired tumor and adjacent peritumoral samples, and macrophage phenotype was assessed using double-stain immunohistochemistry. PCR array analysis was used to evaluate the expression profiles of chemokines and their receptors in tumors grown in nude mice that were challenged with liver cancer (CBRH-7919 cells injected subcutaneously). The expression of some chemokines was regulated in a variety of patterns during the progression of the tumors. The expression of chemokines was confirmed by Western blotting and immunohistochemistry, demonstrating the

change in chemokines and receptors at the protein level in HCC. Additionally, the effect of chemokine expression knockdown by RNA interference on tumor growth was assessed.

Innovations and breakthroughs

Recent studies have highlighted that the tumor inflammatory microenvironment plays an essential role in the progression of HCC. The tumor microenvironment plays a critical role in modulating the process of liver fibrosis, hepatocarcinogenesis, epithelial-mesenchymal transition, tumor invasion, and metastasis. Thus, understanding the inflammatory microenvironment is critical to promote understanding of the molecular, cellular, and pathophysiologic mechanisms of HCC, and is essential for the development of new therapeutic strategies.

Applications

This study provides insight into the roles of CXCL1, CXCL2, and CXCL3 in a proinflammatory peritumoral microenvironment that affect HCC development and progression. Understanding CXCL1 is involved in tumor progression may facilitate the design of new therapeutic approaches that inhibit tumor cell growth.

Terminology

CXCL1 is a member of CXC chemokines family, which is identified as growth-regulated oncogene with melanoma growth-stimulating activity. CXCL1 may regulate tumor epithelial stromal interactions that facilitate tumor growth and invasion. CXCL1 is primarily regulated by growth factors/mediators, such as vascular endothelial growth factor, transforming growth factor-β, c-Jun N-terminal kinase, and phosphoinositol 3-kinase.

Peer-review

This paper brings us very important information about the inflammatory microenvironment and expression of chemokines in HCC. This study found that the inflammation microenvironment and differential expression of chemokines, particularly CXCL1, play critical roles in tumor growth. The result is very important for advanced research of HCC.

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ORIGINAL ARTICLE

Basic Study

Comparison of two different laparotomy methods for modeling rabbit VX2 hepatocarcinoma

Zhu Chen, Zhen Kang, En-Hua Xiao, Min Tong, Yu-Dong Xiao, Hua-Bing Li

Zhu Chen, Zhen Kang, En-Hua Xiao, Yu-Dong Xiao, Hua-Bing Li, Department of Radiology, the Second Xiangya Hospital, Central South University, Changsha 410011, Hunan Province, China

Min Tong, Experimental Animal Center, the Second Xiangya Hospital, Central South University, Changsha 410011, Hunan Province, China

Author contributions: Xiao EH and Chen Z designed the research; Chen Z, Kang Z, Tong M and Li HB performed the research; Chen Z, Kang Z and Xiao YD analyzed the data and wrote the paper.

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Ethics approval: The study was reviewed and approved by the Second Xiangya Hospital Institutional Review Board.

Institutional animal care and use committee: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Second Xiangya Hospital (SYXK 2012-003).

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Data sharing: Participants gave informed consent for data sharing. No additional data are available.

Correspondence to: En-Hua Xiao, MD, Professor, Department of Radiology, the Second Xiangya Hospital, Central South University, No. 139 Renmin Zhong Lu, Fu Rong District, Changsha 410011, Hunan Province, China. cjr.xiaoenhua@vip.163.com
Telephone: +86-731-85292116
Fax: +86-731-85292116

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Abstract

AIM: To compare two different laparotomy methods for modeling rabbit VX2 hepatocarcinoma.

METHODS: Thirty New Zealand rabbits were randomly divided into two groups: A and B. Group A was assigned a traditional laparotomy method (embedding tumor fragments directly into the liver with tweezers). Group B was subjected to an improved laparotomy method (injection of tumor fragments into the liver through a 15 G syringe needle). The operation time, incision length, incision infection rate, and mortality rate were compared between the two groups after laparotomy. Magnetic resonance imaging (MRI) was performed to evaluate tumor formation rates and the characteristics of the tumors 2 wk after laparotomy.

RESULTS: The mean operation times for the two groups (Group A *vs* Group B) were 23.2 ± 3.4 min *vs* 17.5 ± 2.9 min ($P < 0.05$); the incision length was 3.3 ± 0.5 cm *vs* 2.4 ± 0.6 cm ($P < 0.05$); and the mortality rate after 2 wk was 26.7% *vs* 0% ($P < 0.05$); all of these outcomes were significantly different between the two groups. The incision infection rates in the two groups were 6.7% *vs* 0% ($P > 0.05$), which

were not significantly different. MRI performed after 2 weeks showed that the tumor formation rates in the two groups were 90.9% *vs* 93.3% ($P > 0.05$). These rates were not significantly different between the two groups. The celiac implantation rate and abdominal wall metastasis rate in the two groups were 36.4% *vs* 13.3% ($P < 0.05$) and 27.2% *vs* 6.7% ($P < 0.05$), respectively, which were significantly different between the two groups.

CONCLUSION: The tumor formation rates were not significantly different between the two methods for modeling rabbit VX2 hepatocarcinoma. However, the improved method is recommended because it has certain advantages.

Key words: Rabbit VX2 hepatocarcinoma; Laparotomy; Modeling; Magnetic resonance imaging

Core tip: A crucial issue in studying liver cancer is the establishment of an animal model to simulate human liver cancer. There are various ways to establish rabbit VX2 hepatocarcinoma, and using an open laparotomy implant is a widely adopted classical method. We injected tumor fragments into the left lobe of the rabbit liver, deviating from the abdominal wall using a 15 G syringe needle instead of building a sinus using tweezers. This improved method is recommended because of advantages such as decreased injury to the liver, shorter operation time, lower death rates, reduced abdominal cavity implantation and fewer abdominal wall invasions.

Chen Z, Kang Z, Xiao EH, Tong M, Xiao YD, Li HB. Comparison of two different laparotomy methods for modeling rabbit VX2 hepatocarcinoma. *World J Gastroenterol* 2015; 21(16): 4875-4882 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4875.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4875

INTRODUCTION

Liver cancer, the third leading cause of cancer death worldwide, is a primary malignancy originating in the liver. There are approximately 60-70 million liver cancer deaths each year, 50% of which occur in China. Most patients are diagnosed in intermediate or advanced stages of the disease[1,2]. Early detection and diagnosis of liver cancer are an important issue that must be addressed. It is crucial to establish an animal model simulating human liver cancer to study this disease. VX2 carcinoma is a type of animal tumor that was derived from a rabbit papilloma by Shope and Hurst[3]. Rabbit VX2 hepatocarcinoma is an ideal model of liver cancer and is widely used for imaging and other experimental studies due to the rapid growth of these tumors and their similarity to human hepatocellular carcinoma[4-6]. There are various ways to establish rabbit VX2 hepatocarcinoma, including the widely adopted classical method of open laparotomy implantation. In open laparotomy implantation, the implant substance can be a cell suspension or tumor fragments, with the latter being reported to show a higher success rate[7]. However, traditional laparotomy implantation methods may cause spill-out into the celiac. We developed a modified method that uses a syringe to inject tumor fragments to improve the success rate of implantation. Here we compared the two different methods for implanting VX2 tumors, and the results are presented below.

MATERIALS AND METHODS

Experimental animals

Thirty New Zealand white rabbits of both sexes weighing 2.0-3.0 kg were provided by the experimental animal center of Second Xiangya Hospital of Central South University. The animal experiments were performed in accordance with a protocol approved by the animal care committee and were in compliance with institutional guidelines (Permit Number: 2012-0087).

Surgical instruments

Basic surgical instruments and supplies were used, including a 15 G needle connected to a 5 mL syringe.

Experimental methods

Establishment of the rabbit VX2 liver carcinoma model: The 30 New Zealand white rabbits were divided into two groups using a random number table, with 15 rabbits being placed in each group. Direct embedding of tumor fragments was performed on the rabbits in Group A (traditional open method group). Group B was subjected to the modified method, in which tumor fragments were injected with a 15 G needle connected to a 5 mL syringe. Four hours before the operation, the rabbits were fasted and only given water. The hair around the operation field was shaved using a razor. Intramuscular injections of gentamicin (80000 units) were administered before the operation and once a day after the operation for a total of two days. All rabbits were anesthetized with 2 mL/kg chloral hydrate *via* ear vein injection.

Tumor-bearing rabbits were anesthetized with chloral hydrate. Tissue at the edge of the tumor was removed under sterile conditions. Then, the tumor was placed in physiological saline solution for washing, and necrotic tissue, fascia and other connective tissues were removed. This procedure was followed by soaking the tumor in physiological saline solution and cutting it into small pieces (0.5-1 mm diameter) using scissors. Then, the incision in the tumor-bearing rabbits was sutured, and gentamicin (80000 units) was injected intramuscularly.

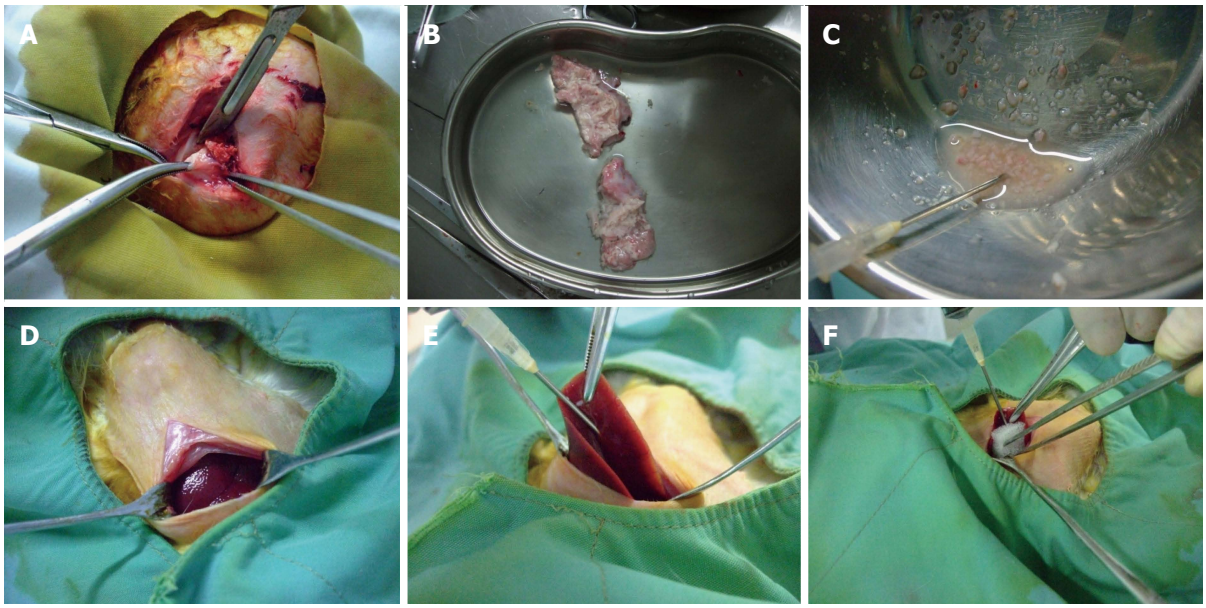

Figure 1 Process of establishing the rabbit VX2 hepatocarcinoma model. Tumor-bearing rabbits were anesthetized with chloral hydrate. A: Fish-like tissue at the edge of the tumor was removed under sterile conditions; B: The tumor tissue was placed in saline for washing and to remove the necrotic tissue, fascia and other connective tissues; C: The tumor tissue was cut into small pieces (0.5-1 mm diameter) using scissors; D: Healthy rabbits were anesthetized and placed on a rabbit operating table with their limbs fixed at the four corners. Routine sterilization of the surgical field was performed, and a vertical incision was made below the xiphoid. Then, the liver was exposed; E: After exposure of the middle lobe of the liver using smooth forceps, a 15 G needle was inserted sideways and upward into the visceral surface of the liver lobe, and 0.5 mL of tumor fragments was injected with a 5 mL syringe. During this process, we ensured that the tumor fragments were injected into the liver parenchyma; F: The puncture site was covered with gelfoam after removal of the needle, and the incision was sutured after confirming that there was no obvious bleeding.

Healthy rabbits were anesthetized and placed on a rabbit operating table with their limbs fixed at the four corners. Routine sterilization of the surgical field was performed, and a vertical incision was made below the xiphoid. When the liver was exposed, the 15 rabbits in Group A were subjected to the following conventional open laparotomy implantation method: stretching of the left lobe using smooth forceps, puncturing of the liver tissue to build a sinus with an ostium diameter of approximately 2 mm using ophthalmic scissors, insertion of tumor fragments into the sinus, covering the sinus with gelfoam, and suturing the incision after confirming that there was no obvious bleeding. Group B was subjected to the following modified implantation method: exposure of the middle lobe of the liver using smooth forceps, insertion of a 15 G needle sideways and upward into the visceral surface of the liver lobe and then injection of 0.5 mL of tumor fragments with a 5 mL syringe. During this process, we made sure that the tumor fragments were injected into the liver parenchyma. We could see that the surface of the puncture site was intumescent and white. Finally, the puncture site was covered with gelfoam after removal of the needle, and the incision was sutured after confirming that there was no obvious bleeding. The modified implantation method is shown in Figure 1.

Postoperative indicators: Observed indicators included the operation time, incision size, infection rate, rabbit survival after 2 wk, and tumor formation as assessed by magnetic resonance imaging (MRI).

MRI detection: All of the rabbits in Groups A and B were subjected to 3 T MRI to assess the implant. Successful implantation criteria were as follows: intrahepatic nodules or space-occupying masses identified *via* MRI with a slightly low signal on T1WI and slightly high signal on T2WI. Celiac implantation and abdominal wall violation were also assessed *via* MRI. All scans were performed on a 3-T MR scanner (Philips Achieva 3.0-T X-series, Phillips Healthcare, The Netherlands) using the 16-element phased array of a SENSEXL Torso coil. The imaging protocol included the following parameters: (1) coronal sense turbo spin-echo T2-weighted fat-suppressed MRI [repetition time (TR)/echo time (TE), 1565/70 ms; average, 3; flip angle, 90 degrees; matrix size, 252 × 193; field of view (FOV), 24 cm × 24 cm; section thickness, 3 mm; and gap, 0.3 mm]; (2) axial plane sense turbo spin-echo T2-weighted fat-suppressed MRI (TR/TE, 1565/70 ms; average, 3; flip angle, 90 degrees; matrix size, 252 × 192; FOV, 24 cm × 24 cm; section thickness, 4 mm and gap, 0.4 mm); and (3) axial plane sense turbo spin-echo T1-weighted MRI (T1WI; TR/TE, 10/2.3 ms; average, 3, flip angle, 15 degrees; matrix, 252 × 193; FOV, 24 cm × 24 cm; section thickness, 4 mm; and gap, 0.4).

Pathological examination

Following MRI examination, the experimental rabbits were sacrificed *via* air embolism, and the liver was anatomized to observe the growth of the tumors. The tumors were removed and fixed in formalin.

Table 1 Comparison of the operation time and incision size between the traditional laparotomy method and the modified laparotomy method (*t* test, mean ± SD)

Group	Mean operation time (min)	Mean incision size (cm)
A (traditional laparotomy method)	21.2 ± 2.6	3.3 ± 0.5
B (modified laparotomy method)	17.6 ± 2.4	2.6 ± 0.4
t test	3.903	2.928
P value	0.001	0.007

Table 2 Comparison of incision infections and the mortality rates between the traditional laparotomy method and the modified laparotomy method (Fisher exact test)

Group	Number of infected rabbits	Number of non-infected rabbits	Postoperative incision infection rate (%)	Mortality in each group	Survival in each group	Mortality rate (%)
A (traditional laparotomy method)	2	9	18.2	5	10	33.3
B (modified laparotomy method)	1	14	6.7	0	15	0
P value			0.556			0.042

Pathological testing was performed to confirm the histopathology of the intrahepatic nodules or space-occupying masses found through MRI.

Statistical analysis

The data were analyzed using SPSS 17.0 software. All of the data for continuous variables are expressed as the median and range. The *t*-test was employed to compare the differences between the medians of continuous variables for operation time and incision size. The Fisher exact test or χ^2 test was used to compare proportions between groups regarding incision infections, death rates, inoculation success rates, celiac implantation and abdominal wall violation. Statistical significance was defined as $P < 0.05$.

RESULTS

Operation time and incision size

Five rabbits in Group A (traditional laparotomy method, 15) died within two weeks. Four of these rabbits died on the day of operation, and coagulated blood that may have resulted from liver rupture was found in their abdominal cavities during autopsy. The other rabbit died four days after the operation. Festering was observed on the abdominal wall, which may have been due to infection.

No death was observed in Group B (modified laparotomy method, 15) within two weeks. Two of the remaining 11 rabbits in Group A and 1 of the 15 rabbits in Group B developed an incision infection. After disinfection of the incision and injection of antibiotic treatment, the infections of 1 of the rabbits in Group A and the 1 rabbit in Group B were relieved. The statistical data showed that the operation time for Group B was shorter than that for Group A. Additionally, the incision length was shorter, and postoperative mortality at 2 wk was lower in Group B than in Group A. There was no obvious difference in the postoperative incision infection rate between the two groups. The data for comparison of the two groups are shown in Tables 1 and 2.

MRI detection of VX2 hepatocarcinoma and the characteristics of tumor formation

MRI examination was performed on the remaining 10 experimental rabbits in Group A and 15 in Group B to evaluate the inoculation success rate and tumor growth. Nine out of 10 of the remaining rabbits in Group A and 14 out of 15 of the rabbits in Group B were implanted successfully (Figure 2). Six of the 10 rabbits in Group A and 2 of the 15 in Group B appeared to exhibit celiac implantation (Figure 3). Five of the 10 rabbits in Group A and 1 of the 15 in Group B appeared to show abdominal wall invasion (Figure 4). There was no significant difference between the two groups in the implantation success rate. The celiac implantation ratio and the frequency of invasion of the abdominal wall in Group A were higher than in Group B. The MRI findings for the two groups are shown in Table 3.

Pathological examination

It was confirmed that the intrahepatic nodules or masses observed by MRI were VX2 hepatocarcinomas and that they shared common characteristics with human hepatic carcinoma. The histological findings are shown in Figure 5.

DISCUSSION

There are many methods for establishing a rabbit VX2 hepatocarcinoma model, such as puncture under computed tomography (CT)[8] or ultrasound (US)[9] guidance, direct percutaneous puncture inoculation[10,11], open implantation[12-15], and perfusion *via* the hepatic artery or portal vein[16]. Because special equipment and laboratory space are needed

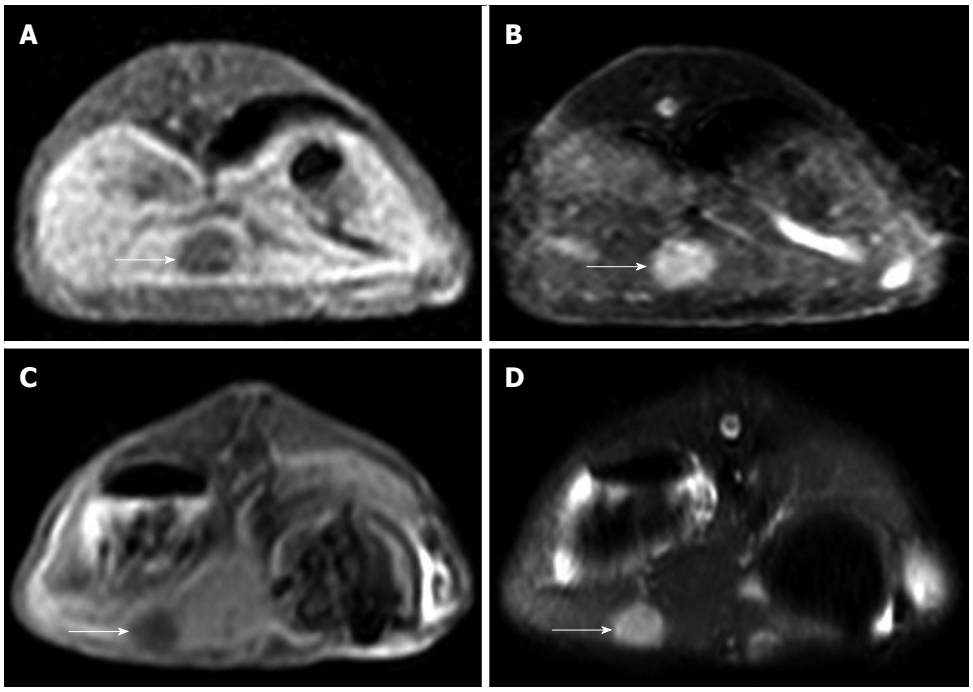

Figure 2 Magnetic resonance imaging of successfully implanted VX2 hepatocarcinoma (shown by arrow). A, B: T1- and T2-weighted images of a successfully implanted VX2 hepatocarcinoma; C, D: T1- and T2-weighted images of another successfully implanted VX2 hepatocarcinoma.

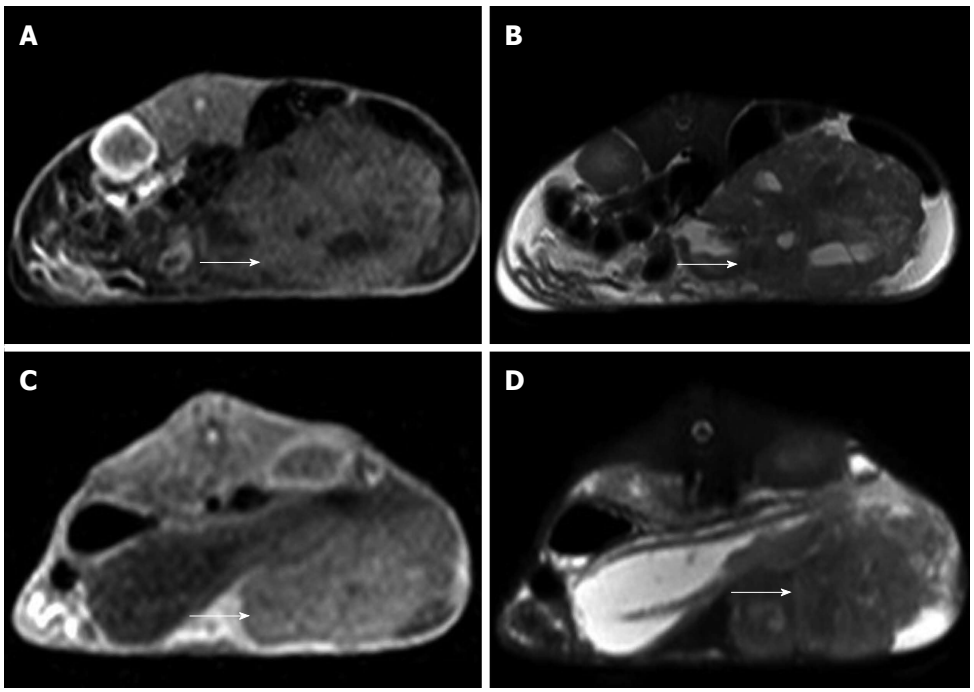

Figure 3 Magnetic resonance imaging of an implanted VX2 hepatocarcinoma with celiac implantation (shown with an arrow). A, B: T1- and T2-weighted images of an implanted VX2 hepatocarcinoma with celiac implantation; C, D: T1- and T2-weighted images of another implanted VX2 hepatocarcinoma with celiac implantation.

for implantation under CT or US guidance, the application of this procedure is limited. In contrast, direct percutaneous puncture inoculation presents the disadvantage of a lower success rate. Therefore, open implantation, which shows an acceptable success rate[17], is now widely used. However, it is extremely easy for invasion of nearby tissues and liver damage to occur after conventional open implantation. To improve the success rate of modeling, researchers have proposed several modifications[18].

The difference between the improved laparotomy method and the conventional method is that im-

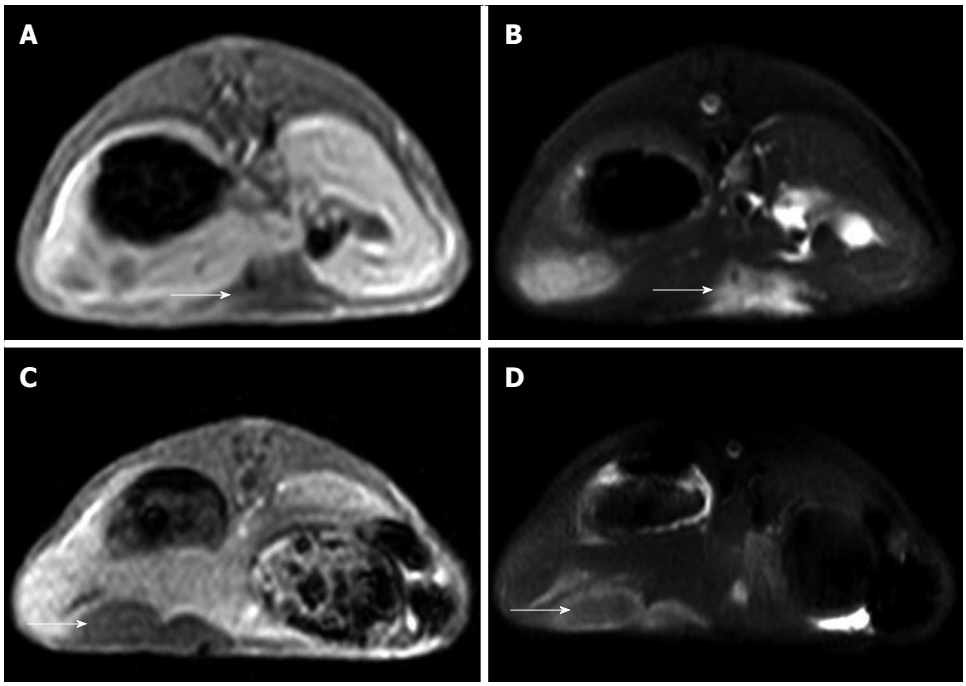

Figure 4 Magnetic resonance imaging of an implanted VX2 hepatocarcinoma with abdominal wall invasion (shown by arrow). A, B: T1- and T2-weighted images of an implanted VX2 carcinoma with abdominal wall invasion; C, D: T1- and T2-weighted images of another implanted VX2 hepatocarcinoma with abdominal wall invasion.

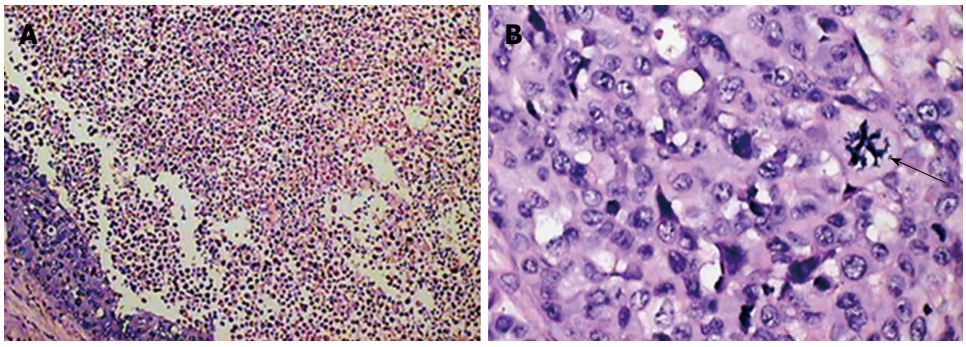

Figure 5 Histological findings for intrahepatic nodules or masses observed through magnetic resonance imaging. HE staining (A: × 100; B: × 400): the tumor cells were morphologically diverse, with obviously heterogeneous and trachychromatic nuclei; karyokinesis could also be found (shown by arrow).

Table 3 Comparison of magnetic resonance imaging findings between the traditional laparotomy method and the modified laparotomy method (Fisher exact test)

Group	Successfully implanted	Unsuccessfully implanted	Successful implantation rate	With celiac implantation	No celiac implantation	Celiac implantation rate	With abdominal wall invasion	No abdominal wall invasion	Abdominal wall invasion rate
A (traditional laparotomy method)	9	1	90%	6	4	60%	5	5	50%
B (modified laparotomy method)	14	1	93.3%	2	13	13.3%	1	14	6.7%
P value			1			0.028			0.023

plantation of tumor fragments is accomplished *via* injection instead of by building a sinus using tweezers. This subtle change has many advantages. First, because the rabbit liver is thin, brittle and fragile, it is likely to bleed after conventional piercing. Once bleeding occurs, it is difficult to stop under experimental conditions. The modified laparotomy method involving injection with a 15 G syringe needle can markedly decrease both

bleeding and the death rate caused by liver rupture. Second, the conventional method can lead to abdominal cavity transplantation[12], as the tumor fragments inserted with tweezers can easily enter the abdominal cavity. Additionally, the sinus becomes wider after tumor insertion, which also facilitates the spilling of tumor tissue out of the sinus. Third, Lee *et al*[19] found that the left lobe of the rabbit liver is preferable for implanting VX2 carcinoma. Yoon *et al*[17] showed that implanting VX2 carcinoma in the left lobe of the rabbit liver, which deviates from the abdominal wall, was effective in preventing tumor invasion in the muscular tissue of the rabbit abdominal wall. We were able to perform implantation effectively using the improved puncture method developed in this study. Fourth, compared with building a sinus in liver tissue using tweezers, injection requires a smaller operating field and surgical incision, decreases injury and shortens the incision and suturing time. Therefore, this method can improve efficiency, decrease surgical risk, and be easily performed.

Both Groups A and B showed a high success rate, as the process of laparotomy occurred under direct vision, which can ensure that the tumor tissue becomes embedded into the liver tissue. The infection rate did not differ between the two groups, due to the aseptic procedure, correct incision and suturing performed by the same skilled operator.

Considering the above-mentioned analysis, we concluded that there was no difference in the tumor formation rate between the improved and conventional laparotomy methods for modeling rabbit VX2 hepatocarcinoma. Nevertheless, the improved method is recommended because it has several advantages, such as decreasing injury to the liver, shorter operation time, a lower death rate, less abdominal cavity implantation and fewer abdominal wall invasions.

In conclusion, we found that the tumor formation rates were not significantly different between the two methods for modeling rabbit VX2 hepatocarcinoma. However, the improved method is recommended because it shows several advantages.

COMMENTS

Background

Early detection and diagnosis of liver cancer are an important issue that must be addressed. It is crucial to establish an animal model simulating human liver cancer to study the disease. Rabbit VX2 hepatocarcinoma is an ideal model of liver cancer that is widely used. There are various ways to establish rabbit VX2 hepatocarcinoma, including the widely adopted classical method of open laparotomy implantation. However, the traditional laparotomy implantation method presents some disadvantages. Therefore, the authors developed a modified method to improve the modeling of this disease.

Research frontiers

To investigate hepatocarcinoma more completely, we needed to establish an ideal liver cancer model that is stable and controllable and in which the implanted lesion is limited.

Innovations and breakthroughs

The authors injected tumor fragments into the left lobe of the rabbit liver, deviating from the abdominal wall, using a 15 G syringe needle, instead of implanting tumor fragments by building a sinus using tweezers.

Applications

The improved method is recommended because of showing advantages such as decreased injury to the liver, shorter operation time, a lower death rate, less abdominal cavity implantation and fewer abdominal wall invasions.

Terminology

Open laparotomy implantation is a widely adopted classical method for establishing rabbit VX2 carcinoma. In this study, the authors improved this method, which could be useful in the study of liver cancer.

Peer-review

This is an important field of study and the authors examine an interesting approach. Overall the writing and phrasing should be improved. In addition, the weaknesses of the various models could be discussed more. Indeed, the most interesting aspect to the study may rather be the description of the method as the results are so dramatically related to sample size. Therefore the text should more closely reflect the procedural aspect of the study.

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ORIGINAL ARTICLE

Basic Study

Intestinal dendritic cells change in number in fulminant hepatic failure

Xu Cao, Mei Liu, Peng Wang, Dong-Yan Liu

Xu Cao, Mei Liu, Peng Wang, Dong-Yan Liu, Research Center, Shengjing Hospital of China Medical University and Key Laboratory of Congenital Malformation Research, Ministry of Health, Shenyang 110004, Liaoning Province, China

Author contributions: Cao X performed the research, analyzed the data and wrote the paper; Liu M analyzed the data and revised the manuscript; Wang P analyzed the data; Liu DY designed the research, performed the research, wrote and revised the manuscript.

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Conflict-of-interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

Correspondence to: Dong-Yan Liu, Professor, Research Center, Shengjing Hospital of China Medical University and Key Laboratory of Congenital Malformation Research, Ministry of Health, No. 36 Sanhao Street, Heping District, Shenyang 110004, Liaoning Province, China. dongyan.liu@aliyun.com
Telephone: +86-24-96615-13427
Fax: +86-24-23929903

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Abstract

AIM: To investigate the change in intestinal dendritic cell (DC) number in fulminant hepatic failure (FHF).

METHODS: An animal model of FHF was created. Intestinal CD11b/c was detected by immunohistochemistry and Western blot. Quantitative real-time polymerase chain reaction (PCR) was used to detect intestinal integrin-α mRNA expression. Intestinal CD83, CD86, CD74, CD3 and AKT were detected by immunohistochemistry, Western blot and PCR. Phosphorylated-AKT (p-AKT) was detected by immunohistochemistry and Western blot.

RESULTS: In the FHF group [D-galactosamine (D-Galn) + lipopolysaccharide (LPS) group], the mice began to die after 6 h; conversely, in the D-Galn and LPS groups, the activity of mice was poor, but there were no deaths. Immunohistochemistry results showed that in FHF, the expression of CD11b/c (7988400 ± 385941 *vs* 1102400 ± 132273, $P < 0.05$), CD83 (13875000 ± 467493 *vs* 9257600 ± 400364, $P < 0.05$), CD86 (7988400 ± 385941 *vs* 1102400 ± 13227, $P < 0.05$) and CD74 (11056000 ± 431427 *vs* 4633400 ± 267903, $P < 0.05$) was significantly increased compared with the normal saline (NS) group. Compared with the NS group, the protein expression of CD11b/c (5.4817 ± 0.77 *vs* 1.4073 ± 0.37, $P < 0.05$) and CD86 (4.2673 ± 0.69 *vs* 1.1379 ± 0.42, $P < 0.05$) was significantly increased. Itg-α (1.1224 ± 0.3 *vs* 0.4907 ± 0.19, $P < 0.05$), *CD83* (3.6986 ± 0.40 *vs* 1.0762 ± 0.22, $P < 0.05$) and *CD86* (1.5801 ± 0.32 *vs* 0.8846 ± 0.10, $P < 0.05$) mRNA expression was increased significantly in the FHF group. At the protein level, expression of CD74 in the FHF group (2.3513 ± 0.52) was significantly increased compared with the NS group (1.1298 ± 0.33), whereas in the LPS group (2.3891 ± 0.47), the level of CD74 was the highest ($P < 0.05$). At the gene level, the relative expression of *CD74* mRNA in the FHF group (1.5383 ± 0.26) was also significantly increased in comparison to the NS group (0.7648 ± 0.22; $P < 0.05$). *CD3* expression was the highest in the FHF group ($P < 0.05$). In the FHF, LPS and D-Galn groups, the expression of AKT at the protein and mRNA levels was elevated compared with the NS group, but there was

no statistical significance ($P > 0.05$). The p-AKT protein expression in the FHF (1.54 ± 0.06), LPS (1.56 ± 0.05) and D-Galn (1.29 ± 0.03) groups was higher than that in the NS group (1.07 ± 0.03) ($P < 0.05$).

CONCLUSION: In FHF, a large number of DCs mature, express CD86, and activate MHC class Ⅱ molecular pathways to induce a T cell response, and the AKT pathway is activated.

Key words: Fulminant hepatic failure; Intestinal dendritic cells; MHC Ⅱ; CD3; AKT/Phosphorylated-AKT

Core tip: In this study, we showed that the expression of CD11b/c, CD83, CD86, CD74 and CD3 in the fulminant hepatic failure (FHF) group [D-galactosamine (D-Galn) + lipopolysaccharide (LPS) group] was significantly higher compared with the normal saline (NS) group. Additionally, the phosphorylated-AKT protein expression in the FHF, LPS and D-Galn groups was higher than that in the NS group. This result showed that a large number of dendritic cells mature, express CD86, and activate MHC class Ⅱ molecular pathways to induce a T cell response in FHF. In addition, the AKT pathway in FHF was activated.

Cao X, Liu M, Wang P, Liu DY. Intestinal dendritic cells change in number in fulminant hepatic failure. *World J Gastroenterol* 2015; 21(16): 4883-4893 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4883.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4883

INTRODUCTION

Fulminant hepatic failure (FHF) is a clinical syndrome resulting from a rapid loss of hepatocyte function with acute morbidity. A lack of effective treatment has resulted in high mortality so far[1,2]. During the progression of FHF, bacterial overgrowth in the small intestine affects intracellular signal transduction pathways, leading to the disruption of epithelial tight junctions and an increase in the paracellular permeability to macromolecules. Furthermore, intestinal bacterial translocation (BT) through the gastrointestinal barrier into the blood is associated with decreased intestinal resistance. This mechanism may lead to primary peritonitis. Clinically, this event is diagnosed as spontaneous bacterial peritonitis (SBP). Therefore, a decrease in intestinal permeability would prevent BT, suppress intestinal endotoxemia, prevent the occurrence of SBP, and thus enhance recovery from liver injury.

In the gut, dendritic cells (DCs), which are professional antigen presenting cells (APC), play a crucial role in both innate and adaptive immunity against microbial antigens. Activated mature intestinal DCs are the main stimulators of naive T cells, ultimately shaping the adaptive mucosal immune system[3]. Additionally, the invariant natural killer T cells regulate DC numbers[4]. T cell responses are initiated with specific peptide-MHC protein complexes on the surface of APC and through signals provided by co-stimulators expressed on APC[5]. In response to BT, gut epithelial cells release chemokines that induce the recruitment of DCs to the mucosa[6]. DCs that underlie the epithelium may open tight junctions between epithelial cells, sending dendrites into the lumen to directly sample bacteria[7,8]. Previous studies have shown that both viable and killed probiotic bacteria had strain-specific effects on the phenotype of human and murine DCs[9-11]. For example, lamina propria DCs can be distinguished according to CD11c (high and low), CD11b, CD103, CX3CR1, and CD70[12] expression. LP-DCs can be divided into two different subsets: $CD103^+CX3CR1^-$ DCs that induce the development of regulatory T cells and $CD103^-CX3CR1^+$DCs with features of macrophages, which promote TNF production and the development of Th1/Th17 T cells. It has been reported that bacterial translocation in rats with cirrhotic ascites was associated with an increase in the total number of intestinal $CD103^+$DCs in the lamina propria and in mesenteric lymph nodes[13].

Preventing the occurrence of SBP is essential to avoid liver damage and reduce mortality in patients with FHF. In this study, the intestinal CD11b/c, CD83, CD86, the major histocompatibility complex Ⅱ-associated invariant chain Ii (also known as CD74), the T cell marker (CD3), and AKT/ phosphorylated-AKT (p-AKT) were assessed to gain a better understanding of how the distribution of DCs and the corresponding immune response change after the onset of FHF.

MATERIALS AND METHODS

All animals in this study were from the Animal Center of Shengjing Hospital of China Medical University. Adult wild-type rats were anesthetized and killed by cervical dislocation. All studies were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee of the China Medical University for Basic Research in Developmental Disabilities. All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

Animal model

A mouse model of FHF was established as described previously[14]. BALB/C mice housed in a specific-pathogen-free environment, weighing 18-22 g, were purchased from the Experimental Animal Center of China Medical University. All pups were randomly

divided into four groups: normal saline (NS, 15 mice), lipopolysaccharide (LPS, 30 mice), D-galactosamine (D-Galn, 30 mice), and FHF (LPS + D-Galn, 100 mice). Mice were injected intraperitoneally with LPS (10 μg/kg, Sigma, United States) and/or D-Galn (800 mg/kg, Sigma, United States). Mice were sacrificed *via* decapitation 9 h after the injection of NS, LPS, or D-Galn.

Tissue preparation

BALB/C mice were sacrificed by decapitation. To standardize analysis, half of the small intestine was fixed in situ with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) and then embedded in paraffin. The rest of the small intestine was harvested for mRNA and protein analysis.

Immunohistochemistry for the detection of CD11b/c, CD83, CD86, CD74, CD3, AKT, and p-AKT

Paraffin-embedded sections of intestinal tissues were deparaffinized, rehydrated, and incubated with mouse anti-mouse CD11b/c (Abcam, United Kingdom), mouse anti-mouse CD86 (Santa Cruz Biotechnology, United States), rabbit anti-mouse CD3 (Sigma, United States), rabbit anti-mouse AKT (Thermo Fisher Scientificm, United States), rabbit anti-mouse CD74 (Santa Cruz Biotechnology, United States), rabbit anti-mouse p-AKT(Santa Cruz Biotechnology, United States) and goat anti-mouse CD83 (Santa Cruz Biotechnology, United States). Slides were rinsed three times with PBS between incubations, and sections were incubated with biotinylated secondary antibodies and horseradish peroxidase labeled avidin (ZSGB-BIO, China). Slides were rinsed three times with PBS after each incubation, and sections were counterstained with hematoxylin. For negative controls, the primary antibody was replaced with PBS. After scanning, the median absorbance values were determined using Image-Pro analysis software (Media Cybernetics, United States).

Protein determination

A BCA Protein Assay Kit (Beyotime, Shanghai, China) was used to determine the protein concentration of intestinal tissue according to the manufacturer's instructions.

Western blot for analysis of CD11b/c, CD83, CD86, CD74, CD3, AKT and p-AKT protein in intestinal tissues

Proteins extracted from intestinal tissues were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinyl fluoride membranes. Membranes were blocked with Tris-buffer containing 5% skim milk and probed with anti-CD11b/c, -CD86, -CD74, -CD3, -AKT and -p-AKT antibodies or anti-GAPDH followed by a peroxidase-conjugated secondary antibody. They were then incubated with an enhanced chemiluminescent substrate and exposed to X-OMAT film (Perkin Elmer, American). Images were scanned, and densitometry was analyzed for protein levels normalized to GAPDH using the Image-pro plus 6.0 software (Media Cybernetics, American).

Quantitative real-time polymerase chain reaction of intestinal integrin-α, CD83, CD86, CD74, CD3, and AKT mRNAs

Total RNA was extracted from the intestinal tissue of FHF mice using an RNA Mini Kit from Takara (Takara Biotechnology Co., Dalian, China). The quality of extracted RNA was determined by the number and size of the bands obtained using agarose gel electrophoresis. cDNA (Takara Biotechnology Co., Dalian, China) was synthesized using 100 ng RNA. The levels of individual RNA transcripts were quantified using quantitative real-time polymerase chain reaction (PCR). The primers used were as follows: CD3ε-Forward (F): 5′-TCACGGAGATGTCGCCAGA-3′, CD3ε-Reverse (R): 5′-TTGCCATCCAGTTTGCCCTTA-3′; integrin-α (Itg-α)-F: 5′-ATGGCTCCGGTAGCATCAACA-3′, Itg-α-R: 5′-CTCGTCCGAGTACTGCATCAAAG-3′; CD74-F: 5′-AGCCAGATGCGGATGGCTA-3′, CD74-R: 5′-TCCTGGGTCATGTTGCCGTA-3′; CD86-F: 5′-ATATGACCGTTGTGTGTGTTCTGGA-3′, CD86-R: 5′-AGGGCCACAGTAACTGAAGCTGTAA-3′; CD83-F: 5′-AAGTACAGGGCAGAAGCTGTGTTG-3′, CD83-R: 5′-AAGCTTGTTCCGTACCAGGTTTAGA-3′; AKT-F: 5′-GAGGTTGCCCACACGCTTA-3′, AKT-R: 5′-GGCGTACTCCATGACAAAGCAG-3′; GAPDH-F: 5′-GGTGAAGGTCGGTGTGAACG-3′, GAPDH-R: 5′-CTCGCTCCTGGAAGATGGTG-3′.

The primers and fluorescent probes were purchased from Takara. The PCR conditions were as follows: a preliminary cycle at 95 ℃ for 10 s, followed by 45 cycles at 95 ℃ for 5 s and at 60 ℃ for 20 s, followed by 1 min at 60 ℃ and 5 s at 95 ℃. We also confirmed that the efficiency of amplification for GAPDH was 100% in the exponential phase of PCR. The mRNA levels were normalized to *GADPH* mRNA according to the following formula: relative levels of target mRNA = $2^{-\Delta\Delta CT} \times 100$ %, where ΔΔ CT = ($CT_{FHF, LPS, D\text{-}Galn\ groups}$ - CT_{GAPDH}) - ($CT_{NS\ group}$ - CT_{GAPDH}). The intestinal mRNA levels of the FHF groups were compared with those of other groups.

Statistical analysis

For each experiment, at least 15 mice were tested per group. Data regarding group differences were reported as mean ± SD. Significant differences between treatment groups were determined using one-way analysis of variance with the Dunnett test.

RESULTS

Number of mature DCs

The DC surface markers, CD11b/c (Figure 1A) and CD83 (Figure 1B), were detected using immunohistochemistry.

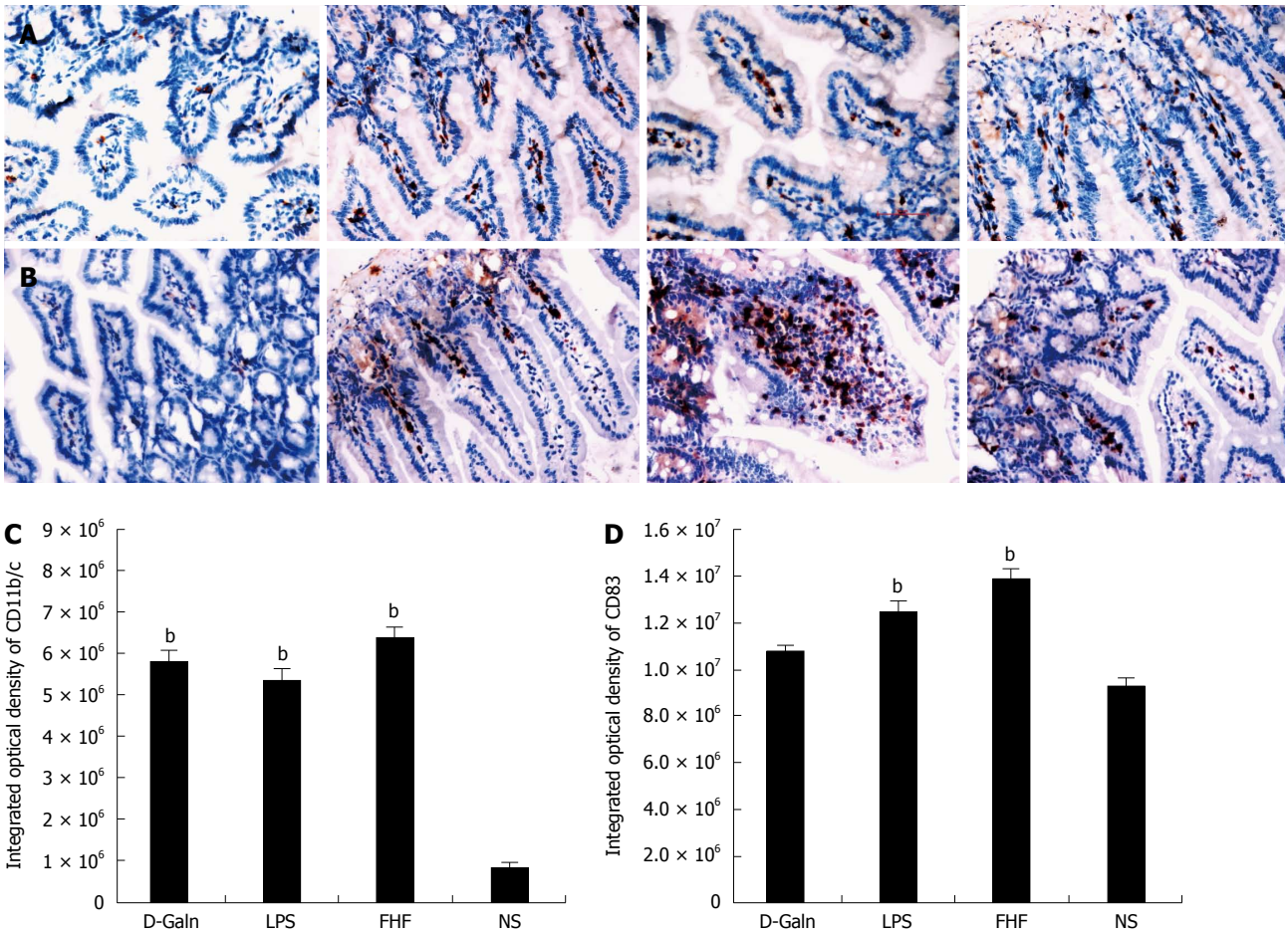

Figure 1 Immunohistochemical staining of CD11b/c (A) and CD83 (B) and integrated optical density of CD11b/c (C) and CD83 (D). From left to right panel: Normal saline (NS) group, D-galactosamine (D-Galn) group, lipopolysaccharide (LPS) group, and fulminant hepatic failure (FHF) group (A and B). The integrated optical density of CD11b/c (C) and CD83 (D) in the FHF group was notably increased compared with those of the NS group, but there was no significant difference compared with the LPS and D-Galn groups. The integrated optical density of CD11b/c in the LPS and D-Galn groups, as well as CD83 in the LPS group, was notably increased compared with that of the NS group ($^{b}P < 0.001$ *vs* the NS group, one way ANOVA with Dunnett test, $n \geqslant 15$).

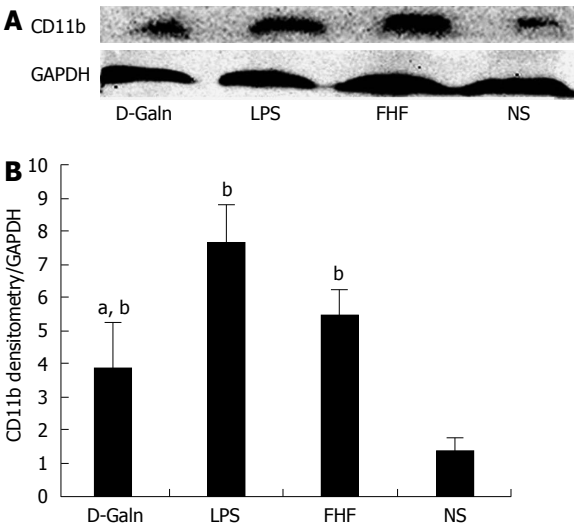

Figure 2 Intestinal CD11b protein expression. A: CD11b protein was detected by Western blot; B: Densitometric analysis using Image-Pro software. The absorbance value of CD11b/absorbance value of GAPDH in the fulminant hepatic failure (FHF) was notably increased compared with those of the normal saline (NS) and D-galactosamine (D-Galn) groups, but there was no significant change compared with that of the lipopolysaccharide (LPS) group ($^{b}P < 0.001$ *vs* the NS group, $^{a}P < 0.05$ *vs* the D-Galn group, one-way ANOVA with Dunnett test; $n \geqslant 15$).

Mature DC markers were expressed on the cytoplasmic membrane and in the cytoplasm of intestinal mucosal cells. In the FHF group, DCs were found to be distributed throughout the basal layer and in the lymphatic vessels, but they only appeared on the basal layer in the NS group (Figure 1A). In the FHF, LPS, and D-Galn groups, CD11b/c staining (Figure 1C) was significantly higher than that of the NS group. CD83 staining (Figure 1D) in the other three groups was higher than that of the NS group. To further confirm DC maturation, CD11b protein expression was measured (Figure 2A). The results demonstrated that the CD11b protein expression in the FHF, D-Galn and LPS groups was higher than that of the NS group (Figure 2B). At the genetic level, *CD11b* is encoded by the *Itg-α*, which is part of the integrin family. The *Itg-α* (Figure 3A) and *CD83* (Figure 3B) mRNA expression changes were consistent with the changes in their protein levels; the protein levels observed in each of the three treatment groups were higher than those of the NS group. Additionally, an increase in protein expression was more pronounced in the LPS and FHF groups. These data indicated that a greater number of DCs matured in the intestinal tissues of the FHF mice.

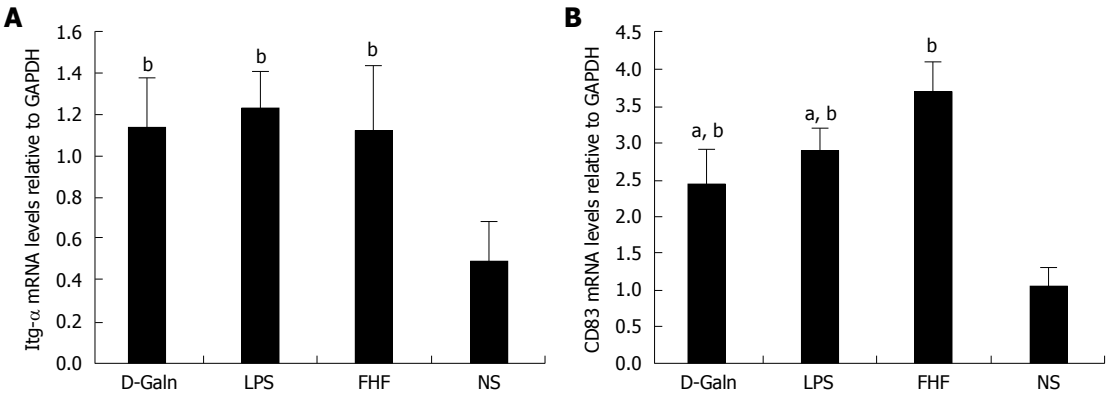

Figure 3 Relative expression of intestinal integrin-α (A) and CD83 (B) mRNAs. The relative levels of intestinal integrin-α (Itg-α) mRNA in the fulminant hepatic failure (FHF) group were significantly increased compared to the normal saline (NS) group, but there was no significant difference compared with those of the lipopolysaccharide (LPS) and D-galactosamine (D-Galn) groups. The relative levels of intestinal CD83 mRNA in the FHF group were significantly increased compared with the NS group, LPS group and D-Galn group ($^{b}P < 0.001$ *vs* the NS group, $^{a}P < 0.05$ *vs* the LPS and D-Galn groups, one-way ANOVA with Dunnett test; $n \geqslant 15$).

Mature DCs express CD86 and induce the activation of MHC Ⅱ (CD74) to elicit a T cell response

To explore how DCs might influence the development of FHF, the co-stimulatory molecule, CD86, was tested. The staining results (Figure 4A and D) showed that CD86 expression in the FHF, LPS and D-Galn groups was significantly higher than that of the NS group. The CD86 protein (Figure 5A and C), approximately 56 kDa, was remarkably increased in the FHF group compared with the other groups. Similarly, CD86 mRNA (Figure 6A) in the three treatment groups was much higher than in the NS group. To determine whether the MHC Ⅱ molecular pathway was activated, CD74 was measured. As shown in Figure 4B, CD74 was expressed both on the cell membrane and in the cytoplasm and was significantly increased (Figure 4E) in the FHF group. In all the three treatment groups, CD74 protein was expressed at a significantly higher level compared with the NS group (Figure 5A), and *CD74* mRNA was significantly increased as well (Figure 6B). To determine whether there was an increased number of T cells present in intestinal tissue samples from the FHF group, we measured CD3 staining. CD3 was distributed in the intestinal mucosal tissue (Figure 4C) and in the FHF group its number was the highest (Figure 4F). The CD3 protein expression (Figure 5B and 5) in the FHF group was also much higher than in other groups. The *CD3* mRNA (Figure 6C) of the three treatment groups was still far higher than that of the NS group. However, there was no difference among the three treatment groups. Therefore, we concluded that in the FHF group, the number and activity of intestinal mature DCs were significantly increased and that the T cells were also increased. Thus, these data suggested that the entire intestinal mucosal immune barrier was influenced.

Alterations in the AKT signaling pathway

The AKT signal transduction pathway is an important mechanism by which cells avoid apoptosis because it promotes the progression of the cell cycle and thus cell proliferation and activation. The activation of AKT is usually characterized by phosphorylation. AKT is primarily expressed in the basal layer of the intestinal mucosa. The results of immunohistochemistry staining showed that in the FHF, LPS and D-Galn groups, AKT (Figure 7A and C) was not significantly changed, but p-AKT (Figure 7B and D) was higher than that of the NS group. Compared with the NS group, AKT protein (Figure 8A and B) was only non-significantly increased in the FHF, LPS and D-Galn groups. However, the expression of p-AKT protein (Figure 8A and C) showed a significant difference. In addition, the *AKT* mRNA (Figure 9) levels were not significantly different among these groups. We concluded that the AKT signaling pathway was activated in the FHF group.

DISCUSSION

The gut microbiome plays both physiological and pathological roles in intestinal homeostasis and the pathogenesis of inflammatory bowel diseases[15]. As effector cells of both innate and adaptive immune responses, DCs are central not only for maintaining protective immunity against pathogens but also for preventing inflammatory intestinal immune responses against the microbiota and food antigens. Similar effector functions to those involved in protective immunity against pathogens are engaged during inappropriate inflammatory responses against harmless antigens[16]. The pathological changes of other organs often affect the gut, as occurs in many hepatic diseases. In the case of FHF, SBP is a common complication caused by pathological BT. The clinical pathological causes of BT are the increase in intestinal permeability and the disruption of the intestinal mucosal barrier[17,18]. These changes indicate that DCs may be involved in FHF due to their role as

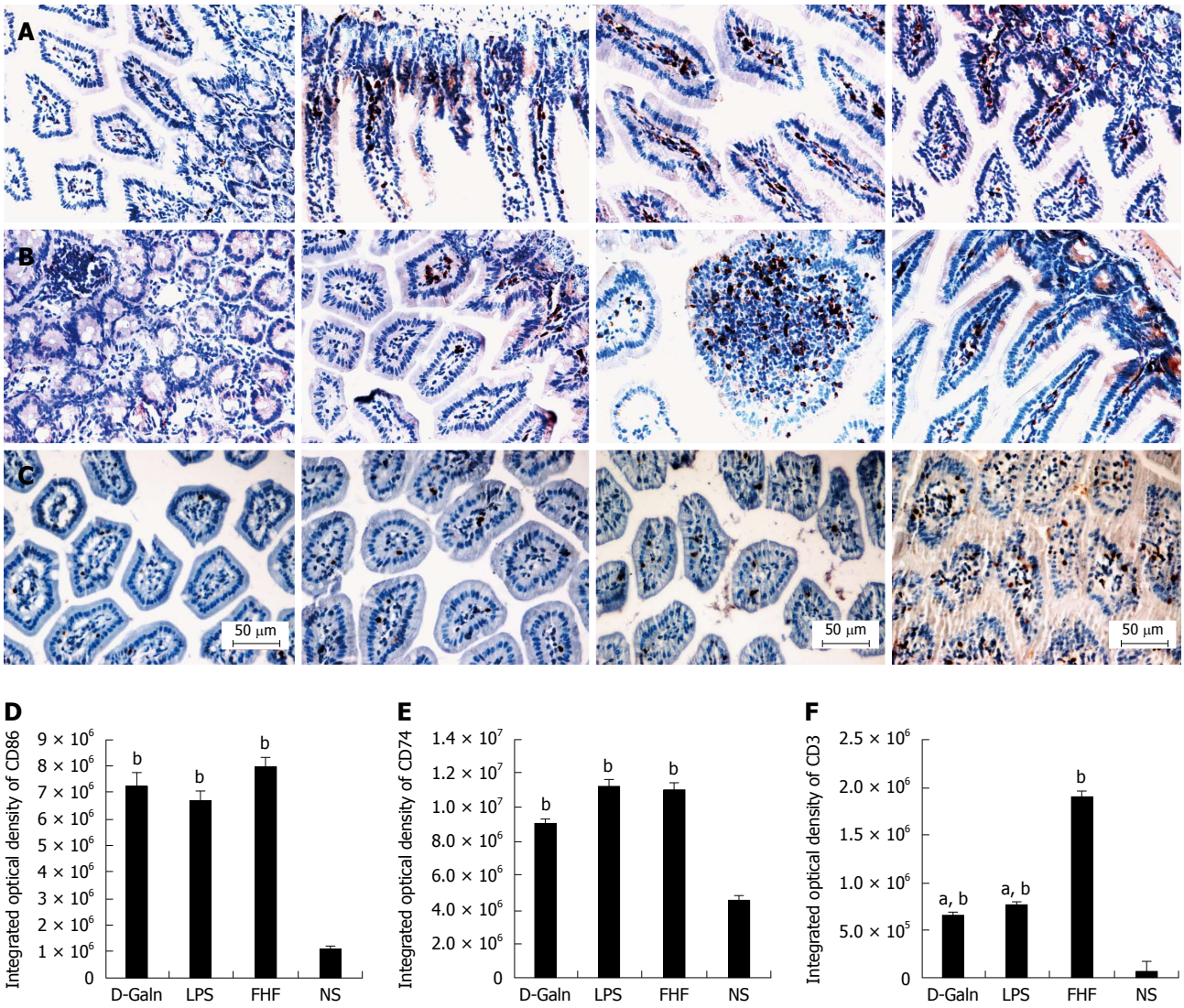

Figure 4 Immunohistochemical staining of CD86 (A), CD74 (B), and CD3(C) and integrated optical density of CD86 (D) CD74 (E), and CD3 (F). From left to right panel: Normal saline (NS) group, D-galactosamine (D-Galn) group, lipopolysaccharide (LPS) group, and fulminant hepatic failure (FHF) group (A, B and C). The integrated optical density of CD86 (D) and CD74 (E) in the FHF group was notably increased compared to the NS group. However, CD86 and CD74 were not significantly different among the FHF, LPS and D-Galn groups. The integrated optical density of CD3 in the FHF group was notably increased compared with the NS, LPS and D-Galn groups ($^{b}P < 0.001$ *vs* the NS group, $^{a}P < 0.05$ *vs* LPS and D-Galn groups, one-way ANOVA with Dunnett test; $n \geqslant 15$).

professional APCs present in the gut and their ability to disrupt tight junctions to sample bacteria in the lumen.

The intestinal mucosa contains many subtypes of DCs, which are present in two functionally distinct states; *i.e.*, immature and mature cells. The subtypes of DCs vary based on their typical tissue localization. For example, DCs isolated from intestinal tissues are endowed with unique mucosal functions that are specific to these DC subsets. It was originally thought that mature DCs induced protective immunity to infectious agents but that immature DCs induced tolerance to innocuous antigens[19]. CD83 has been implicated in the regulation of inflammation, and it is expressed on a number of cell types[20]. In this study, by using CD11b/c and CD83 as DC maturation markers, we demonstrated that mature intestinal DCs in the FHF group showed a significantly increased trend. These data were in agreement with other studies in which CD83 expression was upregulated in a chemical-induced murine colitis model[21]. Some studies showed that LPS injection can influence the expression of MCH-Ⅱ and CD11b in brain sections[22]. Our results might also be associated with the concentration of LPS used *in vivo*[23].

DCs have roles in both the innate and adaptive immune responses. DCs are sentinels of the immune system recognizing and translating pathogenic signals into immune responses[24]. Thus, an increase of intestinal DCs suggested that the intestinal mucosa had been injured in FHF. Some studies have shown that the presence of CD83 on mDC membranes enhanced T lymphocyte proliferation[25]. For instance, CD80/CD86, expressed in DCs, plays a central role in T cell activation by delivery of co-stimulatory

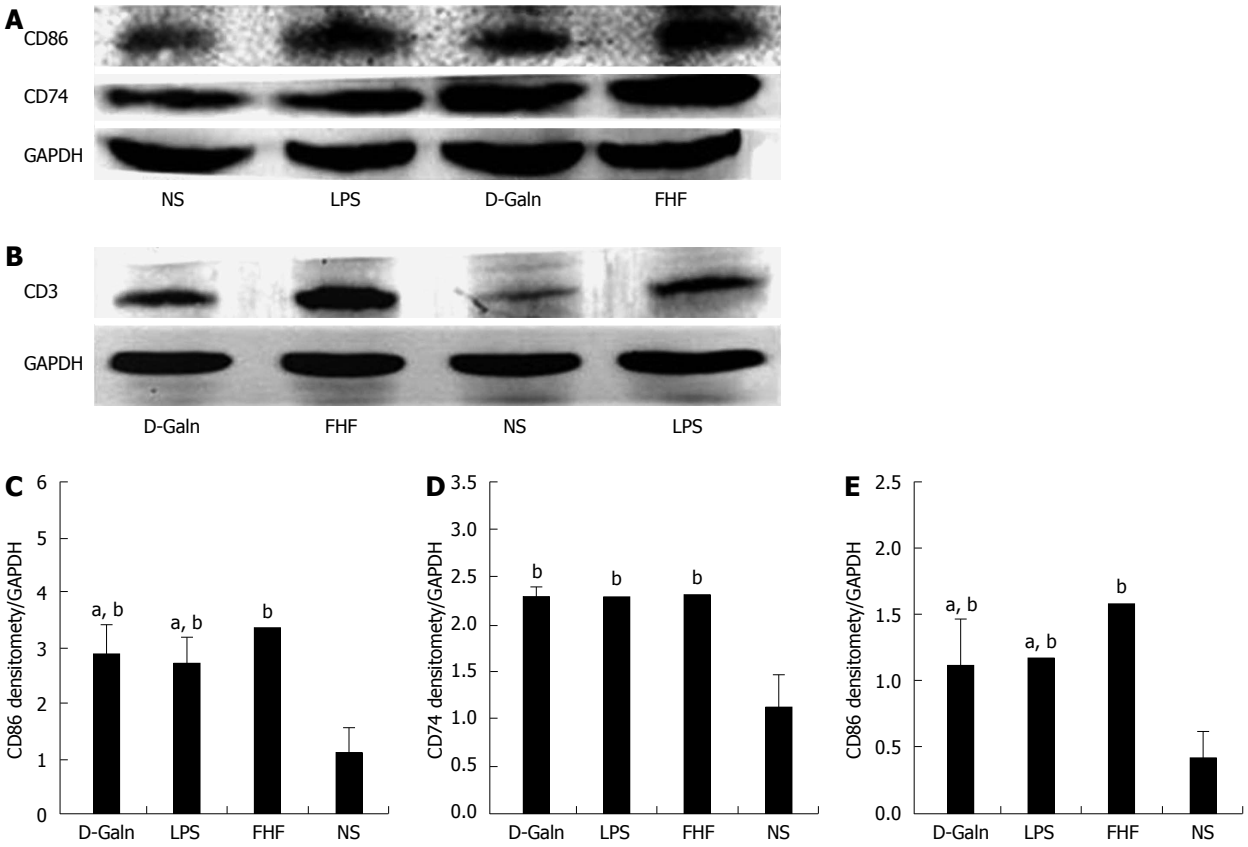

Figure 5 Intestinal CD86 (A), CD74 (B), and CD3 (C) protein expression. A and B: CD86, CD74, and CD3 protein expression was detected by Western blot; C, D and E: Densitometric analysis using Image-Pro software. The absorbance values for CD86 and CD74 and the CD3/absorbance value of GAPDH in tissues from the fulminant hepatic failure (FHF) group were notably increased compared with those of the normal saline (NS) group. The CD86 and CD3 levels were increased, but for CD74 there were no significant differences compared with the lipopolysaccharide (LPS) and D-galactosamine (D-Galn) groups ($^{b}P < 0.001$ *vs* the NS group, $^{a}P < 0.05$ *vs* the LPS and D-Galn groups, one-way ANOVA with Dunnett test; $n \geqslant 15$).

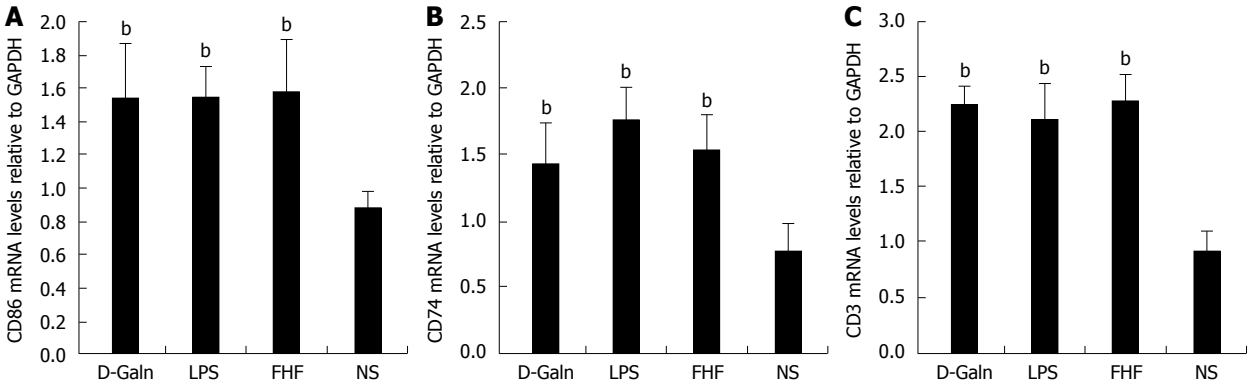

Figure 6 Relative levels of intestinal CD86 (A), CD74 (B), and CD3 (C) mRNAs. Levels of intestinal CD86, CD74 and CD3 mRNAs in tissues from the fulminant hepatic failure (FHF) group were significantly increased compared with those of the normal saline (NS) group, but there were no significant differences compared with tissues from the lipopolysaccharide (LPS) and D-galactosamine (D-Galn) groups ($^{b}P < 0.001$ *vs* the NS group, one-way ANOVA with Dunnett test; $n \geqslant 15$).

signals to naïve T cells, activating them to become effector T cells[26]. The report showed that the expression intensity of CD86 with LPS stimulation was significantly higher[27]. The results of the current study demonstrated that CD86 and CD3 were significantly increased in FHF tissues. These data indicated that intestinal T cell activation was increased in FHF as a result of ligation with CD80/CD86 on DCs.

CD74 is a type Ⅱ integral membrane protein and acts as a chaperone for MHC Ⅱ protein expression[28]. It is a key substrate that contributes to the survival of DCs[29,30]. Downregulation of CD74 has been shown to regulate DC migration *in vitro* and *in vivo*[31,32]. It was also reported that post-infectious intestinal lamina propria DCs displayed increased CD86 and MHC class Ⅱ, resulting in the enhanced induction of T cell proliferation[33]. Other reports suggested that DCs displayed higher expression of MHC Ⅱ and CD86 in

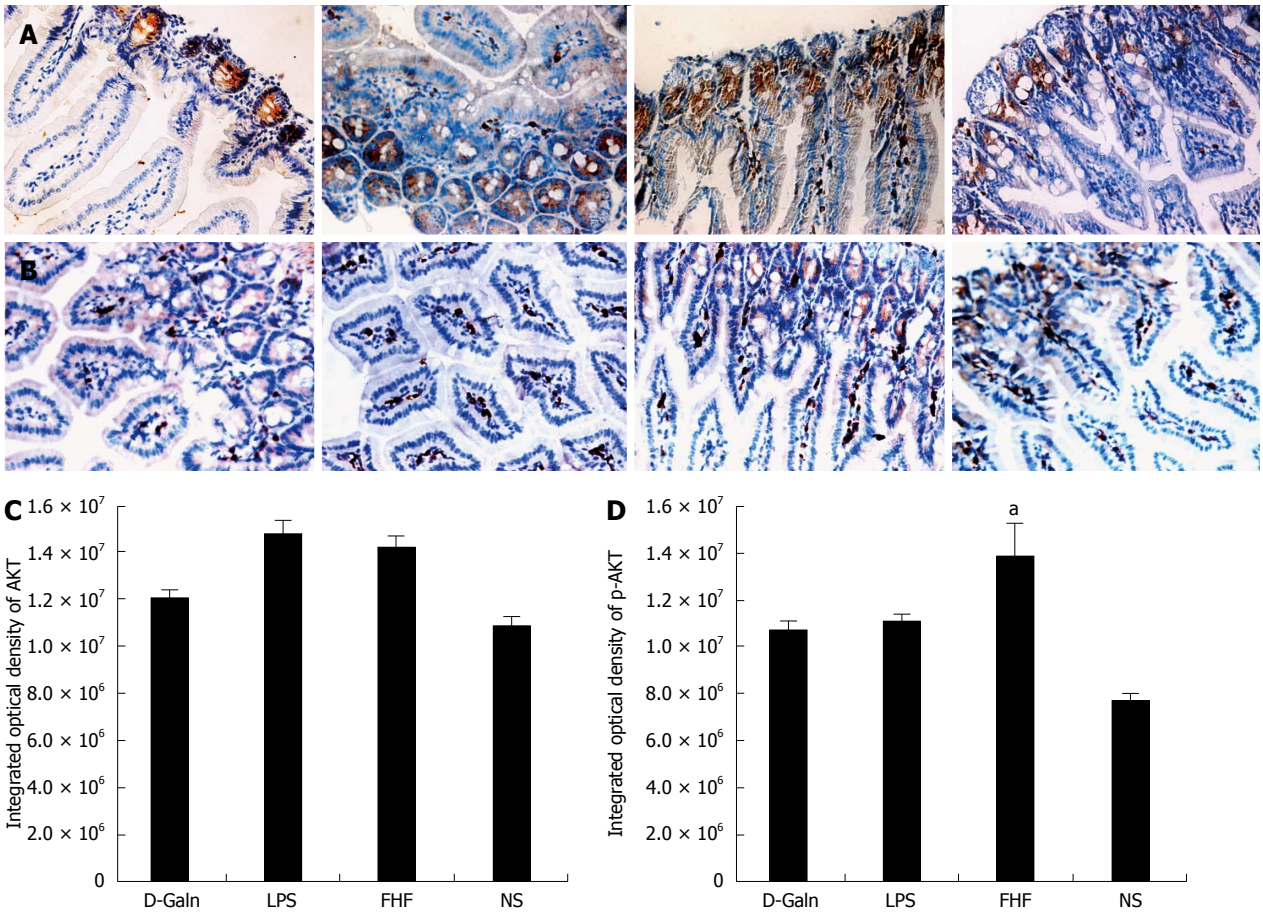

Figure 7 Immunohistochemistry staining of AKT (A) and phosphorylated-AKT (B) and integrated optical density of AKT (C) and phosphorylated-AKT (D). From left to right panel: Normal saline (NS) group, D-galactosamine (D-Galn) group, lipopolysaccharide (LPS) group, and fulminant hepatic failure (FHF) group (A and B). The integrated optical density of AKT (C) in the FHF group was not significantly different compared to the NS group, LPS group and D-Galn group The integrated optical density of phosphorylated-AKT (p-AKT) (D) in the FHF was notably increased compared with the NS group but not significantly different compared with the LPS and D-Galn groups ($^aP < 0.05$ *vs* the NS group, one-way ANOVA Dunnett test; $n \geqslant 15$).

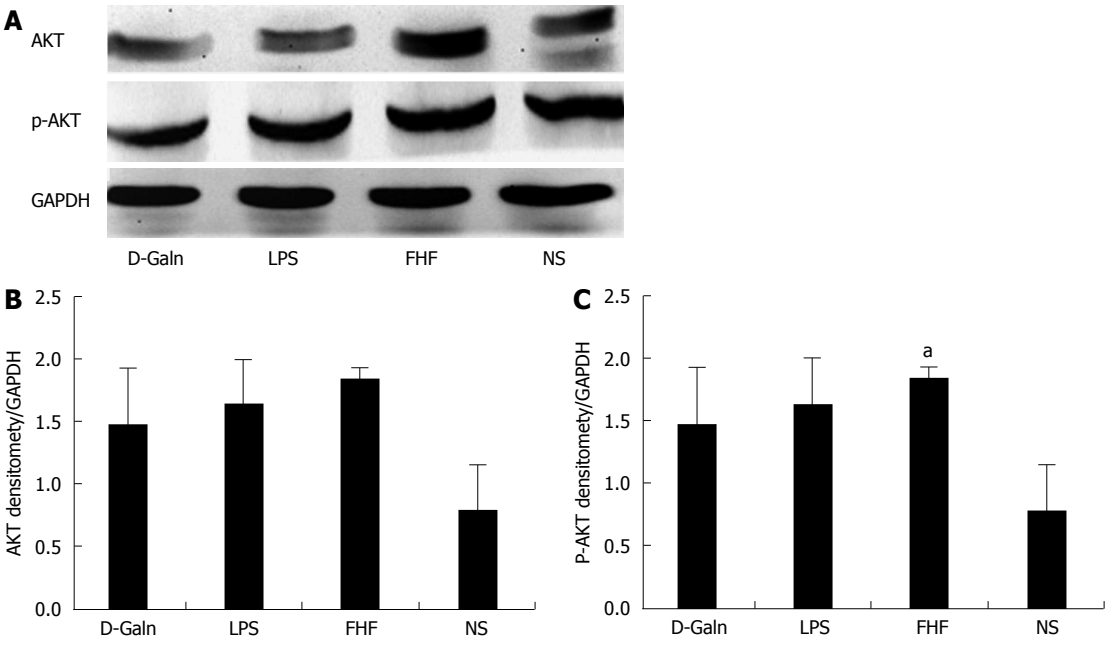

Figure 8 Intestinal AKT and phosphorylated-AKT protein expression. A: AKT and p-AKT protein expression was detected by Western blot; B and C: Densitometric analysis using the Image-Pro software. The ratio of the absorbance of AKT/absorbance of GAPDH in the fulminant hepatic failure (FHF) group was not significantly different compared with those of normal saline (NS), lipopolysaccharide (LPS) and D-galactosamine (D-Galn) groups. The ratio of the absorbance of phosphorylated-AKT (p-AKT)/absorbance of GAPDH in the FHF group was notably increased compared with that of NS group, but was not significantly different compared with those of the LPS and D-Galn groups ($^aP < 0.05$ *vs* the NS group, one-way ANOVA with Dunnett test; $n \geqslant 15$).

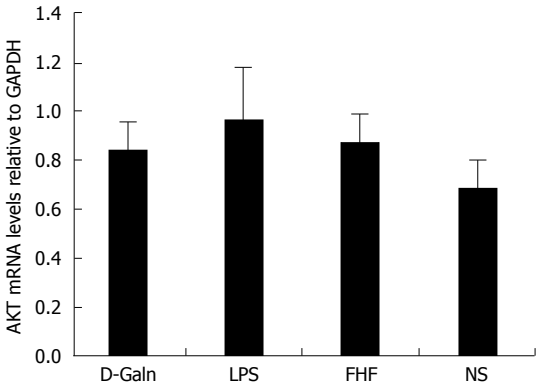

Figure 9 Relative levels of intestinal AKT mRNA. The relative levels of intestinal AKT mRNA among the four groups were not significantly different ($P > 0.05$, one-way ANOVA with Dunnett test; $n \geqslant 15$). D-Galn: D-galactosamine; FHF: Fulminant hepatic failure; LPS: Lipopolysaccharide; NS: Normal saline.

LPS-induced liver injury[34]. In this study, CD74 was significantly increased in FHF tissue. Collectively, these results imply that in FHF, SBP would lead to the rapid maturation of DCs and the subsequent activation of T cells to produce an adaptive immune response.

AKT signaling has been reported to play a key role in regulating cell growth, survival, and metabolism in a variety of apoptotic paradigms[35]. Activated AKT, which in turn phosphorylates and inactivates components of the apoptotic machinery, participates in cell survival, proliferation and apoptosis inhibition[36,37]. Abnormal activation of the AKT pathway may lead to disease and dysfunction. Studies have found that crosslinking CD80/CD86 in human DCs activated the PI3K/AKT pathway[38]. AKT activation has also been shown to be an early sign of intestinal microenvironmental change and is closely related to the treatment of gut injury[39]. Therefore, we measured p-AKT as a first attempt to understand the role of the AKT pathway in FHF. Our results demonstrated that p-AKT was significantly increased in FHF. In addition, other researchers have shown that activation of the AKT pathway might be involved in the pathogenesis of Crohn's disease[40]. Moreover, AKT activation might be involved in the transition from intestinal inflammation to cancer[41]. Taken together, these results suggested that the AKT pathway was activated in FHF and might participate in early intestinal mucosal injury.

DCs play an important role in intestinal mucosal health because they can induce a T cell adaptive immune response. In this study, a large number of DCs matured, expressed CD86, and activated MHC class Ⅱ molecular pathways to induce a T cell response in FHF. In addition, the AKT signaling pathway was activated in FHF.

COMMENTS

Background

Fulminant hepatic failure (FHF) is often associated with spontaneous bacterial peritonitis (SBP), which is caused by bacterial translocation from the gut after serious liver damage and is associated with significantly increased mortality. Dendritic cells (DCs) play an important role in the generation and regulation of immune responses and oversee intestinal immune homeostasis in SBP. Studies on intestinal DCs were performed to understand the mechanisms of SBP.

Research frontiers

DCs are sentinels of the immune system that recognize and translate pathogenic signals into immune responses. Intestinal DC numbers and function in liver diseases have become a topic of interest. This study is basic to understanding the mechanisms of SBP.

Innovations and breakthroughs

To date, many studies have been carried out primarily on bacterial translation and cytokine expression by T cells and DCs. In this study, the authors carefully studied changes in the number of intestinal DCs by employing immunohistochemistry, Western blot and polymerase chain reaction and found that a large number of DCs matured, expressed CD86, and activated the MHC Ⅱ molecular pathway to induce a T cell response in FHF. Furthermore, the AKT signaling pathway was activated in FHF.

Applications

By identifying the intestinal DCs in FHF, the authors evaluated CD86, CD83 and CD74 in intestinal DCs, which could improve our understanding of the mechanisms of SBP in FHF.

Terminology

Integrin-α (Itg-α): At the genetic level, *CD11b* is encoded by the *Itg-α* gene, which is part of the integrin family.

Peer-review

The authors performed experiments to detect CD86, CD83 and CD74 in the intestinal DCs in FHF and found that a large number of DCs matured, expressed CD86, and activated the MHC Ⅱ molecular pathway to induce a T cell response in FHF. Furthermore, the authors detected AKT and phosphorylated-AKT, and the results showed that the AKT signaling pathway was activated in FHF.

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ORIGINAL ARTICLE

Case Control Study

Estimating steatosis and fibrosis: Comparison of acoustic structure quantification with established techniques

Thomas Karlas, Joachim Berger, Nikita Garnov, Franziska Lindner, Harald Busse, Nicolas Linder, Alexander Schaudinn, Bettina Relke, Rima Chakaroun, Michael Tröltzsch, Johannes Wiegand, Volker Keim

Thomas Karlas, Nikita Garnov, IFB Adiposity Diseases, Leipzig University Medical Center, 04103 Leipzig, Germany
Thomas Karlas, Joachim Berger, Franziska Lindner, Michael Tröltzsch, Johannes Wiegand, Volker Keim, Department of Medicine, Neurology and Dermatology, Division of Gastroenterology and Rheumatology, University Hospital Leipzig, 04103 Leipzig, Germany
Nikita Garnov, Harald Busse, Nicolas Linder, Alexander Schaudinn, Department of Diagnostic and Interventional Radiology, University Hospital Leipzig, 04103 Leipzig, Germany
Bettina Relke, Internistische Hausarzt- und Diabetologische Schwerpunktpraxis Dr. Relke, 04249 Leipzig, Germany
Rima Chakaroun, Department of Medicine, Neurology and Dermatology, Division of Endocrinology and Nephrology, University Hospital Leipzig, 04103 Leipzig, Germany
Author contributions: Karlas T and Berger J contributed equally to this work; Karlas T, Berger J, Garnov N, Wiegand J and Keim V designed the research; Karlas T, Berger J, Garnov N, Lindner F, Busse H, Linder N, Schaudinn A, Relke B, Chakaroun R, Tröltzsch M, Wiegand J and Keim V performed the research; Karlas T, Berger J, Garnov N, Busse H, Linder N, Schaudinn A, Wiegand J and Keim V analyzed the data; Karlas T, Berger J, Garnov N, Wiegand J and Keim V wrote the manuscript; Lindner F, Busse H, Linder N, Schaudinn A, Relke B, Chakaroun R and Tröltzsch M revised the manuscript.
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Data sharing: Technical appendix, statistical code, and dataset are available from the corresponding author at thomas.karlas@medizin.uni-leipzig.de. No additional data are available.

Correspondence to: Thomas Karlas, MD, Department of Medicine, Neurology and Dermatology, Division of Gastroenterology and Rheumatology, University Hospital Leipzig, Liebigstrasse 20, 04103 Leipzig, Germany. thomas.karlas@medizin.uni-leipzig.de
Telephone: +49-341-9712200
Fax: +49-341-9712209
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Abstract

AIM: To compare ultrasound-based acoustic structure quantification (ASQ) with established non-invasive techniques for grading and staging fatty liver disease.

METHODS: Type 2 diabetic patients at risk of non-alcoholic fatty liver disease (n = 50) and healthy volunteers (n = 20) were evaluated using laboratory analysis and anthropometric measurements, transient elastography (TE), controlled attenuation parameter (CAP), proton magnetic resonance spectroscopy (^{1}H-MRS; only available for the diabetic cohort), and ASQ. ASQ parameters mode, average and focal disturbance (FD) ratio were compared with: (1) the extent of liver fibrosis estimated from TE and non-alcoholic fatty liver disease (NAFLD) fibrosis scores; and (2) the amount of steatosis, which was classified according to CAP values.

RESULTS: Forty-seven diabetic patients (age 67.0 ± 8.6 years; body mass index 29.4 ± 4.5 kg/m²) with reliable CAP measurements and all controls (age 26.5 ± 3.2 years; body mass index 22.0 ± 2.7 kg/m²) were included in the analysis. All ASQ parameters showed differences between healthy controls and diabetic patients (P < 0.001, respectively). The ASQ FD ratio (logarithmic) correlated with the CAP (r = -0.81, P < 0.001) and ^{1}H-MRS (r = -0.43, P = 0.004) results. The FD ratio [CAP < 250 dB/m: 107 (102-109), CAP between 250 and 300 dB/m: 106 (102-114); CAP between 300 and 350 dB/m: 105 (100-112), CAP ⩾ 350 dB/m: 102 (99-108)] as well as mode and average parameters, were reduced in cases with advanced steatosis (ANOVA P < 0.05). However, none of the ASQ parameters showed a significant difference in patients with advanced fibrosis, as determined by TE and the NAFLD fibrosis score (P > 0.08, respectively).

CONCLUSION: ASQ parameters correlate with steatosis, but not with fibrosis in fatty liver disease. Steatosis estimation with ASQ should be further evaluated in biopsy-controlled studies.

Key words: Transient elastography; Non-alcoholic fatty liver disease; Liver stiffness; Non-alcoholic fatty liver disease; Fibrosis score; Controlled attenuation parameter

Core tip: Non-invasive characterization of hepatic steatosis and fibrosis is becoming important for the screening, diagnosis, and monitoring of patients with chronic liver diseases. This work compared acoustic structure quantification (ASQ) and established non-invasive methods to characterize fatty liver disease. ASQ parameters differed between healthy controls and diabetic patients with fatty liver disease independent of the extent of fibrosis. The focal disturbance ratio and further ASQ parameters correlated with the severity of steatosis. Therefore, ASQ could be used to evaluate steatosis and merits further investigation; however, ASQ seems to be impractical to characterize fibrosis in patients with fatty liver disease.

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INTRODUCTION

Non-invasive characterization of hepatic steatosis and fibrosis are attracting growing scientific and medical interest for the screening, diagnosis, and monitoring of patients with chronic liver diseases[1-3]. In particular, fibrosis staging by means of elastography is highly accurate for detecting advanced liver injury and has, therefore, been progressively implemented in clinical practice[1].

In addition to fibrosis characterization, non-invasive quantification of hepatic fat content has attracted increasing attention in terms of both risk assessment and monitoring of patients with non-alcoholic fatty liver disease (NAFLD) and liver diseases from other etiologies[1,4]. The controlled attenuation parameter (CAP) - computed by commercial software that comes with the transient elastography device (Fibroscan) - correlates steatosis with the signal attenuation during liver stiffness measurement (LSM), and is very accurate to detect advanced hepatic steatosis[5-9]. Moreover, magnetic resonance based techniques (*e.g.*, proton magnetic resonance spectroscopy, ^{1}H-MRS) allow quantification of the hepatic lipid fraction, an additional parameter of the severity of steatosis[10,11]. However, these approaches are affected by high costs and limited availability (^{1}H-MRS) or by anthropometry (CAP)[4,8].

Analysis of B-Mode ultrasound may represent a further option for the non-invasive grading and staging of liver damage, as conventional sonography is highly sensitive to subtle changes in tissue texture[12,13]. However, comparison of gray scale images is operator-dependent and also varies with some technical parameters[12]. Computerized analysis of acoustic tissue properties may overcome these limitations: acoustic structure quantification (ASQ) software analyzes the characteristic intensity pattern ("speckles") of conventional B-Mode ultrasound and compares its distribution histogram with a theoretical probability density function (PDF, Rayleigh distribution) of the echo amplitude[13,14]. Histology-based studies have reported that ASQ parameters correlate with the degree of fibrosis in patients with liver diseases of different etiologies[13-15]. Moreover, a pilot study revealed strong agreement between ASQ values and hepatic fat accumulation in an animal model[16].

ASQ results have not yet been compared with those of established non-invasive techniques. Therefore, we prospectively compared ASQ measurements in healthy controls and diabetic patients at risk for NAFLD or associated liver injury with TE and CAP values (reference standards) as well as ^{1}H-MRS results and serum based NAFLD fibrosis scores.

MATERIALS AND METHODS

Patients and controls

The study was reviewed and approved by the local ethics committee (University of Leipzig, register no. 358/08-B-ff and No. 419-12-17122012) and performed in accordance with the ethical guidelines of the Helsinki

Declaration. Written informed consent was obtained from all participants before study enrollment.

Between April 2013 and March 2014, outpatients with type 2 diabetes mellitus at risk of NAFLD and without other liver diseases were recruited. For the control group, healthy volunteers with no history of any chronic liver disease, diabetes mellitus, or metabolic syndrome, were recruited. Increased ultrasound echogenicity of the liver parenchyma (compared with the right renal cortex) or CAP > 252 dB/m were regarded as exclusion criteria for the control group[5]. Significant alcohol intake (weekly consumption > 210 g for men and 140 g for woman, respectively) was ruled out for the total study cohort before inclusion[17].

For all participants, anthropometric examination, ultrasound, LSM (transient elastography, M probe), CAP assessment and laboratory parameters (patients only) were performed on the same day after a fasting period of at least three hours. ^{1}H-MRS was performed either on the same day or after 6 mo during follow-up visits.

Laboratory assessment and NAFLD fibrosis score

Albumin, blood count, and serum levels of amino-transferases (ALT and AST) were determined for all diabetic patients. Individuals with highly elevated aminotransferases (at least five times the upper normal limit) were excluded from further examinations because of the risk of inaccurate LSM.

The NAFLD fibrosis score was calculated according to Angulo *et al*[18]: Score = -1.675 + 0.037 × age (years) + 0.094 × body mass index (kg/m^2) + 1.13 × diabetes (yes 1, no 0) + 0.99 × AST/ALT ratio - 0.013 × platelet (Gpt/l) - 0.66 × albumin (g/dL). A score of at least 0.676 indicated high risk of advanced liver fibrosis[18].

Ultrasound, elastography, and controlled attenuation parameter

All subjects underwent conventional ultrasound to rule out mechanical cholestasis. LSM was performed using the M probe of transient elastography (TE; Fibroscan, Echosens, Paris, France). According to the manufacturer's recommendation, examinations with fewer than 10 valid measurements or an interquartile range > 30% of the median LSM value (only in cases with liver stiffness ⩾ 7.1 kPa) were excluded from further analysis[19]. TE values ⩾ 7.9 kPa indicated the presence of advanced fibrosis[20].

The CAP gives additional information about the attenuation of ultrasonic signals during TE examination. CAP computation is included in the TE software, and the results (CAP, LSM) are shown together[21]. CAP was only considered when TE measurements were valid and reliable[5,21]. CAP served as reference method; therefore, only cases with valid CAP results were included in the final analysis. CAP values ⩾ 252 dB/m indicated fatty liver disease[5], and values ⩾ 300 dB/m were regarded as advanced steatosis[8]. No patient had values between 250 and 252 dB/m; therefore, the diabetic subjects were classified into four groups to better present the data: (1) CAP < 250 dB/m; (2) CAP ⩾ 250 and < 300 dB/m; (3) CAP ⩾ 300 and < 350 dB/m; and (4) CAP ⩾ 350 dB/m.

Acoustic structure quantification

Analytical method: The ASQ software analyzes the linear raw data from ultrasound B-mode images. It provides a number of tissue parameters that are related to the scattering of echoes in user-defined region of interests (ROI). The histogram of the measured intensity distribution in the B-mode image ROI is essentially compared with the PDF of an ideal, homogenous scatterer (Rayleigh distribution)[22]. ASQ subdivides the primary ROI into a large number of (up to 1000) secondary ROIs and displays the so-called C^2-histogram of the distribution of frequency ratios given by frequency of the ratio

$C^2 = \sigma^2/\sigma_R^2$, where σ and σ_R stand for the standard deviations of measured and estimated (Rayleigh) PDFs, respectively[13]. In the liver, ASQ uses an empirical cut-off parameter α and reports slightly modified parameters σ_m and C_m^2 instead to minimize the influence of individual strong scatterers[13].

Mode (value with highest appearance, "peak value"), average and SD are then derived from the C^2 or C_m^2 histogram. A more recent ASQ implementation computes two curves (displayed in red and blue), depending on whether the variation in α quantitatively changes the σ parameter by less (depicted in red) or more (depicted in blue) than an empirical percentage (20%), respectively. The focal disturbance (FD) ratio is then defined as the ratio of the areas under the curve between the red and blue curve[16].

ASQ imaging: In the present study, B-mode images of the right liver lobe were recorded in the region of LSM and CAP acquisition using a curved array transducer (PVT-375 BT 3.5 MHz) and an Aplio 500 ultrasound scanner (Toshiba Medical Systems, Otawara, Japan). Display depth and focus were fixed at 10 and 5.5 cm, respectively. The ultrasound signal gain was set to 90%. A raw data loop of 2-3 s was recorded using the ASQ preset mode and files were exported to a PC workstation in DICOM format. ASQ was analyzed with the vendor's software (PC-ASQR, Aplio500, Version 1.01R000). Mode, average and FD ratio were computed in four ROIs covering most of the liver parenchyma, but avoiding larger vessels and bile ducts (Figure 1). The respective mean ASQ values of five separate frames were computed to obtain more reliable measures for each subject.

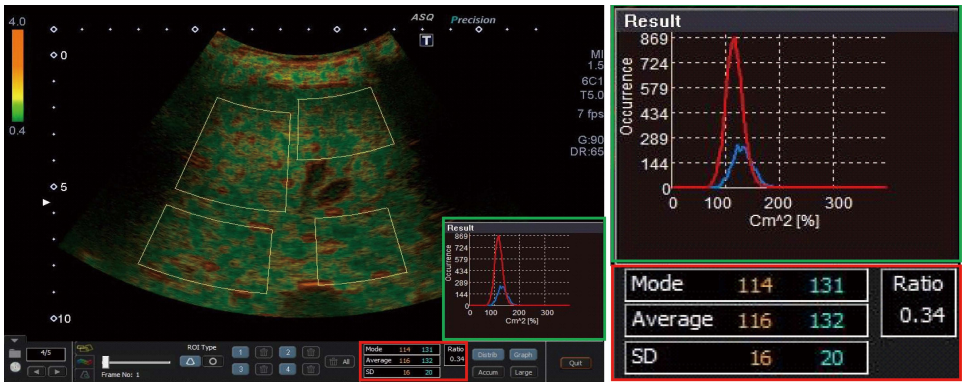

Figure 1 Acoustic structure quantification analysis of liver tissue. Four separate regions of interest were drawn to cover most of the parenchyma, but avoiding large vessels. The acoustic structure quantification software automatically calculated the parameters mode, average and standard deviation for both curves of the histogram (green frame), as well as the ratio of the areas under both curves (focal disturbance ratio, red frame).

Table 1 Clinical characteristics of the study cohort *n* (%)

Parameter	Healthy controls	Type 2 diabetes mellitus
Sex (F/M)	10/10	23/24
Age (yr)	26.5 ± 3.2	67.0 ± 8.6[b]
Body mass index (kg/m²)	22.0 ± 2.7	29.4 ± 4.5[b]
< 25 kg/m²	19 (95)	7 (15)
25-30 kg/m²	1 (5)	21 (45)
> 30 kg/m²	-	19 (40)
Waist-to-hip ratio	0.85 ± 0.08	0.97 ± 0.08[b]
< 1.0	19 (95)	27 (57%)
⩾ 1.0	1 (5)	20 (43%)
CAP (dB/m)	192 (151-237)	329 (100-396)[d]
< 250 dB/m	20 (100)	6 (13)
250-300 dB/m	-	10 (21)
300-350 dB/m	-	19 (40)
> 350 dB/m	-	12 (26)

[b]$P < 0.01$ *vs* controls; [d]$P < 0.01$ *vs* controls by design. Controls were excluded if CAP > 252 dB/m. CAP: Controlled attenuation parameter.

Magnetic resonance spectroscopy

The majority of patients (79%) underwent ^{1}H-MRS on the same or the following day of ASQ and TE examinations. In 21% of the cases, the time interval between ultrasound examination and ^{1}H-MRS was 6.5 mo (range: 6.2-7.3 mo) for technical reasons.

^{1}H-MRS was performed as described previously with some technical modifications[8]. In brief, T2-corrected, single-voxel MR spectra were acquired on a 1.5-T scanner (Achieva XR, Philips Healthcare, Best, Netherlands) using local shimming and a stimulated-echo acquisition method (STEAM). Voxels sized 20 mm × 20 mm × 20 mm were placed in liver segment Ⅶ, avoiding larger bile ducts and vessels. Spectroscopic data were acquired without water suppression, using the following sequence parameters: repetition time, *TR* = 3.000 ms; number of echoes, 5; echo times 10-50 ms; 2048 data points; bandwidth 1.000 Hz/pixel; 40 averages; total acquisition time 270 s. MR spectra were analyzed using a commercial tool (LCModel 6.3, Oakville, Canada) that determines the relative hepatic lipid concentrations. Calculated areas of water and fat peaks were corrected for T2 relaxation (using MR spectra at different echo times) and were used to calculate the hepatic fat fraction[8].

Data processing and statistical methods

All parameters were recorded in a spreadsheet file (Microsoft Excel, Microsoft). Statistical testing was carried out using commercial software (MedCalc 14.12, MedCalc Software, Ostend, Belgium). Data were expressed either as mean ± SD or median and range, as appropriate.

Fisher's exact test and χ^2 tests were used to test for the association of variables. Nonparametric tests (Mann-Whitney *U* test, Kruskal-Wallis test) were used to compare median values of independent samples, where post-hoc pair-wise comparisons were performed according to Conover[23]. For mean values, the *t*-test was applied. The Pearson correlation coefficient was calculated to analyze the degree of association between two variables. *P* values < 0.05 indicated a significant difference. Diagnostic performance of ASQ parameters was analyzed using receiver operating characteristic (ROC) curves.

The statistical methods of this study were reviewed by PD Dr. David Petroff (IFB Adiposity Diseases, Leipzig University Medical Center/Clinical Trial Center, University of Leipzig, Leipzig, Germany).

RESULTS

Clinical characteristics of the study cohort

Fifty patients with type 2 diabetes mellitus and 20 healthy volunteers were recruited. TE and CAP were available in 47 of the diabetic patients (94%): three subjects had an invalid measurement (two males, all cases with fewer than ten valid shots) and where excluded from further analysis. ^{1}H-MRS was available for *n* = 43 diabetic patients because of contraindications and technical reasons in four cases. The characteristics of the analyzed cohort are displayed in Table 1.

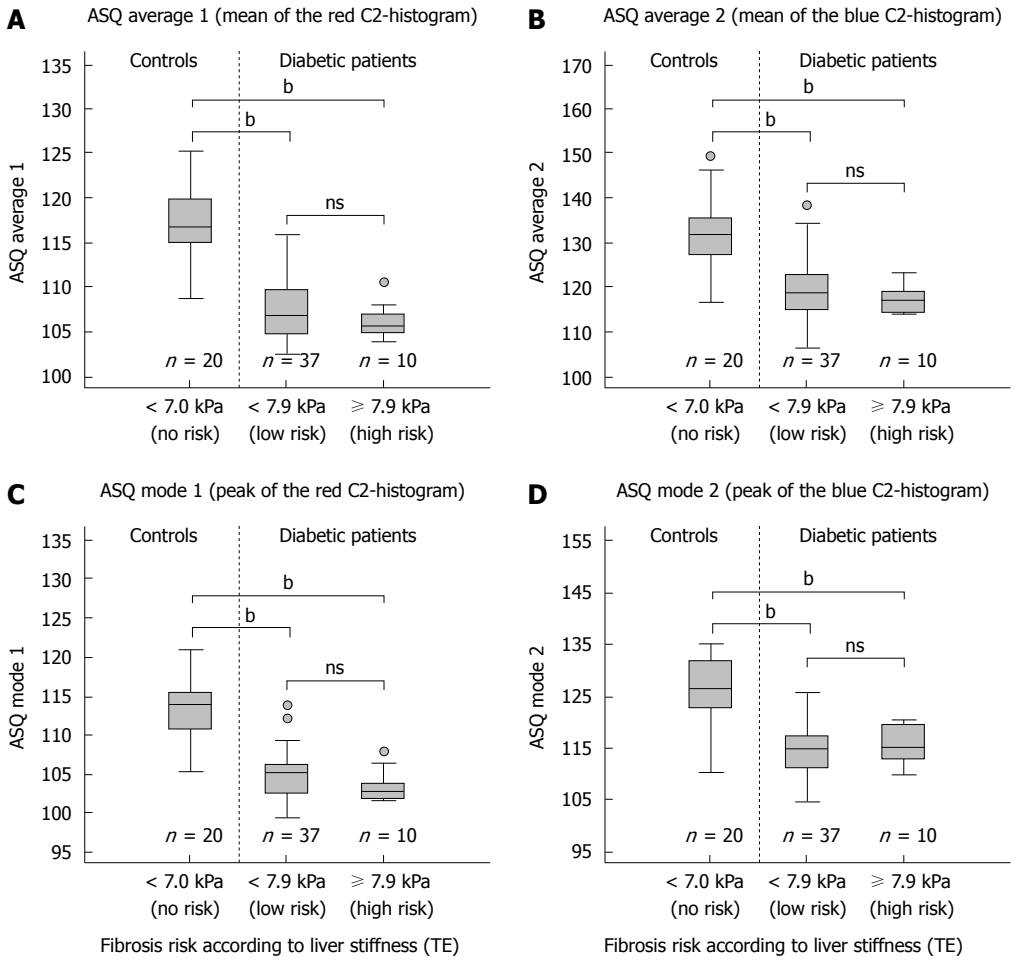

Figure 2 Correlation of acoustic structure quantification parameters with liver stiffness. Acoustic structure quantification (ASQ) parameters mode and the average of the "red" (A, C) and "blue" C2-histogram (B, D) show no significant differences between diabetic patients with and without increased liver stiffness (transient elastography, TE). The differences of all parameters between both patient groups and healthy controls ($^{b}P < 0.01$ *vs* control, Mann-Whitney *U* test) suggest an influence of diabetes-related anthropometric and metabolic factors, *e.g.*, steatosis, on ASQ analysis.

Non-invasive fibrosis estimation with ASQ vs TE and NAFLD score

LSM values (logarithmic) showed good correlation with NAFLD fibrosis scores: $r = 0.46$ (0.20; 0.66), $P = 0.0012$. None of the healthy controls had elevated TE, whereas advanced hepatic fibrosis was considerable in the diabetic cohort: ten patients (21%) had LSM > 7.9 kPa and 12 patients (26%) had NAFLD scores > 0.676.

The association of ASQ parameters mode, average, and FD ratio was analyzed according to the risk of hepatic fibrosis. All parameters showed differences between healthy controls and diabetic patients (Figures 1 and 2). No significant association of mode and average for both (blue and red) C^2-histogram curves was observed in diabetic patients, independent of the presence of fibrosis as defined by TE (Figure 2) or NAFLD score (*P*-values >0.08, respectively). In addition, FD ratios did not differ between both groups of diabetic patients: 0.060 (0.044; 0.076) *vs* 0.053 (0.037; 0.089) (TE cut-off, $P = 0.640$) and 0.059 (0.030; 0.125) *vs* 0.057 (0.047; 0.071) (NAFLD fibrosis score cut-off, $P = 0.946$), respectively.

Non-invasive steatosis characterization with ASQ vs CAP and ^{1}H-MRS

Diabetic patients were classified according to the degree of fatty liver disease, defined by CAP values: only $n = 6$ cases were at low risk for fatty liver (CAP < 250 dB m), whereas $n = 33$ were above the highly specific cut-off level (CAP > 300 dB/m) for advanced hepatic steatosis[8]. Gender distribution did not differ between healthy controls and the four diabetes subgroups, whereas BMI was increased in patients with advanced hepatic steatosis (Table 2). Furthermore, ^{1}H-MRS revealed a positive correlation between CAP and the hepatic fat fraction [$n = 43$; $r = 0.5$ (0.31; 0.73), $P < 0.001$] (cf. also Table 2).

ASQ parameters mode and average of both C^2-histogram curves and FD ratio differed between healthy controls and diabetic patients (*P*-values < 0.001). They also showed a stepwise decrease according to the CAP-defined classification of steatosis in diabetic patients (Table 2, Figure 3). ROC analysis for the detection of advanced steatosis (defined by CAP > 300 dB/m) in diabetic patients by the FD ratio

Table 2 Distribution of anthropometry, hepatic lipid content, fibrosis risk and acoustic structure quantification parameters in different grades of fatty liver *n* (%)

Parameter	Controls	Type 2 diabetes mellitus			
		CAP < 250 dB/m	CAP 250-< 300 dB/m	CAP 300-< 350 dB/m	CAP ≥ 350 dB/m
Sex (F/M)	10/10	2/4	6/4	8/11	7/5
Body mass index (kg/m²)[b]	22.0 ± 2.7	26.7 ± 4.6	28.3 ± 5.1	29.2 ± 4.3	32.1 ± 3
Waist-to-hip ratiob	0.85 ± 0.08	1.00 ± 0.03	0.91 ± 0.13	0.98 ± 0.06	1.01 ± 0.05
Liver stiffness (kpa)[a]	4.9 (2.9-6.8)	3.5 (3-6.1)	5.1 (3.5-11.7)	5.7 (3.4-12.3)	6.9 (4.1-70.6)
⩾ 7.9 kpa[c]	0 (0)	0 (0)	1 (10)	5 (26)	4 (33)
NAFLD fibrosis score	-	-0.68 (-2.45-1.25)	0.29 (-0.3-1.26)	-0.4 (-3.04-1.1)	0.11 (-1.68-2.28)
⩾ 0.676		1 (17)	3 (30)	2 (11)	4 (33)
^{1}H-MR spectroscopy (relative lipid signal, %)	-	4.19 (2.69-16.62)	9.77 (1.56-15.71)	18.99 (5.66-35.55)	18.38 (9.42-41.11)
Asq					
Mode 1 (red histogram)[b]	114 (105-121)	107 (102-109)	106 (102-114)	105 (100-112)	102 (99-108)
Average 1 (red histogram)[a]	117 (109-125)	110 (104-116)	108 (106-114)	106 (103-112)	105 (102-112)
Mode 2 (blue histogram)[b]	126 (110-135)	117 (114-126)	119 (105-125)	114 (107-119)	114 (105-121)
Average 2 (blue histogram)[b]	132 (116-149)	122 (116-139)	123 (114-130)	118 (109-134)	115 (106-123)
Focal disturbance (FD) ratio[b]	0.34 (0.12-0.71)	0.16 (0.03-0.36)	0.07 (0.03-0.18)	0.06 (0.02-0.09)	0.04 (0.02-0.13)

[a]P < 0.05 (Kruskal-Wallis test), [b]P < 0.01 (Kruskal-Wallis test) *vs* healthy controls; [c]P < 0.05 (exact test for count data), *vs* healthy controls. ASQ: Acoustic structure quantification; CAP: Controlled attenuation parameter.

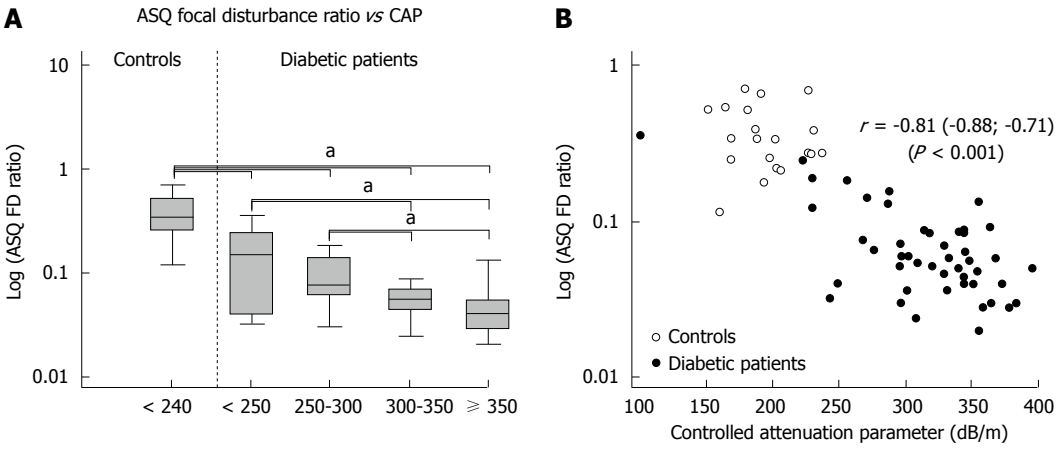

Figure 3 Correlation of acoustic structure quantification focal disturbance ratio and controlled attenuation parameter values in healthy controls and diabetic patients. Acoustic structure quantification (ASQ) focal disturbance (FD) ratio decreased in patients with advanced steatosis compared with healthy controls and patients with mild steatosis ([a]P < 0.05, *vs* control, Kruskal-Wallis test) (A). Logarithmic ASQ values correlated strongly with controlled attenuation parameter (CAP) values (B).

revealed an area under the curve (accuracy) of 0.76 (0.61; 0.87) (sensitivity 97%, specificity 50%) at a cut-off value of 0.092. With the addition of control subjects, the accuracy increased to 0.89 (sensitivity 97%, specificity 78%) with the same cut-off.

As with the CAP results (Figure 3B), ASQ parameters were negatively correlated with ^{1}H-MRS in the diabetic cohort (Table 2), *e.g.*, r = -0.43 (-0.65; -0.15), P = 0.004 for the FD ratio. Agreement between both methods increased when only cases with a hepatic fat fraction < 25% were considered (n = 36): r = -0.64 (-0.80; -0.40), P < 0.001.

DISCUSSION

The present study provided a comprehensive comparison of the ASQ technique with established non-invasive methods for the grading and staging of fatty liver disease. Our data underline the value of ASQ for the quantification of liver fat: the FD ratio showed a strong correlation with the CAP method, which in turn achieved high diagnostic accuracy for the grading of liver steatosis compared with liver histology[1,4-9]. Furthermore, the FD ratio also correlated with the hepatic lipid fraction, as quantified by ^{1}H-MRS, especially in cases with low to moderate hepatic fat content. These results confirm data from a mouse model, which demonstrated the potential value of the refined ASQ algorithm for non-invasive liver fat quantification[16]. Our findings are also in line with preliminary observations on the regression of FD ratios in NAFLD patients treated for morbid obesity[24]. Therefore, ASQ may become a novel tool for the estimation of hepatic steatosis that is equally as

accurate as other non-invasive methods[4].

ASQ was originally developed as an alternative to liver biopsy for fibrosis staging. Three biopsy-controlled, cross-sectional studies have shown a correlation of ASQ parameters (mode and average) with the extent of liver fibrosis[13-15]. However, these ASQ parameters correlated with CAP results and thus with the degree of liver steatosis in our cohort (Table 2). All ASQ parameters also differed between healthy controls and patients at risk of fatty liver disease, independent of the extent of fibrosis. By contrast, none of these parameters were associated with either the NAFLD fibrosis score or liver tissue stiffness, although both approaches represent well-established surrogates of liver fibrosis[3,4]. We therefore assumed that anthropometric factors and advanced steatosis, which were associated with a higher frequency of increased liver stiffness in our cohort, interfered with fibrosis in the B-mode speckle pattern and thus limited the prognostic value of ASQ for fibrosis in such cases. The three previous studies where ASQ correlated with fibrosis were performed either in lean cohorts without advanced steatosis and cirrhosis[13], in patients with homogeneous steatosis for all fibrosis stages[14], or did not analyze the impact of steatosis on ASQ results in detail[15]. Furthermore, the study of Ricci *et al*[15] reported low diagnostic accuracy of ASQ for fibrosis detection (AUROC 0.71 for any grade) in patients with viral hepatitis, which may be associated with steatosis especially for cases with progressive fibrosis[25]. Thus, our results may demonstrate a limited value of ASQ for characterizing fibrosis in the presence of steatosis. This finding is supported by recent data from a histology-controlled cohort where ASQ proved imprecise in assessing liver fibrosis[26].

Our study had some limitations: quantitative analysis of ASQ parameters is still an experimental approach and the method has not yet been standardized (*e.g.*, position and size of B-Mode ROIs, number of measurements, technical ultrasound parameters). Most previous ASQ studies did not adequately describe their data acquisition[13,15], which prevented a proper comparison with our results. Another source of discrepancy in fibrosis detection is a vendor's update to the ASQ data processing[16] that came with the introduction of the FD ratio[13-15]. Furthermore, we only correlated ASQ results with other non-invasive detection techniques because liver histology was ethically prohibited in this pilot study and only provides a semi-quantitative estimate of steatosis[8]. However, an impact of the non-invasive reference methods on our study findings cannot be ruled out. As has been discussed previously[8], anthropometric factors, as well as the extent of liver fat, can alter liver stiffness, CAP and MR spectroscopic results[27-31]. In particular, MR techniques are susceptible to fibrosis-related iron deposits and may not properly discriminate between advanced grades of hepatic steatosis[27,28]. This could explain the moderate correlation between ^{1}H-MRS and ASQ in our cohort, where advanced steatosis and fibrosis was highly prevalent. Accordingly, the findings of our pilot study should be further verified in histology-controlled studies.

In conclusion, our results provided the first evidence that ASQ FD ratio could be used for non-invasive evaluation of hepatic steatosis in patients at risk of fatty liver disease, and merits further investigation. In its current implementation, the ASQ algorithm seems to be impractical to characterize fibrosis in patients with fatty liver disease. There is also a need for biopsy-controlled studies to further validate ASQ parameters and to evaluate critical factors, such as data acquisition or patient anthropometry.

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COMMENTS

Background

Non-invasive characterization of hepatic fat content and fibrosis is becoming important for the care of patients with chronic liver diseases. Among different approaches, liver stiffness measurement combined with ultrasound attenuation analysis represent the current non-invasive references standards to estimate fibrosis and steatosis.

Research frontiers

Computerized analysis of acoustic tissue properties may represent an alternative for grading and staging of liver damage. To this end, acoustic structure quantification (ASQ) software analyzes the intensity pattern of B-Mode ultrasound. Several ASQ parameters correlate with the extent of fibrosis in histology-controlled studies. However, ASQ has not yet been compared with established non-invasive techniques, and the impact of hepatic steatosis on ASQ has not yet been studied.

Innovations and breakthroughs

These results provide the first evidence that ASQ parameters correlate with the extent of hepatic steatosis. However, ASQ may be impractical to characterize fibrosis in patients with fatty liver disease.

Applications

If further proved in biopsy-controlled studies, ASQ may complement ultrasound assessment of patients with chronic liver diseases, especially for the non-invasive estimation of hepatic fat content.

Terminology

Non-alcoholic fatty liver disease (NAFLD): obesity, nutrition and further factors can lead to fat deposition in hepatocytes, which is called NAFLD. This process may lead to hepatic inflammation and ultimately to fibrosis and cirrhosis. Elastography: measurement of tissue stiffness by means of mechanical and ultrasound impulses. Liver stiffness correlates with the extent of fibrosis.

Peer-review

The authors compared ultrasound-based ASQ with established non-invasive techniques for grading and staging fatty liver disease. The topic is relevant because NAFLD is perhaps the commonest liver disease. The authors claim that this study provides the first evidence that the ASQ FD ratio could be used

for non-invasive evaluation of hepatic steatosis in patients at risk for fatty liver disease. Overall, this is a well written manuscript.

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ORIGINAL ARTICLE

Case Control Study

Clinical impact of endoscopy position detecting unit (UPD-3) for a non-sedated colonoscopy

Masakatsu Fukuzawa, Junichi Uematsu, Shin Kono, Sho Suzuki, Takemasa Sato, Naoko Yagi, Yuichiro Tsuji, Kenji Yagi, Chika Kusano, Takuji Gotoda, Takashi Kawai, Fuminori Moriyasu

Masakatsu Fukuzawa, Junichi Uematsu, Shin Kono, Sho Suzuki, Takemasa Sato, Naoko Yagi, Yuichiro Tsuji, Kenji Yagi, Chika Kusano, Takuji Gotoda, Fuminori Moriyasu, Department of Gastroenterology and Hepatology, Tokyo Medical University, Tokyo 160-0023, Japan

Masakatsu Fukuzawa, Takashi Kawai, Endoscopy Center, Tokyo Medical University Hospital, Tokyo 160-0023, Japan

Author contributions: Fukuzawa M planned this work and wrote the manuscript; Fukuzawa M, Uematsu J, Kono S, Suzuki S, Sato T, Yagi N, Tsuji Y and Yagi K performed the endoscopic procedure and supported the research; Kusano C and Fukuzawa M performed the statistical analysis; Gotoda T, Kawai T and Moriyasu F drafted and revised the manuscript.

Ethics approval: The study was not reviewed and approved by the Institutional Review Board.

Informed consent: All study participants, or their legal guardian, provided informed written consent prior to examination.

Conflict-of-interest: The authors have no conflict of interest directly relevant to the contents of this article.

Data sharing: Participants gave informed consent for data sharing. No additional data are available.

Correspondence to: Masakatsu Fukuzawa, MD, PhD, Department of Gastroenterology and Hepatology, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjukuku, Tokyo 160-0023, Japan. masakatu8055@yahoo.co.jp

Telephone: +81-3-33426111

Fax: +81-3-53816654

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Abstract

AIM: To evaluate whether an endoscopy position detecting unit (UPD-3) can improve cecal intubation rates, cecal intubation times and visual analog scale (VAS) pain scores, regardless of the colonoscopist's level of experience.

METHODS: A total of 260 patients (170 men and 90 women) who underwent a colonoscopy were divided into the UPD-3-guided group or the conventional group (no UPD-3 guidance). Colonoscopies were performed by experts (experience of more than 1000 colonoscopies) or trainees (experience of less than 100 colonoscopies). Cecal intubation rates, cecal intubation times, insertion methods (straight insertion: shortening the colonic fold through the bending technique; roping insertion: right turn shortening technique) and patient discomfort were assessed. Patient discomfort during the endoscope insertion was scored by the VAS that was divided into 6 degrees of pain.

RESULTS: The cecum intubation rates, cecal intubation times, number of cecal intubations that were performed in < 15 min and insertion methods were not significantly different between the conventional group and the UPD-3-guided group. The number of patients who experienced pain during the insertion was markedly less in the UPD-3-guided group than in the conventional group. Univariate and multivariate analysis showed that the following factors were associated with lower VAS pain scores during endoscope insertion: insertion method (straight insertion) and UPD-3 guidance in the trainee group. For the experts group, univariate analysis showed that only the insertion method (straight insertion) was associated with lower VAS pain scores.

CONCLUSION: Although UPD-3 guidance did not shorten intubation times, it resulted in less patient pain

during endoscope insertion compared with conventional endoscopy for the procedures performed by trainees.

Key words: Colonoscopy; Training; Endoscopy position detecting unit

Core tip: Non-sedated colonoscopy may be an uncomfortable or painful examination. It is very important for the colonoscopist to understand the shape of the endoscope during its insertion to successfully accomplish cecal intubation with minimal pain. ScopeGuide endoscopy position detecting unit (UPD-3) is designed to provide real-time three-dimensional images of the shape and configuration of the colonoscope inside the body. This study was conducted to evaluate the clinical impact of UPD-3, regardless of the level of experience of the colonoscopist. According to this study, ScopeGuide UPD-3 is useful for reducing patient abdominal discomfort during colonoscopies performed by trainees.

Fukuzawa M, Uematsu J, Kono S, Suzuki S, Sato T, Yagi N, Tsuji Y, Yagi K, Kusano C, Gotoda T, Kawai T, Moriyasu F. Clinical impact of endoscopy position detecting unit (UPD-3) for a non-sedated colonoscopy. *World J Gastroenterol* 2015; 21(16): 4903-4910 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4903.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4903

INTRODUCTION

Colorectal cancer is one of the major malignant cancers in Western countries and its incidence is rapidly increasing in other countries, including Japan. Colonoscopy has been considered the standard screening method for colorectal neoplasms[1,2]. However, expertise is required to correctly perform colonoscopy. Accumulation of a considerable amount of experience is necessary to be able to perform cecal intubation in a short time without causing pain to the non-sedated patient[3-7]. Previous studies have reported that trainees should perform more than 150 colonoscopy examinations to become technically competent in diagnostic colonoscopy[8]. Complete examination by colonoscopy may be difficult technically because of the shape of the endoscope, which may form a loop during insertion into the sigmoid or transverse colon. Furthermore, this looping and resultant stretching of the colon is a major source of pain for patients during colonoscopy and contributes to unsuccessful cecal intubation. Recent studies have reported low success rates of cecal intubations performed by inexperienced colonoscopists[4,9] and have pointed out the risk of complications due to the increasing number of colonoscopies being performed[4,10]. Several factors, such as female sex and old age, have been associated with the difficulty of cecal intubation[9,11-20], which may particularly apply to inexperienced colonoscopists. However, the resources for total colonoscopy, *i.e.*, the number of expert colonoscopists, are limited.

Patient comfort during and after colonoscopy is an important factor in determining patients' compliance with the procedures for the screening and surveillance of colorectal cancer. A number of techniques and endoscopes have been reported to reduce patient abdominal discomfort during difficult colonoscopies, such as the use of a pediatric colonoscope[21], double balloon endoscope[22] and attachment of a transparent hood to the tip of the endoscope[23,24]. Sedation during colonoscopy is commonly performed in many clinics and hospitals to reduce the discomfort of insertion but there is growing recognition of the benefits of non-sedated colonoscopy. The patient who receives a non-sedated colonoscopy does not have to be in the hospital after examination for a long time and it costs less than sedated colonoscopy.

There have been few reports[25-27] on the effect of using an Endoscope Position Detecting Unit (UPD) during colonoscopy on the level of pain in non-sedated patients. The aim of this study was to evaluate whether UPD-3 can improve cecal intubation rates, cecal intubation times and Visual Analog Scale (VAS) pain scores, regardless of the level of experience of the colonoscopist.

MATERIALS AND METHODS

Patients

All patients were informed of the risks and benefits of colonoscopy and all patients provided written informed consent to undergo a colonoscopy. Between February 2012 and June 2012, a total of 260 patients (170 men and 90 women) who underwent a colonoscopy were divided into the UPD-3-guided group or the conventional group (no UPD-3 guidance). All colonoscopies were performed in four rooms. Conventional colonoscopies were performed in three rooms and UPD-3 guided colonoscopies were performed in one room in parallel. Finally, the number of patients was paired between the 2 groups and retrospective analyses were performed (Figure 1). Procedures were performed by 7 colonoscopists who were divided into 2 groups according to their colonoscopy experience: the expert colonoscopists (EC) group, which included colonoscopists with more than 10 years of experience involving more than 1000 procedures (MF, KY and MN), and the trainee colonoscopists (TC) group, with less than 3 years of colonoscopy practice involving less than 100 procedures (SA, YK, MA and MH). If an examining colonoscopist from the TC group failed to pass the endoscope through the sigmoid-descending colon junction within 15 min and a patient complained

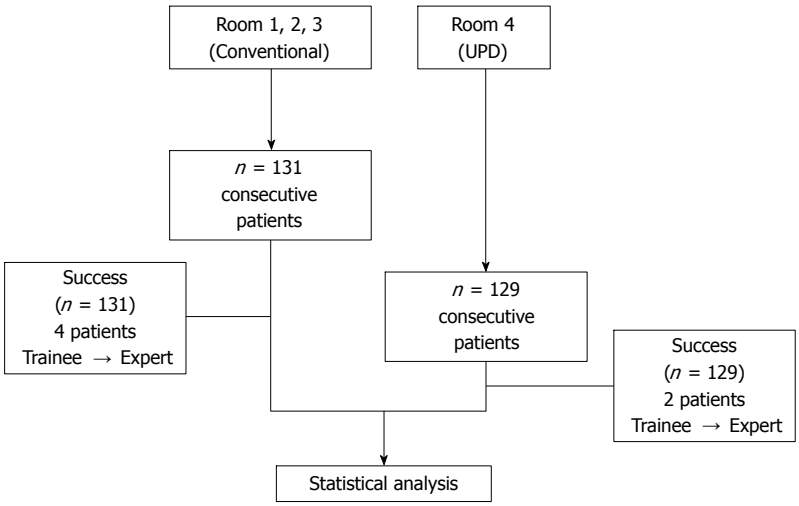

Figure 1 Inclusion schema of study. UPD: Position detecting unit.

of severe pain, a colonoscopist from the EC group replaced the initial examiner before midazolam was administered and the insertion was continued to the cecum.

We examined the association between patient discomfort during endoscope insertion and sex (male/female), age (< 65 years old/> 64 years old), UPD-3 use, experience of the colonoscopist (expert/trainee), insertion method (straight insertion: shortening the colonic fold by the bending technique; roping insertion: right turn shortening technique), past experience of abdominal surgery and use of antispasmodics. Patients recorded their pain level during insertion of the endoscope until it reached the cecum base. Another member of the medical staff, who did not know how the procedures were performed, interviewed the patients immediately after completion of their colonoscopies. Patients' pain and discomfort during endoscope insertion was scored by the VAS that was divided into 6 degrees (from 0 to 5). The VAS scores 0 and 1 were defined as painless and 2 to 5 were defined as painful. We then compared the various clinical characteristics and results of colonoscopy between the conventional and UPD-3-guided group and further investigated the factors affecting VAS pain scores during endoscope insertion by all colonoscopists, as well in the TC group only and in the EC group only. The study protocol was prepared in accordance with the Helsinki Declaration of 1975, as revised in 2008. Written informed consent to participate in the study was given by all subjects.

Colonoscopy

The indications for colonoscopy examination were the standard clinical criteria as follows: colorectal cancer screening, surveillance for polyps, a positive fecal occult blood test, abdominal symptoms or anemia. Exclusion factors included severe heart or lung disease, prior colorectal resection, inflammatory bowel disease, severe hematochezia, age younger than 18 years and prior experience of repeated colonoscopies for therapeutic procedures, including polypectomy.

Patients underwent bowel preparation, taking sennoside on the day before their examination and 2 liters of polyethylene glycol solution in the morning of their colonoscopy. Scopolamine butylbromide (20 mg) was administered intramuscularly to suppress bowel movement, whereas patients with cardiac disease or benign prostatic hypertrophy or glaucoma received glucagon (1 IU) intramuscularly. Sedatives were administered to patients based on the examining colonoscopist's judgment or when requested by the patient due to abdominal pain or distension, and these cases were excluded from the study. Examinations were performed using a CF-Q260DI colonoscope (Olympus Co., Tokyo, Japan) with a distal tip diameter of 12.4 mm in the UPD-3 guided group. Examinations were performed using either a CF-Q260AI colonoscope (Olympus Co.) with a distal tip diameter of 12.2 mm or a CF-H260AI colonoscope (Olympus Co.) with a distal tip diameter of 13.2 mm in the conventional group. Carbon dioxide insufflation was available for all procedures.

Endoscopy position detecting unit

It is very important for the colonoscopist to understand the shape of the endoscope during its insertion to successfully accomplish cecal intubation with minimal pain. Traditionally, an X-ray image of the area of interest in the patient was obtained with the endoscope insertion unit inserted to check the insertion state, such as the insertion position and the orientation of the insertion unit in the body cavity. However, such X-ray imaging is not completely harmless to the body and is restricted to the area of irradiation and is thus not always suitable as a detection method for the insertion state of the endoscope insertion unit.

A magnetic imaging system of colonoscope (Unit

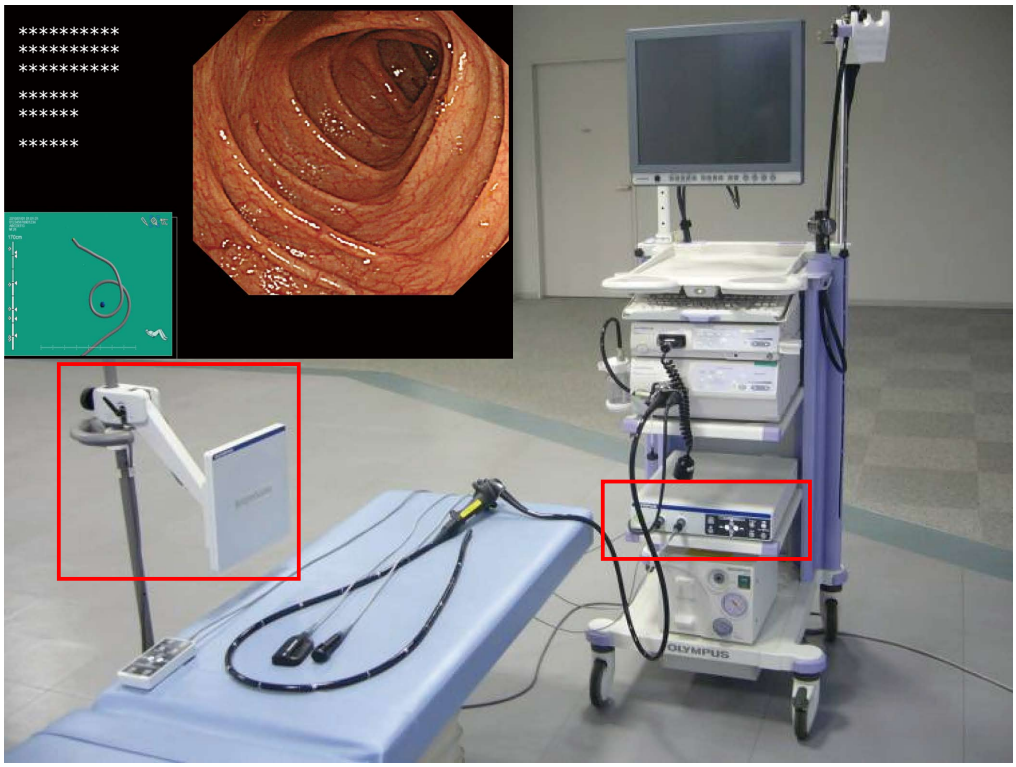

Figure 2 Position detecting unit system. The new Scope Guide receiver dish is compact and thin. Faster frame rate for enhanced image quality with picture in picture.

of Position Detection: UPD, Olympus Optical Co., Ltd.) provides a new facility for viewing real-time three-dimensional (3D) images of the shape and configuration of the colonoscope inside the body, without exposing patients or medical staff to radiation. Electromagnetic coils incorporated along the length of the colonoscope's insertion tube generate a pulsed low-intensity magnetic field that is picked up by the receiver dish. The magnetic pulses are used to calculate the precise position and orientation of the colonoscope. A new, improved UPD-3 model generates 3D images faster than ever. When used in conjunction with a monitor with picture-in-picture functionality, the ScopeGuide image is viewed alongside the endoscopic image (Figure 2).

Statistical analysis

Continuous variables were expressed as the mean ± SD. Categorical variables were expressed as frequencies and percentages. Differences between the 2 groups of patients (conventional group *vs* UPD-3-guided group) were detected using an independent *t*-test or Mann-Whitney *U* test for continuous data, and the χ^2 test or the Fisher's exact test for categorical data, as appropriate. Univariate and multivariate linear regression models were used to identify factors affecting VAS pain scores during endoscope insertion. Multivariate linear regression with stepwise selection was applied; variables that did not improve the model fit at $P < 0.05$ were discarded. A P-value < 0.05 was considered to indicate a statistically significant difference between groups. All statistical evaluations were performed using SPSS version 15.0 J software (SPSS Japan Inc., Tokyo, Japan).

RESULTS

A total of 260 patients (170 men and 90 women) underwent colonoscopy during the study period. The mean age of the patients was 62.5 years (range 23-89; > 65: 133 patients; < 65: 127 patients). The patients were assigned to either the conventional group (n = 131) or the UPD-3-guided group (n = 129). One-hundred and sixty-six patients were examined by expert colonoscopists and 94 patients were examined by trainee colonoscopists. There were 20 post-abdominal operation cases (7.7%) and 239 cases using antispasmodics (91.9%). The baseline characteristics of the patients are summarized in Table 1.

The cecum-intubated proportions were the same in patients in the conventional group and those in the UPD-3-guided group (both 100%). In 6 cases (4 patients from the conventional group and 2 patients from the UPD-3-guided group), the trainee colonoscopist initially performing the procedure was replaced by an experienced colonoscopist due to technical difficulties resulting in intolerable pain for the patient.

The average cecal intubation time was 13.2 ± 4.1 min in the conventional group and 12.5 ± 2.3 min in the UPD-3-guided group. The time taken for the

Table 1 Baseline characteristics of the patients *n* (%)

	Overall	Conventional	UPD	*P* value
Patients	260	131	129	-
Female	90 (34.6)	48 (36.6)	42 (32.6)	0.726
Age (yr, mean ± SD)	62.5 ± 4.8	62.5 ± 5.3	62.9 ± 6.5	0.455
Abdominal surgery	20 (7.7)	9 (6.9)	11 (8.5)	0.334
Examination by an expert colonoscopist	166 (63.8)	83 (63.4)	83 (64.3)	0.768
Antispasmodics use	239 (91.9)	120 (91.6)	119 (92.2)	0.849

UPD: Position detecting unit.

Table 2 Results of colonoscopy *n* (%)

	Overall	Conventional	UPD	*P* value
Cecal intubation rate	100%	100%	100%	-
Cecal intubation time (min, mean ± SD)	12.9 ± 4.3	13.2 ± 4.1	12.5 ± 2.3	0.455
Cecal intubation time (< 15 mn)	182 (70.0)	92 (70.2)	90 (69.8)	0.935
Straight insertion methods	120 (46.1)	58 (44.3)	62 (48.1)	0.540
Change of the colonoscopist	6 (2.3)	4 (3.1)	2 (1.6)	0,420
Absence of pain	143 (55.0)	64 (48.9)	79 (61.2)	0.045

UPD: Position detecting unit.

endoscope to reach the cecum was not significantly different between patients in the conventional group and those in the UPD-3-guided group. The number of cecal intubations that were performed in < 15 min, the insertion methods used and the VAS pain scores were comparable between the patients examined by a trainee colonoscopist and those examined by an expert colonoscopist. Moreover, there were no significant differences in these parameters between the conventional group and the UPD-3-guided group. The number of patients who experienced pain during the insertion was markedly less in the UPD-3-guided group than in the conventional endoscopy group (Table 2).

To investigate the factors affecting VAS pain scores during insertion of the colonoscope, we performed univariate and multivariate linear regression analyses (Table 3). Univariate analysis showed that the following factors were related to lower VAS pain scores during colonoscope insertion: straight insertion methods, UPD-3 guidance, examination by an expert colonoscopist and absence of abdominal surgery. After controlling for other covariates in the multivariate model, the same 4 factors were found to significantly affect VAS pain scores during colonoscope insertion. However, univariate analysis showed that only for the TC group, straight insertion methods and UPD-3 guidance were related to lower VAS pain scores during colonoscope insertion. After controlling for other covariates in the multivariate model, the same 2 factors were found to significantly affect VAS pain scores during colonoscope insertion (Table 4). For the EC group, univariate and multivariate analysis showed that only the insertion method (straight insertion methods) was related to lower VAS pain scores during colonoscope insertion (Table 5).

DISCUSSION

In the present study, there were no differences between the conventional endoscopy and UPD-3-guided groups in cecal intubation rates, mean cecal intubation time, number of cecal intubation procedures that were performed in < 15 min and insertion methods. The number of patients who experienced pain during the insertion was significantly lower in the UPD-3-guided group than in the conventional endoscopy group.

Based on the results of univariate and multivariate analyses for factors influencing VAS pain scores during colonoscope insertion, the straight insertion technique was found to be associated with less pain when experts performed the procedure. When trainees performed the procedure, however, UPD-3 guidance in combination with the straight insertion technique was found to be associated with less pain.

Because colonoscopy is generally regarded as an examination often involving pain, various approaches to reduce the pain have been reported. In Western countries, colonoscopy is frequently performed under sedation, based on a large number of reports supporting its safety and effectiveness[28,29]. In contrast, in Japan, colonoscopy is most commonly performed without sedation owing to its safety and effectiveness and it is frequently difficult to ensure sufficient numbers of recovery beds for patients undergoing endoscopy under sedation. Furthermore, in recent years, the use of an endoscope with a hood attached to its tip for colonoscopy without sedation has been reported to shorten the time required to intubate the cecum, thus reducing the amount of pain compared with conventional endoscopy (without a hood)[23,24]. On the other hand, some reports pointed out that colonoscopy with carbon dioxide insufflation is indispensable for treatment, such as for colorectal endoscopic submucosal dissection[30], as it reduces the feeling of fullness after surgery. However, this does not alleviate the pain experienced during insertion[31]. For pain reduction, selecting an appropriate endoscope insertion technique is an important factor. At present, a method in which the endoscope is made to form a loop at the sigmoid colon and is withdrawn before intubating the cecum is considered a common technique on a worldwide basis. However, in the case of loop formation, the length of the endoscope may be insufficient, leading to difficulties in inserting it into the cecum and completing the examination procedure. One of the most important points regarding examination techniques such as colonoscopy is the education of trainees. Longer procedure times and

Table 3 Univariate and multivariate analysis of the factors affecting visual analog scale pain scores for colonoscope insertion

Factors		Pain	Univariate analysis	Multivariate analysis		
			P value	*P* value	OR	95%CI
Insertion methods (straight *vs* roping)	No	88/55	< 0.001	< 0.001	4.1	2.3-7.3
	Yes	32/85				
UPD *vs* Conventional	No	79/64	0.045	0.041	1.8	1.1-3.2
	Yes	50/67				
Expert *vs* Trainee	No	111/32	< 0.001	< 0.001	4.2	2.3-7.6
	Yes	55/62				
Abdominal surgery (+ *vs* -)	No	5/138	0.005	0.006	4.7	1.5-15.0
	Yes	15/102				
Gender (Male *vs* Female)	No	98/45	0.238	0.231	1.4	0.8-2.6
	Yes	72/45				
Antispasmodic (+ *vs* -)	No	132/11	0.801			
	Yes	107/10				
Age (≥ 65 *vs* < 64)	No	72/71	0.774			
	Yes	61/56				

UPD: Position detecting unit.

Table 4 Univariate and multivariate analysis of factors affecting visual analog scale pain scores for colonoscope insertion by trainees

Factors		Pain	Univariate analysis	Multivariate analysis		
			P value	*P* value	OR	95%CI
Insertion methods (straight *vs* roping)	No	22/10	< 0.001	< 0.001	6.8	2.6-18.2
	Yes	14/48				
UPD *vs* conventional	No	21/11	0.020	0.043	2.7	1.1-7.2
	Yes	25/37				
Abdominal surgery (+ *vs* -)	No	1/31	0.252	0.429	2.6	0.2-28.8
	Yes	6/56				
Antispasmodic (+ *vs* -)	No	30/2	0.773			
	Yes	59/3				
Age (≥ 65 *vs* < 64)	No	16/16	0.767			
	Yes	33/29				
Gender (Male *vs* Female)	No	21/11	0.961			
	Yes	41/21				

UPD: Position detecting unit.

Table 5 Univariate and multivariate analysis of factors affecting visual analog scale pain scores for colonoscope insertion by experts

Factors		Pain	Univariate analysis	Multivariate analysis		
			P value	*P* value	OR	95%CI
Insertion methods (straight *vs* roping)	No	66/45	0.001	0.002	3.0	1.5-5.9
	Yes	18/37				
Gender (Male *vs* Female)	No	77/34	0.098	0.486	1.3	0.6-2.8
	Yes	31/24				
UPD *vs* Conventional	No	58/53	0.410			
	Yes	25/30				
Abdominal surgery (+ *vs* -)	No	4/107	0.422			
	Yes	9/46				
Antispasmodic (+ *vs* -)	No	102/9	0.343			
	Yes	48/7				
Age (≥ 65 *vs* < 64)	No	84/27	0.958			
	Yes	28/27				

UPD: Position detecting unit.

increased abdominal pain and discomfort are caused by both the patient's condition and the colonoscopist's skills and experience[8-32]. However, training methods for colonoscope insertion using UPD-3 have not yet been established.

Unlike conventional colonoscopy under fluoroscopy, the UPD-3 apparatus used in the present study does not involve the risk of radiation exposure and allows

colonoscopists to view the 3D shape of the endoscope similarly to X-ray imaging. It is also very compact and provides clear images without a time delay following actual endoscope handling. In the present study, the use of a UPD-3 by experts was not a pain-reducing factor, whereas its use in combination with the straight insertion technique by trainees resulted in reduced pain.

One limitation of this study is that because it is a case study rather than a randomized control study, the data obtained from this study may be biased in several respects and therefore it may be necessary to conduct prospective comparative studies in the future.

Considering the increasing incidence of colon cancer, reducing the pain involved in colonoscopy is crucial for the promotion of fecal occult blood tests, as well as for increasing the rate of patients undergoing colonoscopy. At the same time, training for those in charge of the examination procedures in educational institutions, such as university hospitals, is also an important issue. As an initial approach, it may be possible to reduce the pain involved in colonoscope insertion by providing trainees with the opportunity to master the straight insertion technique combined with a UPD-3.

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COMMENTS

Background

Achievement of a high success rate for cecal intubation with minimal patient discomfort depends on the shape of the inserted endoscope during the examination, which in turn is affected by the colonoscopist's level of experience.

Research frontiers

This retrospective study was conducted to evaluate the clinical impact of an endoscopy position detecting unit (UPD-3), regardless of the level of experience of the colonoscopist.

Innovations and breakthroughs

Although UPD-3 guidance did not shorten intubation times, it resulted in less patient pain during endoscope insertion compared with conventional endoscopy for the procedures performed by trainees.

Applications

As an initial approach, it may be possible to reduce the pain involved in colonoscope insertion by providing trainees with the opportunity to master the straight insertion technique combined with a UPD-3.

Peer-review

This article seems to have some novelties and the concept of study is interesting. The results are interesting and suggest that UPD-3 guidance did not shorten intubation times but it resulted in less patient pain during endoscope insertion compared with conventional endoscopy for the procedures performed by trainees.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Palliative chemotherapy for gastroesophageal cancer in old and very old patients: A retrospective cohort study at the National Center for Tumor Diseases, Heidelberg

Anne Katrin Berger, Stefanie Zschaebitz, Christine Komander, Dirk Jäger, Georg Martin Haag

Anne Katrin Berger, Stefanie Zschaebitz, Dirk Jäger, Georg-Martin Haag, National Center for Tumor Diseases, Heidelberg University Hospital, 69120 Heidelberg, Germany
Christine Komander, NCT Clinical Cancer Registry, German Cancer Research Center, 69120 Heidelberg, Germany
Author contributions: Berger AK participated in study concepts and design, data acquisition, data analysis and interpretation, manuscript preparation and editing; Haag GM participated in data analysis and interpretation, manuscript preparation, quality control of data and algorithms and statistical analysis; Komander C participated in data acquisition and manuscript preparation; Jäger D and Zschaebitz S participated in data analysis and interpretation and manuscript preparation and review; all authors read and approved the final manuscript.
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Biostatistics Statement: The statistical methods of this study were reviewed by Georg Martin Haag from the NCT Heidelberg.
Informed consent: According to local ethics policy for retrospective analysis of own anonymized clinical data, IC was not obtained.
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Correspondence to: Anne Katrin Berger, National Center for Tumor Diseases, Heidelberg University Hospital, Im Neuenheimer Feld 460, 69120 Heidelberg, Germany. anne.berger@med.uni-heidelberg.de
Telephone: +49-6221-567229
Fax: +49-6221-567225

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Abstract

AIM: To investigate the outcome of palliative chemotherapy in old patients with gastroesophageal cancer at the National Center for Tumor Diseases, Heidelberg.

METHODS: Using a prospectively generated database, we retrospectively analyzed 55 patients ⩾ 70 years under palliative chemotherapy for advanced gastroesophageal cancer at the outpatient clinic of the National Center for Tumor Diseases Heidelberg, Germany between January 2006 and December 2013. Further requirements for inclusion were (1) histologically proven diagnosis of gastroesophageal cancer; (2) advanced (metastatic or inoperable) disease; and (3) no history of radiation or radiochemotherapy. The clinical information included Eastern Cooperative Oncology Group performance status (ECOG PS), presence and site of metastases at diagnosis, date of previous surgery and perioperative chemotherapy, start and stop date of first-line treatment, toxicities and consecutive dosage reductions of first-line treatment, response to first-line therapy, date of progression, usage of second-line therapies and date and cause of death. Survival times [progression-free survival (PFS), overall survival (OS) and residual survival (RS)] were calculated. Toxicity and safety were examined. Prognostic factors including ECOG PS, age and previous

perioperative treatment were analyzed.

RESULTS: Median age of our cohort was 76 years. 86% of patients received a combination of two cytotoxic drugs. 76 percent of patients had an oxaliplatin-based first-line therapy with the oxaliplatin and 5-fluorouracil regimen being the predominantely chosen regimen (69%). Drug modifications due to toxicity were necessary in 56% of patients, and 11% of patients stopped treatment due to toxicities. Survival times of our cohort are in good accordance with the major phase Ⅲ trials that included mostly younger patients: PFS and OS were 5.8 and 9.5 mo, respectively. Survival differed significantly between patient groups with low (≤ 1) and high (≥ 2) ECOG PS (12.7 mo *vs* 3.8 mo, $P < 0.001$). Very old patients (≥ 75 years) did not show a worse outcome in terms of survival. Patients receiving second-line treatment (51%) had a significantly longer RS than patients with best supportive care (6.8 *vs* 1.4 mo, $P = 0.001$). Initial ECOG PS was a strong prognostic factor for PFS, OS and RS.

CONCLUSION: Old patients with non-curable gastro-esophageal cancer should be offered chemotherapy, and ECOG PS is a tool for balancing benefit and harm upfront. Second-line treatment is reasonable.

Key words: Gastroesophageal cancer; Old patients; Palliative chemotherapy; Toxicity; Eastern Cooperative Oncology Group performance status

Core tip: Data concerning efficacy and safety of palliative chemotherapy for gastroesophageal cancer in patients ≥ 70 years are scarce. Concerns about poor tolerability due to reduced functional status are common, and older patients are at risk for undertreatment. In our analysis of 55 patients ≥ 70 years, the survival times were in good accordance to the results of the landmark phase Ⅲ trials including younger patients. Except for increased polyneuropathy, toxicity rates were also comparable. Initial Eastern Cooperative Oncology Group performance status was a strong prognostic factor for PFS, OS and RS.

Berger AK, Zschaebitz S, Komander C, Jäger D, Haag GM. Palliative chemotherapy for gastroesophageal cancer in old and very old patients: A retrospective cohort study at the National Center for Tumor Diseases, Heidelberg. *World J Gastroenterol* 2015; 21(16): 4911-4918 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4911.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4911

INTRODUCTION

For older adults, cancer incidence rates are higher than for younger persons[1]. The current epidemiologic development in the western world will therefore result in a rising incidence of cancer, and an increasing proportion of old and very old patients among all cancer cases[2]. Despite this development for most malignancies, older cancer patients remain underrepresented in clinical studies[3], and age above 70 years often is an exclusion criterion. There is widespread critique concerning this issue, but to date, extrapolation of trial results to the older population remains questionable[4]. When treating elderly cancer patients, medical comorbidities, impaired organ function and reduced functional status are frequently found[5]. Ensuring an adequate antitumor treatment while avoiding toxicity is a pivotal question of geriatric oncology in daily routine.

Gastric cancer and cancer of the gastroesophageal junction belong to the most common cancer forms worldwide[6]. In the United States, there were diagnosed approximately 40000 new cases of esophageal and gastric cancer with estimated 26000 deaths in 2013[7]. While incidence rates for distal gastric cancer keep declining continuously over the past decades[8], the incidence for cancer of the esophagogastric junction remains increasing[9]. In the United States, the 5-year relative survival for all stages in patients aged 65 or more is 26%[8], and for metastasized disease it is less than 5% for all patients. To date, palliative chemotherapy is the accepted standard of care for patients with metastasized disease since benefits in terms of survival times and quality of life have been widely shown[10,11]. Still, there is no internationally accepted standard therapy for the first-line situation[12]. Combination therapies are considered to be more active than single-agent approaches[11]. For cytotoxic double combinations, infusional or oral fluoropyrimidines in combination with platin-derivates have been shown to be active[13-15]. The addition of docetaxel as a cytotoxic triplet combination showed improved efficacy in terms of response rate and overall survival but is associated with increased toxicity[16]. For HER2-positive patients, trastuzumab is known to significantly improve overall survival in combination with a fluoropyrimidine and cisplatin[17]. Although gastroesophageal carcinomas predominantely occur in elder patients[8], the knowledge and recommendations about an adequate first-line treatment for older persons are sparse. There is some evidence that for patients older than 65 years survival times are comparable to those of younger ones, but grade 3 or higher toxicities might be more frequent for currently used regimens[18]. Oxaliplatin-based combinations might have a better toxicity profile and seem to be associated with an improved efficacy in contrast to cisplatin based regimens[14], whereas the benefit of using three-drug cytostatic combinations in elderly patients is questionable[19].

Since most patients develop disease progression

under first-line therapy, options for second-line treatment are frequently needed. Recently, the value of a second-line therapy with either docetaxel or irinotecan was shown[20]. But again, specific data for old patients are rarely found.

In our study, we retrospectively analyzed palliative chemotherapy and outcomes for old and very old patients (≥ 70 years) with advanced gastroesophageal cancer at the outpatient clinic of the National Center for Tumor Diseases (NCT) at Heidelberg University Hospital. In particular, we investigated the characteristics, toxicities and outcomes of cytotoxic treatment strategies in the first-line situation and evaluated the frequency and efficacy of second-line chemotherapy.

MATERIALS AND METHODS

Using a prospectively maintained database (the NCT Clinical Cancer Registry), we retrospectively identified patients aged 70 years or over who started palliative first-line chemotherapy for advanced gastroesophageal cancer at the outpatient clinic of the NCT Heidelberg, Germany between January 2006 and December 2013. Further requirements for inclusion were (1) histologically proven diagnosis of gastroesophageal cancer; (2) advanced (metastatic or inoperable) disease; and (3) no history of radiation or radiochemotherapy. The observation period for each patient started with initiation of palliative first-line treatment (*i.e.,* first systemic chemotherapy after primary diagnosis of metastatic or inoperable disease or, in resected patients, after diagnosis of recurrence). The follow-up period for this analysis ended on July 1, 2014. Survival data were available for all patients.

Via an electronic medical record, the responsible oncologists and medical staff routinely documented the clinical data. The retrieved information included Eastern Cooperative Oncology Group performance status (ECOG PS)[21], presence and site of metastases at diagnosis, date of previous surgery and perioperative chemotherapy, start and stop date of first-line treatment, toxicities and consecutive dosage reductions of first-line treatment, response to first-line therapy, date of progression, usage of second-line therapies and date and cause of death. Toxic effects were registered according to the National Cancer Institute´s common terminology criteria for adverse events[22]. Tumor response was routinely evaluated according to the response evaluation criteria in solid tumors[23]. The attending oncologist decided about the cytotoxic regimen and the start and end of antitumor treatment.

The study was approved by the local Ethics Committee (Ethics Committee University of Heidelberg; S-335/2014, 28.07.2014). According to local ethics policy for retrospective analysis of own anonymized clinical data, IC was not obtained.

Statistical analysis

Man Whitney *U*-Test and Fisher's exact test were used for comparing independent samples of quantitative and binary data, respectively. Progression-free survival (PFS) was defined as time from start of palliative first-line treatment to documented tumor progression or death. Overall survival (OS) was defined as the time from start of palliative first-line treatment to death. Residual survival (RS) was defined as the time from the day of documented disease progression to death. Time-to event data were analyzed using standard methods, including Kaplan-Meier product-limit estimates. Multivariate analysis was performed using cox regression analysis. All analyses of prognostic factors were of an exploratory nature. Statistical analysis was performed using the SPSS statistical software, Version 21. The statistical methods of this study were reviewed by Georg Martin Haag, NCT Heidelberg.

RESULTS

Patients' demographics

We identified 55 patients meeting the inclusion criteria. Median duration of observation was 9.1 mo (range 0.9-45.8). The median age at diagnosis of advanced disease was 76 years (range 70-86), 32 patients (58.2%) were 75 years or older. Fifteen patients (27.3%) with secondary metastases or local recurrent tumor had undergone prior tumor resection. Twelve of these had received perioperative chemotherapies. Thirty-seven patients (67.3%) had an ECOG PS of 0 or 1. Four patients started treatment with an ECOG PS of 3. Complete patient characteristics are outlined in Table 1. At time of analysis, 50 deaths (90.9%) had occurred. Of those, 18 patients had died during first-line treatment before documented tumor progression. In 8 of those cases, tumor-associated factors (*e.g.,* tumor bleeding) or tumor progression were clinically assumed and documented as causes of death. One patient died from pleural empyema and one further patient died from cholangitis. In 8 cases, no further information on the exact mode of death was available.

First-line chemotherapy and toxicities

Median duration of first-line therapy was 105 d (range 6-568). Patients with an ECOG PS ≥ 2 had a median duration of therapy of 70 d compared to 146 d for ECOG PS ≤ 1 (P = 0.006). Forty-seven patients (85.5%) received a combination of two cytotoxic agents (doublet). Three patients (5.5%) were treated with a single-agent therapy (oral or infusional fluoropyrimidine), and five patients (9.1%) underwent therapy with three cytotoxic agents (triplet). Seven of our patients (12.7%) had a cisplatin containing regimen for first-line treatment. In 42 cases (76.4%), oxaliplatin-based regimens were used. Three patients were treated in a clinical trial (Cisplatin-based chemo-

Table 1 Patients demographics

Patient characteristics	*n* (%)
Number of patients	55
Median age (range), yr	76 (70-86)
Gender	
Female	16 (29.1)
Male	39 (70.9)
ECOG PS	
0	10 (18.2)
1	27 (49.1)
2	14 (25.5)
3	4 (7.3)
Metastatic disease	52 (94.5)
Locally advanced tumor	3 (5.5)
Primary palliative treatment	40 (72.7)
Secondary palliative treatment	15 (27.3)
Prior (neo) adjuvant CTX	12 (21.8)
Histology	
Adenocarcinoma	52 (94.5)
Sqaumous cell carcinoma	3 (5.5)
Site of Tumor	
Stomach, distal	25 (45.5)
Cardia	15 (27.3)
Esophagus, distal	9 (16.4)
Other	2 (3.6)
NA	4 (7.3)
Number of deaths	50 (90.9)
Cause of death	
Tumor	40 (80)
Infection	2 (4)
Unknown	8 (16)

ECOG PS: Eastern Cooperative Oncology Group performance status.

Table 2 Characteristics of chemotherapy

Treatment characteristics	*n* (%)
Median duration of first-line therapy (range), d	105 (6-568)
First-line therapy	
Single-agent (Fluoropyrimidine)	3 (5.5)
Doublet	47 (85.5)
FLO	38 (69.1)
FLP	2 (3.6)
XP	2 (3.6)
FOLFIRI	2 (3.6)
Other	3 (5.5)
Triplet	5 (9.1)
EOX	2 (3.6)
FLOT	2 (3.6)
TFLP	1 (1.8)
Oxaliplatin-based therapy	42 (76.4)
Cisplatin-based therapy	7 (12.7)
Other therapy	6 (10.9)
Trastuzumab containing therapy	5 (9.1)
Participation in clinical trial	3 (5.5)
Toxicities with dosage reduction	31 (56.4)
Polyneuropathy	14 (25.5)
Hematotoxicity	6 (10.9)
Leucopenia	3 (5.5)
Thrombopenia	3 (5.5)
Fatigue	4 (7.3)
Nausea	4 (7.3)
Other	3 (5.5)
Toxicities with interruption of treatment	6 (10.9)
Polyneuropathy	2 (3.6)
Fatigue	2 (3.6)
Anaphylaxia	1 (1.8)
Infection	1 (1.8)
Second-line treatment	28 (50.9)

FLO: 5-Fluorouracil (5-FU), leucovorin, oxaliplatin; FLP: 5-FU, leucovorin, cisplatin; XP: Capecitabine, cisplatin; FOLFIRI: 5-FU, leucovorin, irinotecan; EOX: Epirubicin, oxaliplatin, capecitabine; FLOT: 5-FU, leucovorin, oxaliplatin, docetaxel; TFLP: Docetaxel, 5-FU, leucovorin, cisplatin.

therapy +/- an anti EGFR antibody).

For this analysis, we included all toxicities that led to treatment modifications (Grade 3 or worse). In total, 31 patients (56.4%) developed such side effects. In 14 of those cases (45.2% of all relevant side effects or 25.5% of all patients) polyneuropathy (PNP) and in 6 cases (19.4% or 10.9%, respectively) hematotoxicity caused dosage reductions. Six patients (10.9%) discontinued therapy due to toxicity. PNP leading to treatment modifications was significantly more common in patients receiving prolonged firstline therapy (13 of 31 patients with a first-line $\geqslant$ 80 d *vs* 1 of 24 patients with a duration < 80 d (P = 0.002). No significant differences in toxicities requiring discontinuation of therapy were found for patients with an ECOG PS $\geqslant$ 2 or for patients $\geqslant$ 75 years, and we also did not find significant differences for combination regimens (doublet *vs* triplet *vs* monotherapies). A summary of therapies and toxicities is given in Table 2.

Progression and survival

Progression-free survival: Median PFS was 5.8 mo (95%CI: 4.1-7.6 mo). Median PFS for patients with an ECOG PS 0 or 1 was 6.8 mo (95%CI: 5.4-8.1) compared to 2.5 mo (95%CI: 1.5-3.5) for patients with ECOG PS $\geqslant$ 2; (P < 0.001; Figure 1A). Age $\geqslant$ 75 years was not associated with significant different PFS, neither there were significant differences for metastasized compared to locally advanced tumors, previous tumor resection or not.

Overall survival: Median overall survival in our patients was 9.5 mo (95%CI: 5.8-13.2). Median OS for patients with ECOG PS 0 or 1 was 12.7 mo (95%CI: 10.8-14.7) *vs* 3.8 mo (95%CI: 0.9-6.7) for patients with ECOG 2 or higher, (P < 0.001, Figure 1B). There was no apparent association between age at diagnosis and OS. Patients receiving a monotherapy had a median OS of 2.1 (95%CI: 0.2-4.0) mo *vs* 9.5 mo for doublets (95%CI: 6.2-12.7) and 19.0 mo for triplets (95%CI: 5.5-32.5, P < 0.001). OS did not significantly differ between the groups of patients with metastasized compared to irresectable locally advanced tumors or those with or without previous tumor resection.

Second-line chemotherapy and residual survival: Regarding the group of patients being alive at the time point of documented tumor progression, second-line chemotherapy was offered to 28 patients, whereas 7 patients received best supportive care (BSC) only. Patients receiving second-line treatment had a significantly longer RS than the BSC group (6.8 mo *vs*

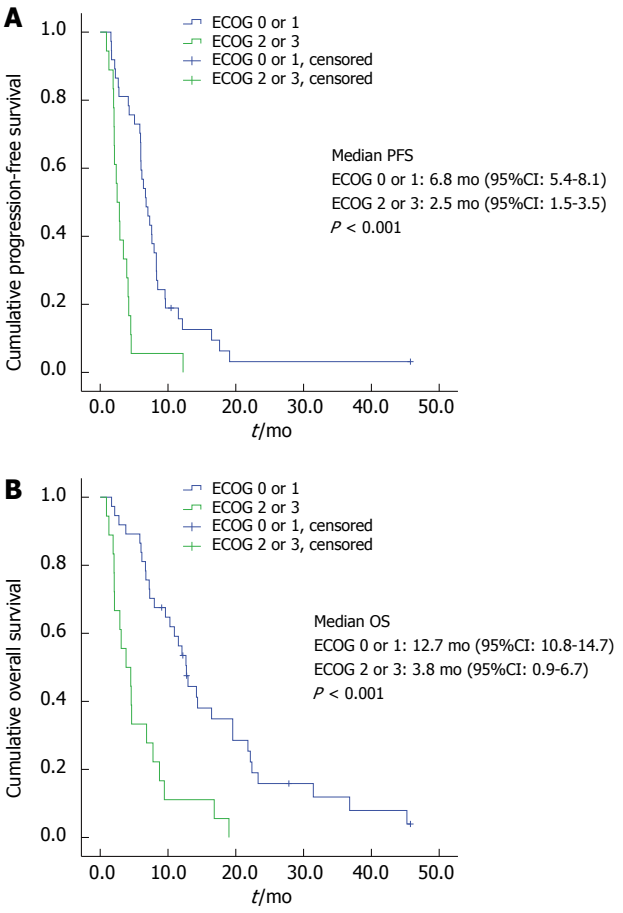

Figure 1 Progression-free survival (A), overall survival (B) by Eastern Cooperative Oncology Group performance status.

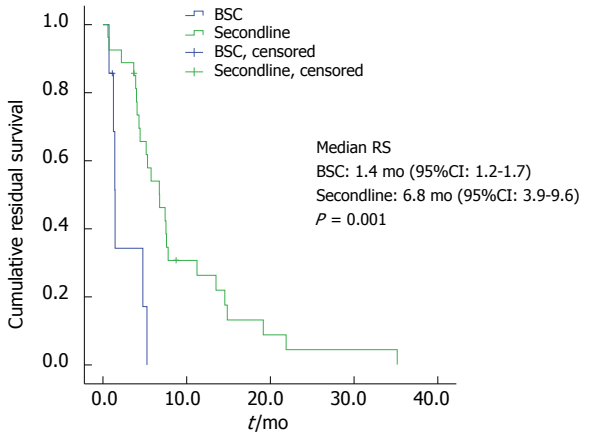

Figure 2 Residual survival by second-line therapy.

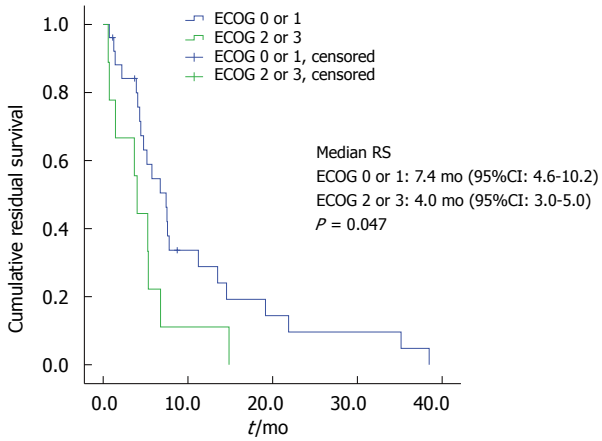

Figure 3 Residual survival by Eastern Cooperative Oncology Group performance status.

1.4. mo, $P = 0.001$; Figure 2). Patients with an ECOG PS of 0 or 1 before first-line therapy had a significantly longer RS (7.4 mo *vs* 4.0 mo, $P = 0.047$; Figure 3). No significant influence on RS was found for the variables age or duration of first-line treatment.

Multivariate analysis of overall survival confirmed the prognostic role of the initial ECOG PS (Hazard Ratio for patients with an ECOG PS of 2/3 3.9) and the application of secondline therapy (Hazard Ratio for patients receiving a secondline therapy 0.44, Table 3).

DISCUSSION

Although gastroesophageal cancer predominantely occurs in patients older than 65 years[24], the landmark phase Ⅲ trials establishing the standard protocols for palliative chemotherapy usually included much younger patients. Recently, some effort has been made to evaluate cytotoxic treatment strategies for patients ⩾ 65 years in the perioperative and palliative setting[19,25,26]. In our study, we analyzed a remarkably old cohort of palliative patients with a median age of 76 years (range 70-86) with the majority of patients (60%) being even older than 75 years. We did not find significant differences for survival times between the patient groups aged 70-74 years and ⩾ 75 years.

Our median times for PFS (5.8 mo) and for OS (9.5 mo) are in very good accordance with the results of the major phase Ⅲ trial collectives including mainly younger patients. For a study collective with a median age of 56 years, Kang *et al*[13] found a PFS of 5.6 mo and an OS of 10.5 mo for a doublet treatment with capecitabine and cisplatin. Concordant data for PFS (5.6 mo) and OS (10.7 mo) are recently reported for the combination of cisplatin and capecitabine[27]. Median age in the latter trial was 59 years with 30% of patients being older than 65 years. Similar results (PFS 5.8 mo, OS 10.7 mo) were described for a doublet treatment with oxaliplatin and 5-fluorouracil (FLO) in a study collective with a median age of 64 years[14]. In a subgroup analysis of this trial's patients older than 65 years, FLO seemed to be associated with improved survival times compared to a cisplatin-containing doublet. Comparable results for a modified FOLFOX regimen (PFS 6.8 and OS 10.5 mo, respectively) are recently reported in a phase Ⅱ study with 43 patients older than 69 years[25]. In a feasibility study, a triple combination (FLOT) failed to improve survival times for patients older than 69 years[19] compared to the FLO regimen.

Table 3 Multivariate analysis of overall survival

Prognostic factor	Hazard ratio	95%CI	Significance
Initial ECOG (2/3 *vs* 0/1[1])	3.9	2.0-7.5	$P < 0.001$
Secondline (Yes *vs* No[1])	0.44	0.2-0.8	$P = 0.006$
Very old (⩾ 75 yr *vs* < 75 yr[1])	1.36	0.7-2.5	$P = 0.314$

[1]Reference. ECOG: Eastern Cooperative Oncology Group.

Surprisingly, only 3 of our patients (5.5%) received single-agent therapy. OS was significantly shorter than for patients under combination therapy. Considering the small number, we do not have enough data to come to firm conclusions regarding the role of less intensive chemotherapies in older patients. Nevertheless, Sun *et al*[28] recently reported significantly reduced toxicities for single-agent treatment in a Korean geriatric collective while survival times did not differ significantly but were generally striking low (OS 6.6 mo for single-agent treatment *vs* 7.6 for doublet combinations). Median age for single-agent treatment was 77 years, and 73 years for patients receiving doublet combinations in this analysis[28]. 90% of our patients initially received cytotoxic doublet combinations. Platin-derivates were used in 90% of all regimens with the FLO regimen being predominant (oxaliplatin usage 76%, FLO regimen 69%). Notably, more than half of our patients needed dose adjustments due to toxicities. One third of the oxaliplatin group developed relevant PNP leading to treatment modifications. This is clearly more than expected from the trials so far that described grade 3/4 PNP in 14%[14], 2%[25] or 20%[19]. Relevant PNP in our cohort was found significantly more frequent when treatment duration exceeded 80 d. Cessation of therapy was necessary in 11% of our patients with 4% caused by PNP. Rates for grade 3/4 hematotoxicity (11%), nausea (7%) and fatigue (7%) were found to be comparable to the results from phase Ⅲ trials.

Thus, in terms of survival, we did not find any evidence that old and even very old patients that have been deemed fit enough for palliative chemotherapy do have an inferior outcome compared to younger patients in clinical studies. These findings are consistent with current own data concerning palliative chemotherapy in old patients with pancreatic cancer[29] and with previous analysis of other solid malignancies[30,31]. Based on the convincing survival times, in our opinion doublet combinations should be considered for first-line treatment whenever possible in old and even very old patients. However, oncologists should be aware that toxicities might appear more frequent. Symptoms of PNP should be monitored closely, and patients and their relatives should be informed in advance. In case of successful and prolonged treatment with oxaliplatin, planning dose reductions upfront might be reasonable.

In our cohort, residual survival after first progression was significantly longer for patients who received second-line therapy than for the BSC group. One possible explanation for this advantage is that only patients in good condition were offered second-line therapy. However, given that our results are comparable to the recent randomized phase Ⅲ trial on second-line therapy[20], we strongly believe that second-line strategies should be offered to old and very old patients in good performance status after first progression.

Fear of increased toxicities and uncertainty concerning both clinical value and physiological resources may cause withholding or limitation of tumor-specific therapies by the attending oncologist. Therefore, old cancer patients are at risk for therapeutic disparity and undertreatment. In our analysis of unselected old patients, higher age was not significantly associated with impaired survival times. Thus, the feasibility and efficacy of systemic treatment in advanced gastroesophageal cancer in the old population seems to be independent of chronological age. The evaluation of the "functional age" rather than biological age is known as more appropriate for assessing the eligibility for chemotherapy, and the usefulness of comprehensive geriatric assessments (CGA) in this setting has been demonstrated[32-34]. CGA has not been established in our outpatient setting yet, and rather in our patients we use the ECOG PS as the initial classification system. In our study population, all survival times were significantly depending on the initial ECOG PS. An ECOG PS ⩾ 2 was strongly associated with a poor clinical outcome with a median OS of only 3.8 mo. Thus, systemic cytotoxic treatment for old patients in reduced performance status should only be offered cautiously. In contrast, treatment decisions based on chronological age only would reflect a form of "ageism" and should be avoided.

COMMENTS

Background

Palliative chemotherapy is the preferred treatment for metastatic esophagogastric cancer. Superiority regarding overall survival and quality of life compared to best supportive care alone has been shown in several trials. Despite the fact that esophagogastric cancer occurs more often in old patients, this group of patients is underrepresented in clinical trials. When treating old and very old cancer patients, medical comorbidities, impaired organ function and reduced functional status are frequently found. Concerns about poor tolerability might prevent physicians from initiating a tumorspecific therapy.

Research frontiers

Within clinical trials representing old patients in a good general state, there is some evidence that for patients older than 65 years survival times are comparable to those of younger ones, but grade 3 or higher toxicities might be more frequent for currently used regimens. Oxaliplatin-based combinations seem to have a better toxicity profile and seem to be associated with an improved efficacy in contrast to cisplatin-based regimens, whereas the additional benefit of using three-drug cytostatic combinations in elderly patients is questionable. Secondline therapy might be an option in selected patients with a good general condition after progression on first-line therapy. In general, data from clinical trials represent a selected cohort of old patients, whereas real-life data of elderly patients are scarce.

Innovations and breakthroughs

In this study, the authors analyzed efficacy and toxicity in old patients with

metastatic esophagogastric cancer. The applied chemotherapeutical regimens were documented, patterns of toxicity were analyzed. Eastern Cooperative Oncology Group (ECOG)-Score and age were analyzed as potential prognostic factors. Within this real-life setting, efficacy in terms of PFS, OS and residual survival after tumorprogression (RS), was comparable to major phase Ⅲ trials with younger patients. Toxicity was within the expected range. Initial ECOG-Score is a strong prognostic marker regarding overall survival and RS.

Applications

Tumorspecific treatment should be offered to old and very old patients in a good general condition regardless of chronological age. Initial ECOG-Score is a major prognostic marker which could help selecting patients for chemotherapeutical treatment.

Terminology

PFS: Progression-free survival is defined as the time between initiation of systemic therapy and tumor progression or death from any course. Overall Survival is defined as the time between initiation of systemic therapy and death from any cause. The ECOG Scale of Performance Status is widely used to quantify the functional status of cancer patients, and is an important factor determining prognosis in a number of malignant conditions.

Peer-review

The manuscript has a high significance and novelty. Generally speaking, the presentation and organization of the manuscripts good, the quality of language is good. This manuscript describes a single-center clinical study on the effects of palliative chemotherapy in old patients with gastroesophageal cancer. The technique is not novel but the subject of research is ignored by academic world.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Proton-pump inhibitors for prevention of upper gastrointestinal bleeding in patients undergoing dialysis

Young Rim Song, Hyung Jik Kim, Jwa-Kyung Kim, Sung Gyun Kim, Sung Eun Kim

Young Rim Song, Hyung Jik Kim, Jwa-Kyung Kim, Sung Gyun Kim, Division of Nephrology, Department of Internal Medicine, Hallym University Sacred Heart Hospital, Kidney Research Institute, Hallym University College of Medicine, Anyang-si 431-070, South Korea
Sung Eun Kim, Division of Gastroenterology, Department of Internal Medicine, Hallym University Sacred Heart Hospital, Anyang-si 431-070, South Korea
Author contributions: Song YR and Kim HJ contributed equally to this work; Song YR and Kim SE designed the research; Song YR, Kim HJ, Kim JK, Kim SG, and Kim SE performed the research; Song YR and Kim SE analyzed the data; and Song YR and Kim HJ wrote the manuscript.
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Correspondence to: Sung Eun Kim, MD, PhD, Division of Gastroenterology, Department of Internal Medicine, Hallym University Sacred Heart Hospital, 896, Pyeongchon-dong, Dongan-gu, Anyang-si 431-070, South Korea. sekim@hallym.or.kr
Telephone: +82-31-3803705
Fax: +82-31-3862269
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Abstract

AIM: To investigate the preventive effects of low-dose proton-pump inhibitors (PPIs) for upper gastrointestinal bleeding (UGIB) in end-stage renal disease.

METHODS: This was a retrospective cohort study that reviewed 544 patients with end-stage renal disease who started dialysis at our center between 2005 and 2013. We examined the incidence of UGIB in 175 patients treated with low-dose PPIs and 369 patients not treated with PPIs (control group).

RESULTS: During the study period, 41 patients developed UGIB, a rate of 14.4/1000 person-years. The mean time between the start of dialysis and UGIB events was 26.3 ± 29.6 mo. Bleeding occurred in only two patients in the PPI group (2.5/1000 person-years) and in 39 patients in the control group (19.2/1000 person-years). Kaplan-Meier analysis of cumulative non-bleeding survival showed that the probability of UGIB was significantly lower in the PPI group than in the control group (log-rank test, $P < 0.001$). Univariate analysis showed that coronary artery disease, PPI use, anti-coagulation, and anti-platelet therapy were associated with UGIB. After adjustments for the potential factors influencing risk of UGIB, PPI use was shown to be significantly beneficial in reducing UGIB compared to the control group (HR = 13.7, 95%CI: 1.8-101.6; $P = 0.011$).

CONCLUSION: The use of low-dose PPIs in patients with end-stage renal disease is associated with a low frequency of UGIB.

Key words: Dialysis; End-stage renal disease; Peptic ulcer; Gastrointestinal hemorrhage; Proton pump inhibitors

Core tip: Patients with end-stage renal disease are at a high risk for upper gastrointestinal bleeding (UGIB). The aim of this study was to assess the effects of low-dose proton-pump inhibitors (PPIs) for the prevention of UGIB in a cohort of patients with end-stage renal disease who began dialysis at our center between 2005 and 2013. The cumulative non-bleeding survival showed that the probability of UGIB was significantly lower in the PPI group than in the controls. PPI use was beneficial in reducing UGIB compared to the control (HR = 13.7, 95%CI: 1.8-101.6; P = 0.011).

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INTRODUCTION

Patients with end-stage renal disease (ESRD) are at high risk for bleeding complications[1-4]. Upper gastrointestinal bleeding (UGIB) occurs most frequently in patients undergoing dialysis and is associated with higher re-bleeding risk and mortality than the general population[5-7]. Neither the origin nor pathogenesis of UGIB has been elucidated, although platelet dysfunction, blood coagulation abnormalities, and anemia may contribute to bleeding tendency[8,9]. Patients undergoing hemodialysis (HD) have increased risk for UGIB due to repeated anti-coagulant exposure compared with peritoneal dialysis (PD) patients[10]. In the general population, the incidence of UGIB has been declining over time; however, UGIB among patients with ESRD has not decreased in the past ten years according to data from the United States Renal Data System[11]. It was estimated that UGIB accounts for 3%-7% of all deaths among patients with ESRD[4], and prevention of UGIB remains a challenge for the nephrologist. There are multiple strategies to reduce UGIB, and proton-pump inhibitors (PPIs) have been demonstrated to reduce the risk of UGIB and are advocated for patients at high risk for UGIB who are taking aspirin, dual anti-platelet therapy, and non-steroidal anti-inflammatory drugs (NSAIDs)[12,13].

Patients with ESRD have a high prevalence of gastrointestinal symptoms with increased use of acid suppressive therapy[14]. According to the 2011 annual report from the Korean registry system, the frequencies of gastrointestinal disease in patients undergoing HD and PD were 10.1% and 9.3%, respectively[15]. Long-term acid suppression with PPIs rarely produces adverse events and PPIs are considered safe in patients with ESRD. When medical insurance coverage of low-dose PPI was instituted in Korea, prescriptions for low-dose PPI increased in patients with gastrointestinal symptoms. We found that about 30% of patients with ESRD who started dialysis at our center were prescribed PPI at discharge between 2010 and 2012. In the present study, we retrospectively investigated the protective effect of low-dose PPIs on UGIB in a cohort of patients with ESRD.

MATERIALS AND METHODS

Study design and patients

The present study was based on a retrospective review of the clinical records of patients with ESRD who began dialysis between January 2005 and May 2013 at Hallym University Sacred Heart Hospital, Anyang, Korea. Patients were excluded if: they had a previous peptic (gastric and/or duodenal) ulcer; were younger than 18 years of age; had a history of gastric surgery, malignancy, or liver cirrhosis; had undergone dialysis for < 3 mo; had a total follow-up duration of < 6 mo; received renal transplantation; or were prescribed a histamine H2-receptor antagonist, corticosteroid, or NSAID. We divided the patients into the those receiving PPIs and those not treated with PPIs (control group). This study was approved by the Investigation and Ethics Committee for Human Research at the Hallym Sacred Heart Hospital, in accordance with the principles of good clinical practice and the Declaration of Helsinki.

Diagnosis of UGIB

UGIB was defined as a diagnosis made by the gastroenterologist in combination with no other defined bleeding cause. A gastroenterologist performed an endoscopy if a patient with ESRD showed a clinical suspicion of bleeding, such as hematemesis, melena, hematochezia, or unexplained hemoglobin decrease of > 2 g/dL. An endoscopic finding of high-risk stigmata, including active bleeding, visible vessel, or adherent clot, and blood in the stomach was defined as bleeding. Patients bleeding from esophageal and/or gastric varices were excluded from the study. Peptic ulcer bleeding was classified as gastric ulcer, duodenal ulcer, or both. Re-bleeding was defined as a repeat endoscopy for a clinical suspicion of re-bleeding and a finding of high-risk stigmata and blood in the stomach.

Statistical analysis

Statistical analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, United States). All data are expressed as mean ± SD or median with ranges. Continuous data were analyzed by Student's t test for equal variance or Mann-Whitney U test for unequal variance, and categorical valuables were investigated

Table 1 Clinical characteristics of patients according to proton-pump inhibitor use

Characteristic	PPI group (n = 175)	Control group (n = 369)
Age, yr	63.9 ± 12.8	62.7 ± 14.5
Follow-up (mo)	55.7 ± 33.6a	66.5 ± 37.5
Sex, male	98 (57.1)	201 (54.5)
Hypertension	99 (56.6)	215 (58.3)
Diabetes	90 (51.4)	203 (55.0)
Smoking	11 (40.7)	4 (12.1)
Hemodialysis	153 (87.4)	333 (90.24)
Body mass index (kg/m^2)	23.4 ± 3.2	23.5 ± 3.9
Previous cardiovascular disease		
Cerebrovascular accident	18 (10.3)	49 (13.3)
Coronary heart disease	28 (16.0)	68 (18.4)
Chronic liver disease	2 (1.1)	3 (0.8)
Aspirin use	82 (46.9)	175 (47.4)
Dual anti-platelet therapy	15 (8.6)	32 (8.7)
Warfarin	9 (5.1)	8 (2.2)
Helicobacter pyroli		
Positive	36 (6.0)	53 (15.0)
No test	139 (79.4)	316 (85.6)
Baseline laboratory findings		
Hemoglobin (g/L)	9.1 ± 1.6	8.8 ± 1.6
Hematocrit (%)	27.3 ± 5.3	26.2 ± 5.1
Creatinine (mmol/L)	6.7 ± 2.9	7.2 ± 3.2
Protein (g/L)	7.3 ± 0.9	7.2 ± 0.9
Albumin (g/L)	4.1 ± 0.9	3.9 ± 0.6
C-reactive protein (mg/L)	11.0 ± 18.8	9.6 ± 17.2
Death	7 (4.0)	18 (4.9)

Data are presented as mean ± SD, or n (%); [a]P < 0.05 *vs* control. PPI: Proton-pump inhibitors.

by Pearson's χ^2 test. The cumulative non-bleeding rates in the two groups were estimated using Kaplan-Meier survival estimates and the log-rank test was used to determine the differences between the curves. Multivariate Cox's proportional hazard model analysis was used to evaluate the independent predictors for UGIB. Differences with P < 0.05 were accepted as statistically significant.

RESULTS

Patients' characteristics

We retrospectively reviewed 544 patients with ESRD who began dialysis at Hallym University Sacred Heart Hospital between January 2005 and March 2013. The demographic and clinical characteristics of the cohort are as follows: mean age, 63.1 ± 14.0 years; male, 54.0% (n = 299); patients undergoing HD, 87.7% (n = 486); diabetes, 52.9% (n = 293); smoking, 2.7% (n = 15). Ninety-six patients had a history of coronary artery disease, 17 patients were treated with warfarin, 257 patients received aspirin, and 42 patients received dual anti-platelet therapy. Among the 544 patients, there were 175 in the PPI group (152 patients received pantoprazole 20 mg orally once a day and 23 received rabeprazole 10 mg orally once a day) and 369 in the control group. The baseline characteristics of the groups are summarized in Table 1. There were no significant differences in age, sex, primary renal disease, mode of dialysis, smoking, warfarin use, anti-platelet use, body mass index, or biochemical parameters between the two groups.

Table 2 Causes of upper gastrointestinal bleeding in patients with end-stage renal disease

Cause	Patients (n)
Gastric ulcer	19
Duodenal ulcer	7
Duodenal and gastric ulcer	1
Dieulafoy's lesion	7
Mallory-Weiss tear	2
Gastric erosion	2
Duodenal erosion	2
Gastric cancer	1

UGIB in patients with ESRD

During the study period, 41 patients developed UGIB, a rate of 14.4 /1000 person-years, and the incidence of UGIB was 20.7/1000 person-years in patients receiving warfarin or anti-platelet therapy. The mean time between the initiation of dialysis and UGIB events was 26.3 ± 29.6 mo. Table 2 shows the sources of UGIB. Gastric lesions were the most common cause of UGIB in patients with ESRD, accounting for 50% of bleeding sources. Eighteen patients presented with melena or hematochezia, 13 patients were admitted with hematemesis, and 10 underwent endoscopy due to unexplained anemia. The hemoglobin level at admission was 6.6 ± 1.8 g/dL and 40 patients received a red blood cell transfusion, with a median of 4.0 units (range: 1-15 units) and average of 4.5 units. Two patients experienced re-bleeding, one in the control group and the other in the PPI group, and there was one death related to UGIB in the control group.

Predictors for UGIB

There were no significant differences in age, sex, primary renal disease, diabetes, body mass index, or biochemical parameters between patients with and without UGIB. Patients with UGIB had significantly higher frequency of warfarin use (P = 0.017) and anti-platelet therapy (P = 0.041) than those without UGIB. Bleeding occurred in only two patients (2.5/1000 person-years) in the PPI group and 39 patients (19.2/1000 person-years) in the control group. Kaplan-Meier analysis of cumulative non-bleeding survival showed that the probability of UGIB was significantly lower in the PPI group than in the control group (log rank test, P < 0.001; Figure 1). In the subgroup analysis of patients treated with warfarin or anti-platelet therapy, PPI use significantly decreased the risk for UGIB (log rank test, P = 0.003; Figure 2).

Univariate analysis showed that coronary artery disease, no use of PPIs, warfarin, and anti-platelet therapy were associated with UGIB. After adjustments for age, sex, diabetes, hypertension, smoking,

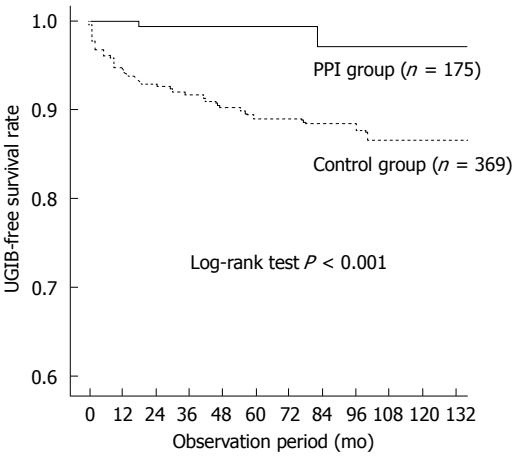

Figure 1 Kaplan-Meier analysis of cumulative non-bleeding survival according to proton-pump inhibitor use in patients with end-stage renal disease. UGIB: Upper gastrointestinal bleeding.

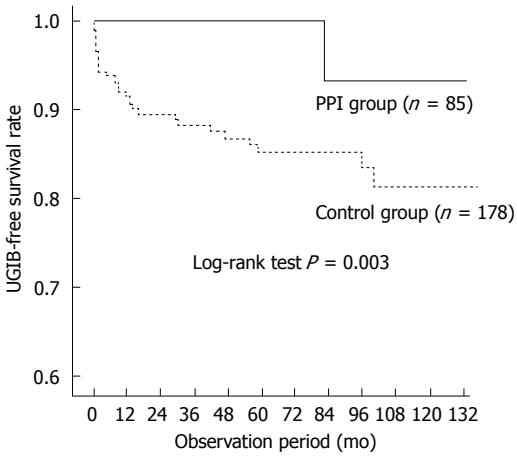

Figure 2 Kaplan-Meier analysis of cumulative non-bleeding survival according to proton-pump inhibitor use in dialysis patients treated with anti-coagulation or anti-platelet therapy. UGIB: Upper gastrointestinal bleeding.

Table 3 Predictors for upper gastrointestinal bleeding in patients with end-stage renal disease

Variable	HR	95%CI	*P* value
PPI (no treatment *vs* treatment)	13.689	1.84-101.64	0.011
Warfarin	4.728	1.57-14.25	0.006
Anti-platelet therapy	2.476	1.38-4.44	0.002
Coronary artery disease	2.079	0.954-4.532	0.094
Cerebrovascular disease	1.492	0.973-2.287	0.067
Smoking	2.154	0.659-7.043	0.204
Diabetes	1.020	0.48-2.30	0.899
Hypertension	1.318	0.59-2.92	0.497
Age	1.002	0.97-1.03	0.870

All models were adjusted for age, sex, diabetes, hypertension, smoking, coronary heart disease, cerebrovascular disease, hemoglobin, albumin and mode of dialysis. PPI: Proton-pump inhibitor.

coronary heart disease, hemoglobin, albumin, and mode of dialysis, PPI use was significantly beneficial in reducing UGIB compared to the control (HR = 13.7, 95%CI: 1.8-101.6, *P* = 0.011; Table 3).

DISCUSSION

In the present study, we found that the incidence of UGIB in patients with ESRD was relatively high, and the use of PPIs was associated with a significant reduction in UGIB events. In our cohort, the incidence of UGIB in patients with ESRD was 14.4/1000 person-years and the mean time from initiation of dialysis to UGIB was 26.3 ± 29.6 mo. The incidence of UGIB was significantly lower in the PPI group than in the control group, and these preventive effects were maintained in patients receiving anti-platelet or warfarin therapy. These results suggest that UGIB may be prevented by PPIs in patients with ESRD.

The risk of UGIB in dialysis patients is thought to be higher than in the general population. In the United States Renal Data System Dialysis Morbidity and Mortality Study, the incidence of UGIB in patients with ESRD was 22.8/1000 person-years[3], similar to the incidence in the control group and higher than that in the PPI group. The incidence of UGIB in HD patients was 42.0/1000 person-years according to data from Taiwan National Health Insurance Research Database[5], which is higher than the incidence in our study. These results may be related to inclusion of PD patients and exclusion of patients with liver cirrhosis and patients receiving NSAIDs or steroids. In the present study, the two- and four-year cumulative incidences of UGIB in the control group were 8.2% and 13.1%, respectively. Although the risk factors related with UGIB in patients with ESRD are uncertain, we found that warfarin therapy and use of anti-platelet agents are significantly associated. Wassel *et al*[3] reported that smoking, history of cardiovascular disease, and inability to ambulate were associated with a higher risk of UGIB. In data from Taiwan HD patients, diabetes, cirrhosis, coronary artery disease, and use of NSAIDs were significant risk factors for UGIB[16]. Chen *et al*[17] found that diabetes, congestive heart failure, albumin, and PD were risk factors for peptic ulcers in patients with ESRD. In the present study, we did not include data regarding ambulatory status and excluded patients with liver cirrhosis and those receiving NSAIDs.

We investigated the efficacy of PPIs in the prevention of UGIB in patients with ESRD. In the Korean National Health Insurance program, low-dose PPIs are covered for prophylaxis against ulcers or to relieve gastric or reflux symptoms. Due to the retrospective observational nature of the present study, patients were not randomly assigned to the PPI or control group. Most of the patients in the PPI group had gastric or reflux symptoms and there were no significant differences in age, sex, diabetes, cardiovascular disease, or smoking between the two groups. Nonetheless, we found a significant preventive effect of PPIs on UGIB in patients with ESRD. PPIs are frequently used to prevent UGIB in patients receiving dual anti-platelet therapy. Liang *et*

al[18] reported that prophylactic use of omeprazole was effective in lowering the incidence of peptic ulcer disease in HD patients. To our knowledge, this is the first report to suggest the preventive efficacy of PPI for UGIB in patients undergoing dialysis. Because several studies have suggested relationships among impaired bone metabolism[19], fractures[20], vascular calcification[21], and PPIs, we evaluated mortality in the two groups. There was no significant difference in mortality according to the use of PPIs. However, we did not assess vascular calcification and bone mineral density.

This study had several limitations. First, as this was not a randomized controlled trial, we can not draw general conclusions. However, in practice, it is difficult to perform a randomized controlled trial due to ethical problems. Second, we examined *Helicobacter pylori* in only a small portion of patients. Third, we excluded patients receiving H_2 receptor antagonists. It has been suggested that H_2 receptor antagonists can be used as alternatives to PPIs to prevent UGIB during dual anti-platelet therapy without an increase in the risk of cardiovascular outcomes. Finally, we did not assess long-term adverse effects of PPIs in patients with ESRD because we did not assess vascular calcification, serum magnesium, mineral bone disease states, or bone fractures.

In conclusion, we found that the risk of UGIB in patients with ESRD was lower in the PPI group than the control group. In addition, PPI did not increase mortality and may be safe and effective for prevention of UGIB in patients undergoing dialysis. Future large-scale controlled trials are necessary to confirm our results.

COMMENTS

Background

Upper gastrointestinal bleeding (UGIB) occurs most frequently in patients undergoing dialysis and is associated with higher re-bleeding risk and mortality in these patients than in the general population. It was estimated that UGIB accounts for 3%-7% of all deaths among patients with end-stage renal disease (ESRD) and prevention of UGIB remains a challenge for the nephrologist. Proton-pump inhibitors (PPIs) have been demonstrated to reduce the risk of UGIB and are advocated for patients at high risk for UGIB who are taking aspirin, dual anti-platelet therapy and non-steroidal anti-inflammatory drugs.

Research frontiers

Recently, a number of practice guidelines recommend administering prophylactic PPI to patients who are at high risk of UGIB. However, there exist limited data regarding the efficacy of PPIs for the prevention of UGIB, and there are no formal guidelines about the use of a PPI for gastroprotection in patients with ESRD. The current research investigated whether PPIs are effective in reducing UGIB in patients undergoing dialysis.

Innovations and breakthroughs

This study revealed a significant preventive effect of PPIs on UGIB in patients with ESRD. PPIs are frequently used to prevent UGIB in patients receiving dual anti-platelet therapy. One study reported that prophylactic use of omeprazole was effective in lowering the incidence of peptic ulcer disease in hemodialysis patients. To our knowledge, this is the first report to suggest the preventive efficacy of PPI for UGIB in patients undergoing dialysis.

Applications

The study results show that the risk of UGIB in patients with ESRD is lower in the PPI group than the control group. In addition, PPI did not increase the mortality and may be safe and effective for prevention of UGIB in patients undergoing dialysis.

Terminology

UGIB is diagnosed by performing an endoscopy if a patient with ESRD shows a clinical suspicion of bleeding, such as hematemesis, melena, hematochezia, or unexplained hemoglobin decrease of > 2 g/dL. In this study, bleeding was used to describe an endoscopic finding of high-risk stigmata, including active bleeding, visible vessel, or adherent clot, and blood in the stomach.

Peer-review

This is an interesting study in which the authors investigated the preventive effect of PPI on UGIB in patients with ESRD, especially patients undergoing dialysis. The results are interesting and suggest that PPI could be used for preventing UGIB in patients undergoing dialysis.

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ORIGINAL ARTICLE

Retrospective Study

Hepatobiliary complications of alveolar echinococcosis: A long-term follow-up study

Tilmann Graeter, Franziska Ehing, Suemeyra Oeztuerk, Richard Andrew Mason, Mark Martin Haenle, Wolfgang Kratzer, Thomas Seufferlein, Beate Gruener

Tilmann Graeter, Department of Interventional and Diagnostic Radiology, University Hospital Ulm, 89081 Ulm, Germany
Franziska Ehing, Suemeyra Oeztuerk, Mark Martin Haenle, Wolfgang Kratzer, Thomas Seufferlein, Department of Internal Medicine I, University Hospital Ulm, 89081 Ulm, Germany
Richard Andrew Mason, Louis Stokes Cleveland Department of Veterans Affairs Medical Center, Cleveland, OH 44106, United States
Beate Gruener, Section of Infectious Diseases and Clinical Immunology, Comprehensive Infectious Diseases Center Ulm, 89081 Ulm, Germany
Author contributions: Graeter T and Ehing F contributed equally to this work; Graeter T, Seufferlein T, Kratzer W and Gruener B designed the research; Graeter T, Ehing F, Oeztuerk S, Kratzer W and Gruener B performed the research; Ehing F, Oeztuerk S, Mason RA, Haenle MM and Kratzer W analyzed the data; and Graeter T, Ehing F and Kratzer W wrote the paper.
Ethics approval: The study was reviewed and approved by the local ethics committee of the University of Ulm.
Informed consent: Because of the retrospective and anonymous character of this study, the need for informed consent was waived by the institutional review board.
Conflict-of-interest: The authors declare that there are no conflicts of interest.
Data sharing: No additional data are available.

Correspondence to: Wolfgang Kratzer, MD, Professor, Department of Internal Medicine I, University Hospital Ulm, Albert-Einstein-Allee 23, 89081 Ulm, Germany. wolfgang.kratzer@uniklinik-ulm.de
Telephone: +49-731-50044730
Fax: +49-731-50044621
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Abstract

AIM: To determine the long-term hepatobiliary complications of alveolar echinococcosis (AE) and treatment options using interventional methods.

METHODS: Included in the study were 35 patients with AE enrolled in the Echinococcus Multilocularis Data Bank of the University Hospital of Ulm. Patients underwent endoscopic intervention for treatment of hepatobiliary complications between 1979 and 2012. Patients' epidemiologic data, clinical symptoms, and indications for the intervention, the type of intervention and any additional procedures, hepatic laboratory parameters (pre- and post-intervention), medication and surgical treatment (pre- and post-intervention), as well as complications associated with the intervention and patients' subsequent clinical courses were analyzed. In order to compare patients with AE with and without history of intervention, data from an additional 322 patients with AE who had not experienced hepatobiliary complications and had not undergone endoscopic intervention were retrieved and analyzed.

RESULTS: Included in the study were 22 male and 13 female patients whose average age at first diagnosis was 48.1 years and 52.7 years at the time of intervention. The average time elapsed between first diagnosis and onset of hepatobiliary complications was 3.7 years. The most common symptoms were jaundice, abdominal pains, and weight loss. The

number of interventions per patient ranged from one to ten. Endoscopic retrograde cholangiopancreatography (ERCP) was most frequently performed in combination with stent placement (82.9%), followed by percutaneous transhepatic cholangiodrainage (31.4%) and ERCP without stent placement (22.9%). In 14.3% of cases, magnetic resonance cholangiopancreatography was performed. A total of eight patients received a biliary stent. A comparison of biochemical hepatic function parameters at first diagnosis between patients who had or had not undergone intervention revealed that these were significantly elevated in six patients who had undergone intervention. Complications (cholangitis, pancreatitis) occurred in six patients during and in 12 patients following the intervention. The average survival following onset of hepatobiliary complications was 8.8 years.

CONCLUSION: Hepatobiliary complications occur in about 10% of patients. A significant increase in hepatic transaminase concentrations facilitates the diagnosis. Interventional methods represent viable management options.

Key words: Alveolar echinococcosis; Endoscopic retrograde cholangiopancreatography; Hepatobiliary complication; Magnetic resonance cholangiopancreatography; Percutaneous transhepatic cholangiodrainage; Stent placement; Treatment

Core tip: Approximately 10% of patients with alveolar echinococcosis experience hepatobiliary complications that occur on average 3.7 years (range: 0-41 years) following first diagnosis. Elevated hepatic transaminases in association with jaundice, abdominal pain, and weight loss are typical symptoms facilitating the diagnosis. Interventional endoscopic methods represent important options in these patients' management. Even in cases of repeated interventions, the rates of complications and treatment-associated mortality are low. The average survival following onset of hepatobiliary complications and interventional treatment stands at 8.8 years.

Graeter T, Ehing F, Oeztuerk S, Mason RA, Haenle MM, Kratzer W, Seufferlein T, Gruener B. Hepatobiliary complications of alveolar echinococcosis: A long-term follow-up study. *World J Gastroenterol* 2015; 21(16): 4925-4932 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4925.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4925

INTRODUCTION

Alveolar echinococcosis (AE) is a rare but potentially life-threatening parasitic disease that results from infection with the larval stage of *Echinococcus multilocularis*[1-3]. Worldwide, the range of the parasite is limited to cold and temperate regions of the northern hemisphere[4]. Although its incidence in endemic regions is generally low (0.03-1.20 per 100000 persons)[1,5], more recent studies suggest that the endemic regions of *E. multilocularis* are larger than previously believed. In addition, increased fox *(Vulpes vulpes)* populations, including in urban areas, have been reported[6-8], with an associated increased infection risk for humans[6]. Despite abundant clinical resources and technical advances, the diagnosis of AE in infected individuals, especially among inexperienced clinicians, remains challenging. With delayed diagnosis, therapy often begins in a late stage of the disease, which significantly limits treatment options. A characteristic feature of AE is its tumor-like growth, which may lead to infiltration of neighboring organs[1]. The liver is the first organ to be affected by larval infestation. Hepatic lesions are localized to the right hepatic lobe in seven of ten cases, while in 40%, the liver hilus is additionally affected. Only in two of ten cases are both hepatic lobes infested[9].

Patients are typically asymptomatic during the initial phase of the infection. The first symptoms may include upper abdominal pain and cholestatic jaundice. The incubation time ranges from five to fifteen years[5]. Radical resection of the echinococcal lesion is the only curative therapy. Untreated patients experience a mortality rate of 95% within ten years of first diagnosis[10]. Only early detection serves to increase the rate of curative resection[11], which is confirmed on the basis of imaging methods and serologic markers[12]. Complications such as biliary obstruction, portal hypertension, and bleeding esophageal varices have been described in advanced disease stages and are ascribed to the invasive growth of the *E. multilocularis* lesion in the liver[13].

Biliary complications such as intrabiliary rupture in combination with obstructive jaundice in cystic echinococcosis, which is caused by *E. granulosus,* has an incidence of 1%-25% and, in cases of biliary fistulae, can often be effectively treated using endoscopic retrograde cholangiopancreatography (ERCP)[14,15]. By contrast, a search of the literature with respect to the use of interventional methods such as ERCP in the treatment of hepatobiliary complications of AE reveals no prospective studies and only a few case studies[13,16] and reports of experience with small numbers of patients[17-20]. A recently published study with 26 cases represents the largest currently available study of patients with hepatobiliary complications in AE[21]. The authors postulate that surgical interventions represent risk factors for the occurrence of such complications. The incidence of biliary complications was reported to be about 30% with an average survival of three years following hepatobiliary complications[21]. The studies by Ozturk *et al*[16] and Sezgin *et al*[13] with 13 and 12 patients, respectively, primarily address the main clinical symptoms and changes in laboratory

parameters associated with biliary complications. Hilmioglu *et al*[17] report on six patients and their ERCP results, which they discuss in terms of characteristic cholangiographic findings. Other authors report single cases, such as Gschwantler *et al*[18] who report on a combined endoscopic and pharmaceutic approach in a patient with rupture of an AE lesion into the bile duct, Koroglu *et al*[19], who describe the complete disappearance of AE following percutaneous drainage, and Rosenfeld *et al*[20], who discuss the onset of painless jaundice and the subsequent therapy. Finally, a few authors mention endoscopic and percutaneous interventions as treatment options for patients in whom the risks of surgery are unacceptably high or in whom the total resection of the AE lesion cannot be guaranteed[22-24].

The objective of the study was to describe the clinical and biochemical outcomes of patients with hepatobiliary complications of AE and to determine treatment options using interventional methods.

MATERIALS AND METHODS

Between 1979 and 2012, AE was diagnosed in 357 patients presenting to the Echinococcosis Specialty Clinic of the University of Ulm. In the present study, we report on 35 patients examined and treated for hepatobiliary complications of AE with interventional methods including ERCP, with or without stent placement, percutaneous transhepatic cholangiodrainage (PTCD), and magnetic resonance cholangiopancreatography (MRCP). Criteria for inclusion in the study included confirmed diagnosis of AE, use of at least one of the above-mentioned methods for treatment of hepatobiliary complications, and availability of pre- and post-procedure data on laboratory findings, medication, and surgery. Not all patients were diagnosed and treated in our clinic; patients' records, however, contained reports and discharge summaries from outside institutions. Patient data was derived retrospectively from the Echinococcus Multilocularis Data Bank of the University Hospital of Ulm. Patients' epidemiologic data, clinical symptoms, and indications for the intervention, the type of intervention and any additional procedures, hepatic laboratory parameters (pre- and post-intervention), medication and surgical treatment (pre- and post-intervention), as well as complications associated with the intervention and patients' subsequent clinical courses were recorded.

In addition, available discharge summaries, digital patient records, and reports from outside institutions were reviewed with retrospective compilation of information regarding the indication for the intervention, biochemical hepatic function parameters before and after the intervention, type of intervention, and prior treatment and medication. The subsequent clinical courses of these patients through the end of 2012 were analyzed. All data were then compared with the clinical courses of 322 patients without history of hepatobiliary complications and, thus, without intervention with respect to prior treatment (surgery, medication) and laboratory parameters including hepatic function tests. Prior to analysis, all patient data were anonymized.

The study was conducted in conformity to the basic principles of Helsinki Declaration. It was approved by the local ethics committee. We declare that none of the authors of the present study has any competing commercial, personal, political, intellectual or religious interest with respect to the present study.

Statistical analysis

Data were analyzed using the SAS 9.2 statistical software package (SAS Institute, Cary, NC, United States). Data were first analyzed descriptively. For quantitative variables, mean, standard deviation, and minimum and maximum values were derived. For qualitative variables, absolute and relative values are given. In order to identify differences between patients with hepatobiliary complications and those without hepatobiliary complications and without intervention, the Wilcoxon rank-sum test was used for quantitative variables; for qualitative variables, the χ^2 test or, when the number of cases was too small, Fisher's exact test was used. All tests were two-sided. Statistical significance was established at $P < 0.05$. The statistical methods of this study were reviewed by Suemeyra Oeztuerk (BSc) from Department of Internal Medicine I, University Hospital Ulm.

RESULTS

Included in the present study were 35 patients (22 male, 13 female), whose average age at the time of intervention was 52.7 years (range: 18-82 years). Age distribution at the time of intervention showed two peaks, at 70-79 years (25.7%) and 40-49 years (22.9%), with fewer interventions in patients over the age of 80 years (5.7%) and under 20 years (2.9%). The time from first diagnosis to the onset of hepatobiliary complications showed high variability (0-41 years), with an average of 3.7 years. In 65%, this period was less than one year (Figure 1).

The most frequent symptom of hepatobiliary complications was jaundice (54.3%) (Table 1). The total number of interventions performed in any individual case ranged from one to ten; more than one method could have been employed in patients undergoing multiple interventions. ERCP was most frequently performed in combination with stent placement (82.9%). In addition to the intervention, 16 patients underwent additional procedures. A stent was placed in 74.3% of patients (n = 26). Twenty-five patients underwent stent exchange at some point in their clinical course, with only one patient not undergoing exchange. The most frequent reason

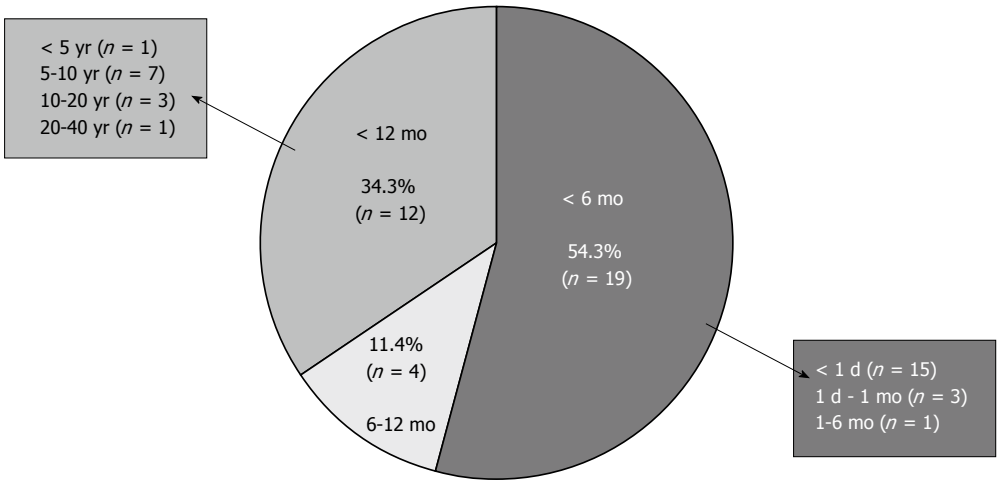

Figure 1 Time from first diagnosis to onset of hepatobiliary complications (average time to onset 3.7 years after first diagnosis).

Table 1 Clinical data of patients with hepatobiliary complications of alveolar echinococcosis

Characteristic	Frequency
Symptoms[1]	
Jaundice	54.3%
Abdominal pain	31.4%
Weight loss	20.0%
Indication for intervention[1]	
Cholestasis	54.3%
Jaundice	25.7%
Stenosis of biliary tract	17.1%
Cholangitis	11.4%
Intervention method[1]	
ERCP + stent replacement	82.9%
PTCD	31.4%
ERCP without stent replacement	22.9%
MRCP	14.3%
Additional procedures[1]	
Papillotomy	68.8%
Dilatation of the bile ducts	50.0%
Gallstone extraction	25.0%
Types of stents	
Plastic stent	30.8%
Pigtail drainage	7.7%
Metal stent	3.9%
Percutaneous drainage	3.9%
Transhepatic jejunal endless drainage	3.9%
No data	50.0%

[1]Multiple indications possible. ERCP: Endoscopic retrograde cholangiop ancreatography; MRCP: Magnetic resonance cholangiopancreatography; PTCD: Percutaneous transhepatic cholangiodrainage.

was cholangitis (*n* = 7), followed by unsuccessful first attempt (*n* = 5), and stent occlusion (*n* = 4).

Of the 35 patients who had undergone a single intervention, 14 (40%) had also been treated surgically, in 12 cases prior to intervention. Procedures included partial resection of the liver (*n* = 7), curative hemihepatectomy (*n* = 2), partial resection of the liver following histological confirmation (*n* = 2), and liver segment resection (*n* = 1). Antihelminthic pharmacotherapy was administered in 23 patients prior to onset of hepatobiliary complications, including albendazole (*n* = 14), mebendazole (*n* = 8), and amphotericin B (*n* = 1). Eleven patients underwent further treatment following the first intervention, including partial liver resection (*n* = 1), curative hemihepatectomy (*n* = 1), and pharmacotherapy with albendazole (*n* = 9).

In the group of patients who had not experienced hepatobiliary complications, 46.3% (149/322) had undergone surgery, whereas 50.9% (164/322) had not. In the remaining 2.8% of patients (*n* = 9), it was not possible on the basis of the available records to retrospectively determine whether or not they had undergone surgery. In the group of patients who had undergone a single intervention, 14/35 (40%) also required surgical treatment, whereas 21/35 (60%) patients did not undergo surgery. There was no significant difference in the rate of surgery between the groups with and without hepatobiliary complications.

Non-resectable AE was diagnosed in 63.0% (225/357) of patients, and hepatobiliary complications were reported in 10.7% (24/225) of these. Patients were considered to have non-resectable AE if they were deemed unsuitable for operation or if curative surgery was unfeasible.

A comparison of biochemical hepatic function parameters at the time of first diagnosis, intervention, and post-intervention in six patients revealed that all parameters increased from the point of first diagnosis to intervention and then declined following intervention. In fact, aspartate transaminase, gamma-glutamyl transpeptidase, alkaline phosphatase, and total bilirubin concentrations fell to levels below those documented at first diagnosis (Table 2).

There was a statistically significant difference in biochemical hepatic function parameters between the group of patients with hepatobiliary complications and subsequent intervention and those who did not experience complications ($P < 0.05$). The same

Table 2 Hepatic function parameters in six patients with alveolar echinococcosis with hepatobiliary complications and intervention

Parameter	First diagnosis	Intervention	Post-intervention
ALT, U/L	109.0 ± 64.3	169.0 ± 87.4	118.5 ± 154.9
AST, U/L	72.7 ± 50.0	108.7 ± 41.9	66.7 ± 50.0
GGT, U/L	301.3 ± 237.3	393.3 ± 242.3	227.3 ± 117.7
AP, U/L	272.3 ± 134.8	300.3 ± 75.5	246.5 ± 95.5
Bilirubin, mmol/L	101.3 ± 164.8	147.1 ± 154.2	83.6 ± 89.8

ALT: Alanine transaminase; AP: Alkaline phosphotase; AST: Aspartate transaminase; GGT: Gamma-glutamyl transpeptidase.

Table 3 Hepatic function parameters in patients with and without hepatobiliary complications of alveolar echinococcosis and subsequent intervention at time of first diagnosis

Parameter	Hepatobiliary complications		*P* value
	No (*n* = 148)	Yes (*n* = 24)	
ALT, U/L	43.2 ± 73.6	59.8 ± 48.7	0.0035
AST, U/L	29.3 ± 35.4	41.4 ± 35.5	0.0431
GGT, U/L	94.4 ± 133.2	186.4 ± 194.1	0.0005
AP, U/L	123.8 ± 88.6	200.3 ± 125.5	0.0016
Bilirubin, mmol/L	9.8 ± 6.2	36.5 ± 86.7	0.0148

ALT: Alanine transaminase; AP: Alkaline phosphotase; AST: Aspartate transaminase; GGT: Gamma-glutamyl transpeptidase.

parameters were significantly higher in patients with hepatobiliary complication compared to the group of patients who had not undergone intervention (Table 3).

Six patients (17.1%) experienced complications during the first intervention (Table 4). Complications occurred in three patients undergoing ERCP and stent placement (8.6%), in two patients undergoing only ERCP (5.7%), and in one patient with PTCD (2.9%). Within the first week after the intervention, an additional 34.4% of patients (*n* = 12) experienced complications.

Antibiotic treatment was administered in ten patients following intervention, with metronidazole and amoxicillin being the two more frequently administered agents (in two patients each).

During follow-up through December 2012, a total of 11 (31.4%) patients died, whereas 23 (65.7%) patients remained alive (Figure 2). The average age at time of death was 75.6 years (range: 18-91 years), whereas the average time from onset of hepatobiliary complications to death was 7.2 years (range: 4 d to 15 years). The average age at the conclusion of the follow-up period of the 23 patients who remained alive was 55.4 years (range: 26-86 years), whereas the average time from onset of hepatobiliary complications to conclusion of follow-up was 9.2 years (range: 3 mo to 34 years). For the entire collective of patients (living and deceased), the average period following onset of hepatobiliary complications was 8.8 years.

Table 4 Type of complications

Complication	Frequency
During first intervention	
Failure visualize bile ducts	66.7%
Bleeding at the papilla of vater	16.7%
Bile leakage	16.7%
Within first week after intervention[1]	
Pancreatitis	41.7%
Cholangitis	33.3%
Hemorrhage	8.3%
Wound infection	8.3%
Fistula formation	8.3%
Bacteremia	8.3%
Abdominal pain	8.3%
Abscess information	8.3%
Pneumoperitoneum	8.3%

[1]Multiple indications possible.

DISCUSSION

To date, only a few studies have investigated the biliary complications of AE. In addition to rare vascular, cerebral, and musculoskeletal complications, AE is associated with hepatobiliary complications, for which the incidence and treatment have been reported in only a few studies with small numbers of patients[13-16,21] and case reports[17-20]. Vascular complications include the Budd-Chiari[25,26] and Vena-cava syndrome[27,28], which are caused by occlusions of the hepatic veins[25] or inferior vena cava[27], respectively. Cerebral involvement is associated with immunosuppression and occurs in 1%-5% of cases[29,30], whereas musculoskeletal manifestation resembles a type of spondylitis when the spinal column is impacted, or an abscess in soft tissue[31,32]. Hepatobiliary complications manifest as cholestatic jaundice, cholangitis, biliary colic, and fever[1,5]. The rate of biliary invasion is given at 11.4%[16]. In our overall collective, hepatobiliary complications occurred in 35 of a total 357 patients. The resulting complication rate of 9.8% is comparable to findings published by Ozturk *et al*[16]. A recent study by Frei *et al*[21] reports a complication rate of 30% in patients with non-resectable AE. In the present study, 24/322 patients with non-resectable AE experienced hepatobiliary complications, corresponding to a rate of 10.7%.

The main symptoms reported by patients in our collective included jaundice, abdominal complaints, and weight loss, which correspond to symptoms reported in other studies[13]. Similarly, the predominant indications of intervention in our study were obstructive jaundice and cholangitis, which agrees with the results of other studies[16,17]. Other indications for intervention reported in the literature, such as biliary fistulae[16], portal vein thrombosis, and esophageal varices[22], were not observed in our collective. On average, hepatobiliary complications occurred 3.7 years following first

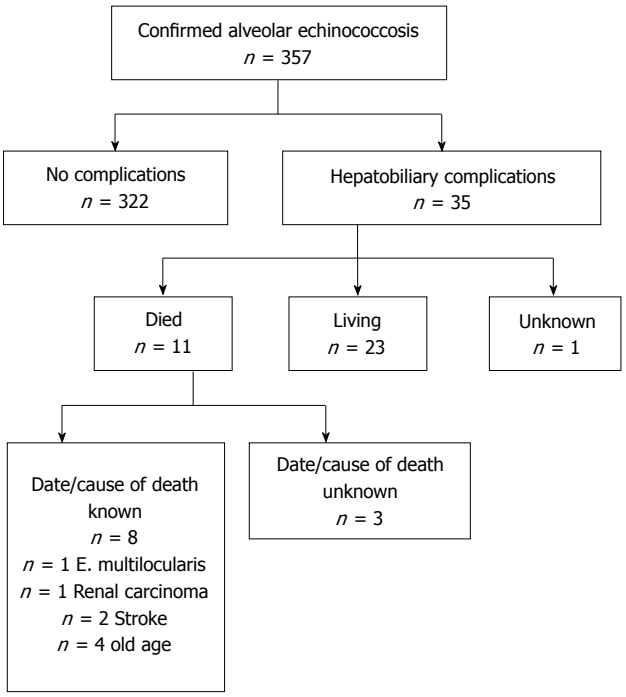

Figure 2 Overview of patients with alveolar echinococcosis through December 2012.

diagnosis. This was more rapid than the average of 15 years reported in the recent study by Frei *et al*[21].

Other data show that only in a few cases (n = 5) did a single interventional procedure suffice in the treatment hepatobiliary complications of AE. This corresponds to experience with other disorders, such as chronic pancreatitis or benign stenosis of the bile duct secondary to iatrogenic injury, in which, on average, two to eight ERCPs are required[32]. With malignant bile duct stenosis, the literature reports not only multiple interventions but, in many cases, also simultaneous double stenting[33,34]. Despite frequent repetition, however, the complication rates associated with interventional procedures is low, reported at 9.8% for ERCP[35] and 7.9% for PTCD[36]. MRCP is generally accomplished without complication[37]. Our rate of complications is comparable to, or lower than, corresponding reports in the literature. Only the subgroup of ten patients in whom symptoms did resolve within one week showed an elevated complication rate of 28.6%; this is most likely due to the complex nature of the clinical situation and the need for stent placement[35]. In order to avoid complications secondary to stent occlusion, stents should be regularly exchanged[32].

As a result of intervention, six patients exhibited improvement in biochemical hepatic function parameters, with concentrations falling to levels lower than those measured at first diagnosis. This improvement is associated with patients' improved clinical conditions[13,16], which is reflected in our study by the fact that, following intervention, only two patients required additional surgery. In our collective, we were not able to identify any difference in the rate of surgery between patients with and without hepatobiliary complications. Our data also confirm that prior liver resection does not represent a risk factor for the development of hepatobiliary complications[21].

To date, only one study by Frei *et al*[21] has investigated survival following onset of hepatobiliary complications. They report a survival of only three years following onset of complications, which, in their series, occurred in about 30% of patients. By contrast, data in the present study show an average survival of 8.8 years following onset of complications. On average, Frei *et al*[21] also report the onset of hepatobiliary complications 15.0 years following first diagnosis of AE compared with the average of 3.7 years in the present study.

The main limitation of this study is its retrospective design. As a result, it was not possible to retrieve more detailed information about the complications occurring in these patients.

The data of the present study allow us to conclude that hepatobiliary complications requiring treatment occur in about 10% of patients with AE. Significant increases in biochemical hepatic function parameters are already present at the time of first diagnosis. On average, hepatobiliary complications occurred 3.7 years following first diagnosis of AE, but this time to occurrence is subject to high variability (0-41 years). Despite the fact that many patients will require multiple treatments, endoscopic interventional methods offer a well-established treatment procedure with a tolerable rate of complications and treatment-associated mortality and an average survival of 8.8 years.

COMMENTS

Background

Despite abundant clinical resources and technical advances, the diagnosis of alveolar echinococcosis in infected individuals, especially among inexperienced clinicians, remains challenging. With delayed diagnosis, therapy often begins in a late stage of the disease, which significantly limits treatment options.

Research frontiers

The liver is the first organ to be affected by larval infestation. Hepatic lesions are localized to the right hepatic lobe in seven of ten cases, whereas in 40%, the liver hilus is additionally affected. Only in two of ten cases are both hepatic lobes infested.

Innovations and breakthroughs

Hepatobiliary complications occur in about 10% of patients. A significant increase in hepatic transaminase concentrations facilitates the diagnosis. Interventional methods represent viable management options.

Applications

Many patients will require multiple treatments, and endoscopic interventional methods offer a well-established treatment procedure with a tolerable rate of complications and treatment-associated mortality and an average survival of 8.8 years.

Peer-review

In this paper, the authors systemically described their own long-term clinical data on alveolar echinococcosis. How to effectively manage post-operative complications was still a challenging problem for the surgeons. This paper was well organized and informative.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic roles of preoperative α-fetoprotein and des-γ-carboxy prothrombin in hepatocellular carcinoma patients

Makoto Meguro, Toru Mizuguchi, Toshihiko Nishidate, Kenji Okita, Masayuki Ishii, Shigenori Ota, Tomomi Ueki, Emi Akizuki, Koichi Hirata

Makoto Meguro, Toru Mizuguchi, Toshihiko Nishidate, Kenji Okita, Masayuki Ishii, Shigenori Ota, Tomomi Ueki, Emi Akizuki, Koichi Hirata, Department of Surgery, Surgical Oncology and Science, Sapporo Medical University School of Medicine, Chuo-ku, Sapporo, Hokkaido 060-8543, Japan

Author contributions: Meguro M and Mizuguchi T contributed equally to this work; Meguro M and Mizuguchi T design this study, analyzed, interpreted and drafted the manuscript; Meguro M and Mizuguchi T, Nishidate T, Okita K, Ishii M, Ota S, Ueki T and Akizuki E acquire the data; Mizuguchi T and Hirata K contributed to statistical advice.

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Ethics approval: The study was reviewed and approved by the institutional review boards of the Sapporo Medical University.

Informed consent: The study design conformed to the ethical guidelines of the Declaration of Helsinki, and all study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Biostatistics: The statistical methods of this study were mainly reviewed by prof. Koichi Hirata from Sapporo Medical University School of Medicine.

Conflict-of-interest: There are no conflict-of-interests for all co-authors.

Data sharing: No additional data are available.

Correspondence to: Makoto Meguro, MD, PhD, Department of Surgery, Surgical Oncology and Science, Sapporo Medical University School of Medicine, South-1, West-16, Chuo-ku, Sapporo, Hokkaido 060-8543, Japan. meguro@sapmed.ac.jp
Telephone: +81-11-6112111
Fax: +81-11-6131678

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Abstract

AIM: To clarify the utility of using des-γ-carboxy prothrombin (DCP) and α-fetoprotein (AFP) levels to predict the prognosis of hepatocellular carcinoma (HCC) in patients with hepatitis B virus (HBV) and the hepatitis C virus (HCV) infections.

METHODS: A total of 205 patients with HCC (105 patients with HBV infection 100 patients with HCV infection) who underwent primary hepatectomy between January 2004 and May 2012 were enrolled retrospectively. Preoperative AFP and DCP levels were used to create interactive dot diagrams to predict recurrence within 2 years after hepatectomy, and cutoff levels were calculated. Patients in the HBV and HCV groups were classified into three groups: a group with low AFP and DCP levels (LL group), a group in which one of the two parameters was high and the other was low (HL group), and a group with high AFP and DCP levels (HH group). Liver function parameters, the postoperative recurrence-free survival rate, and postoperative overall survival were compared between groups. The survival curves were compared by log-rank test using the Kaplan-Meier method. Multivariate analysis using a Cox forward stepwise logistic regression model was conducted for a prognosis.

RESULTS: The preoperative AFP cutoff levels for recurrence within 2 years after hepatectomy in the HBV and HCV groups were 529.8 ng/mL and 60 mAU/mL,

respectively; for preoperative DCP levels, the cutoff levels were 21.0 ng/mL in the HBV group and 67 mAU/mL in the HCV group. The HBV group was significantly different from the other groups in terms of vascular invasion, major hepatectomy, volume of intraoperative blood loss, and surgical duration. Significant differences were found between the LL group, the HL group, and the HH group in terms of both mean disease-free survival time (MDFST) and mean overall survival time (MOST): 64.81 ± 7.47 *vs* 36.63 ± 7.62 *vs* 18.98 ± 6.17 mo (P = 0.001) and 85.30 ± 6.55 *vs* 59.44 ± 7.87 *vs* 46.57 ± 11.20 mo (P = 0.018). In contrast, the HCV group exhibited a significant difference in tumor size, vascular invasion, volume of intraoperative blood loss, and surgical duration; however, no significant difference was observed between the three groups in liver function parameters except for albumin levels. In the LL group, the HL group, and the HH group, the MDFST was 50.09 ± 5.90, 31.01 ± 7.21, and 14.81 ± 3.08 mo (log-rank test, P < 0.001), respectively, and the MOST was 79.45 ± 8.30, 58.82 ± 7.56, and 32.87 ± 6.31 mo (log-rank test, P < 0.001), respectively.

CONCLUSION: In the HBV group, the prognosis was poor when either AFP or DCP levels were high. In the HCV group, the prognosis was good when either or both levels were low; however, the prognosis was poor when both levels were high. High levels of both AFP and DCP were an independent risk factor associated with tumor recurrence in the HBV and HCV groups. The relationship between tumor marker levels and prognosis was characteristic to the type of viral hepatitis.

Key words: Hepatocellular carcinoma; Hepatitis B; Hepatitis C; Des-γ-carboxy prothrombin; α-fetoprotein

Core tip: There is no consensus regarding using cutoff levels of tumor markers to predict survival and recurrence after hepatectomy for hepatocellular carcinoma. Furthermore, the prognostic characteristics of these tumor markers according to hepatitis type remain unclear. The α-fetoprotein (AFP) cutoff level for recurrence within 2 years after surgery was 21.0 ng/mL in the hepatitis C virus (HCV) group compared with 529.8 ng/mL in the hepatitis B virus (HBV) group. Furthermore, patients in the HBV group with high levels of either AFP or des-γ-carboxy prothrombin (DCP) had poor prognoses, as did those patients with high levels of both tumor markers. In contrast, only those patients in the HCV group who had high levels of both AFP and DCP had poor prognoses. We believe that to predict prognosis, preoperative levels of tumor markers should be distinguished and assessed according to the type of viral hepatitis.

Meguro M, Mizuguchi T, Nishidate T, Okita K, Ishii M, Ota S, Ueki T, Akizuki E, Hirata K. Prognostic roles of preoperative α-fetoprotein and des-γ-carboxy prothrombin in hepatocellular carcinoma patients. *World J Gastroenterol* 2015; 21(16): 4933-4945 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4933.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4933

INTRODUCTION

Chronic hepatitis caused by viral hepatitis often progresses to cirrhosis and hepatocellular carcinoma (HCC)[1]. In Asia, HCC is mainly caused by infection with the hepatitis B virus (HBV), whereas in western countries, it is characteristically caused by infection with the hepatitis C virus (HCV)[2]. The oncogenic mechanisms differ between the two virus types[3,4], and these mechanisms should be taken into consideration when evaluating prognosis and establishing treatment regimens.

Various staging systems have been developed to predict the survival for HCC patients, such as the tumor-node-metastasis[5], Okuda *et al*[6], the Cancer of the Liver Italian Program (CLIP)[7], Japan Integrated Staging (JIS)[8], and the Barcelona Clinic Liver Cancer[9] staging systems. These systems classify tumors according to tumor size, tumor number, vascular invasion, and metastatic regions (regardless of whether they are intrahepatic or extrahepatic metastases). Each of these parameters is closely associated with the overall prognosis of HCC patients[5-9].

Liver function parameters are also important prognostic factors for HCC. In fact, the Okuda[6], CLIP[7], and JIS[8] staging systems consider both tumor extension and liver function parameters in tumor classification. Accordingly, it has been reported that tumor-related factors and liver function parameters are also both closely associated with the prognosis of patients with HCC[6-10].

In addition to these factors that impact tumorigenesis and liver function, α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) levels are tumor markers and known prognostic factors for HCC[11,12]. Discovered by Abelev *et al*[13] in 1963, AFP is a glycoprotein with an albumin-like structure produced by liver cells and in the yolk sac during the fetal stage with a half-life of 4-6 d and a molecular weight of 65 kDa. AFP production in the liver is increased in hepatocellular carcinoma as well as in chronic hepatitis and cirrhosis[14]; therefore, AFP is considered to have low specificity for the diagnosis of cancer. In contrast, prothrombin is formed after the γ-carboxylation of vitamin K-dependent propeptides, and DCP is produced as a result of an acquired posttranslational defect in the vitamin K-dependent carboxylase system. According to a 1984 report by Liebman *et al*[15], DCP has a molecular weight of 72 kDa and a half-life of 40-72 h. DCP production does not increase in chronic hepatitis or cirrhosis, and DCP is considered to have high specificity for the diagnosis of cancer. However, DCP has no prognostic value in cases with vitamin K deficiency or vitamin K

function inhibition.

Although the associations between these tumor markers and postoperative prognosis in HCC patients have been reported[16-18], there is no consensus regarding the cutoff levels of these markers to predict survival and recurrence after hepatectomy. Furthermore, the prognostic characteristics of these tumor markers according to hepatitis type remain unclear. Therefore, the aim of the present study was both to compare preoperative tumor marker levels and prognosis after hepatectomy and to clarify the characteristics of these tumor marker levels according to hepatitis type. The prognostic findings associated with these tumor marker levels could inform the development of new selection criteria for living donor liver transplantation candidates with HCC, especially beyond the Milan criteria[19].

MATERIALS AND METHODS

Patients and follow-up

We retrospectively reviewed the medical records of 256 consecutive HCC patients who underwent primary hepatectomy at the Sapporo Medical University Hospital (Sapporo, Japan) from January 2004 to May 2012. Using the Child-Pugh classification system[20], the volume of resectable liver was determined, including the following items: prothrombin time (PT), serum total bilirubin (TBIL) levels, and serum albumin (ALB) levels, all of which are measured on both preoperative function tests and the indocyanine green retention rate at 15 min ($ICGR_{15}$)[21] as measured by three-dimensional computed tomography. AFP and DCP levels were measured immediately before surgery. The present study excluded 40 patients with non-B/non-C hepatitis and six patients with concurrent HBV and HCV infections. In addition, two patients in the HBV group and three in the HCV group receiving warfarin were also excluded. The final sample included 105 patients with HBV infection and 100 with HCV infection (Figure 1).

The operation type was classified as follows: partial hepatic resection including tumor enucleation (Hr0); subsegmentectomy (HrS); monosegmentectomy (Hr1); bisegmentectomy, including right hepatectomy, left hepatectomy, and central bisegmentectomy (Hr2); and trisegmentectomy (Hr3). Hr0, HrS, and Hr1 were defined as minor hepatectomy, whereas Hr2 and Hr3 resections were considered major hepatectomy. Red cell concentrate transfusion was administered at the discretion of the anesthetist on the basis of intraoperative factors, such as systemic hemodynamic factors, hemoglobin levels, and blood lactate levels on blood gas analysis. Following hepatectomy, the number of tumors, tumor size, presence or absence of vascular invasion, and condition of underlying liver tissue (normal liver, chronic hepatitis, and liver cirrhosis) were determined.

All patients were followed up every 3 mo until the end of March 2013, until their last visit to our hospital, or until death. The study design conformed to the ethical guidelines of the Declaration of Helsinki, and informed consent was obtained from each subject before registration.

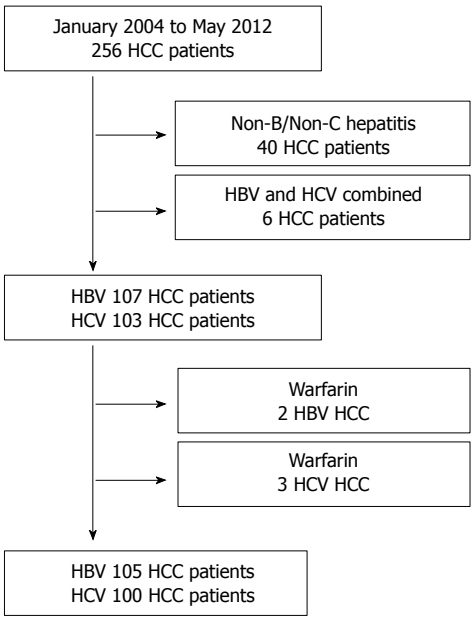

Figure 1 Flowchart of selection of hepatocellular carcinoma patients in this retrospective study. A total of 40 patients with non-B/non-C hepatitis, including those with obstructive jaundice with false-positive preoperative DCP levels, and patients with alcoholic hepatitis were excluded from the study. Six patients with combined HBV and HCV infections were also excluded. Finally, 107 HCC patients with HBV infection and 103 HCC patients with HCV infection were included for analysis. Two patients in the HBV group and three in the HCV group were receiving warfarin and subsequently excluded. Therefore, 105 patients in the HBV group and 100 patients in the HCV group were included in this retrospective study. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; DCP: Des-γ-carboxy prothrombin.

Surgical procedure

The surgery included total and pure laparoscopic procedures and laparoscopically assisted approaches. We used either five or six ports (5-12 mm in diameter) depending on the tumor location, and the first periumbilical port for the laparoscopic camera was inserted using the open technique. If the volume of blood loss exceeded 300 mL from any of these ports, the Pringle maneuver[22] was performed for hepatectomy. The procedures were performed under carbon dioxide pneumoperitoneum, and intra-abdominal pressure was maintained at < 12 mmHg based on electronic readings. We used a variable view angle, high-definition endoscopic camera. Intraoperative ultrasonography (BK Medical, Herlev, Denmark) was performed routinely to examine the location and diameter of the hepatic tumor as well as the positional relationship of the tumor with the main hepatic vessels. Parenchymal transection and hemostasis were performed with a laparoscopic Cavitron ultrasonic surgical aspirator (CUSA; Valleylab, Boulder, CO, United States), a

Table 1 Characteristics of hepatocellular carcinoma patients with hepatitis B virus infection

Variables	All patients (n = 105)	LL group (n = 55)	HL group (n = 34)	HH group (n = 16)	P value
Age (yr)	62 (57-68)	62 (59-66)	64 (56-70)	63 (56-70)	0.962
Sex, male/female	90/15	48/7	29/5	13/3	0.829
Body mass index (kg/m^2)	23.7 ± 3.1	24.0 ± 2.6	23.5 ± 3.3	23.1 ± 4.3	0.691
Open/pure lap/lap-assisted	83/8/14	43/6/6	29/1/4	11/1/4	0.390
Hr					
0/S/1/2/3	50/25/14/14/2	34/13/6/2/0	14/10/4/4/2	2/2/4/8/0	< 0.001
Preoperative laboratory values					
Aspartate transaminase (U/L)	31 (22-46)	31 (22-41)	30 (25-48)	43 (24-62)	0.153
Alanine transaminase (U/L)	29 (20-39)	32 (17-42)	27 (21-42)	27 (19-52)	0.978
Platelets (× 10^4/μL)	16.6 ± 13.3	16.4 ± 12.7	16.2 ± 16.2	18.0 ± 8.6	0.168
Serum ALB (g/dL)	3.95 ± 0.46	3.99 ± 0.46	3.87 ± 0.43	3.98 ± 0.53	0.334
PT (%)	93.3 ± 12.2	95.5 ± 11.1	90.3 ± 14.1	92.2 ± 10.6	0.062
Serum TBIL (mg/dL)	0.79 ± 0.44	0.80 ± 0.45	0.85 ± 0.49	0.65 ± 0.24	0.681
ICGR15	9.6 (5.7-14.9)	9.4 (5.9-14.0)	12.5 (5.7-22.5)	8.5 (5.0-14.6)	0.334
AFP (ng/mL)	24.2 (3.8-165.0)	5.2 (2.4-31.1)	45.0 (5.1-246.5)	1950 (753.5-2827.0)	< 0.001
DCP (mAU/mL)	38 (20-364)	22 (15-28)	399 (90-1,667)	4460 (223-34373)	< 0.001
Intraoperative data					
Blood loss (mL)	400 (130-660)	360 (110-560)	435 (100-1,040)	880 (130-1330)	0.042
Surgical duration (min)	332 (243-411)	313 (236-370)	361 (243-450)	432 (270-604)	0.020
Blood transfusion					
RCC: yes/no (%)	21/84 (20.0%)	4/51 (7.2%)	10/24 (29.4%)	7/9 (43.8%)	0.001
Pathological results					
Tumor size (cm)	4.27 ± 3.21	3.26 ± 2.56	4.97 ± 2.71	6.24 ± 4.77	< 0.001
Multiple tumors: yes/no (%)	39/66 (37.1%)	19/36 (34.5%)	15/19 (44.1%)	5/11 (31.3%)	0.575
Vascular invasion: yes/no (%)	34/71 (32.4%)	10/45 (18.2%)	14/20 (41.1%)	10/6 (62.5%)	0.002
Remnant liver: NL/CH/LC	13/47/41	6/23/26	3/17/14	4/7/5	0.464
Histological tumor differentiation					
Well/moderately/poorly	14/63/28	11/35/9	3/18/13	0/10/6	0.048
Recurrence within 2 yr after liver resection					
Yes/no (%)	50/55 (47.6%)				

Data are presented as medians (25th-75th percentile range) for skewed distribution and mean ± SD for normal distribution. LL group: AFP values < 529.8 ng/mL and DCP values < 60 mAU/mL; HL group: AFP values > 529.8 ng/mL or DCP values > 60 mAU/mL; HH group: AFP values > 529.8 ng/mL and DCP values > 60 mAU/mL; Open group: Open laparotomy hepatectomy group; Lap group: laparoscopic hepatectomy group; Pure Lap: Pure laparoscopic hepatectomy; Lap-assisted: Laparoscopy-assisted hepatectomy. Hr0: Partial hepatectomy; HrS: Subsegmentectomy; Hr1: Sectionectomy; Hr2: Bisegmentectomy; Hr3: Trisegmentectomy; ALB: Albumin; PT: Prothrombin; TBIL: Total bilirubin; ICGR15: Indocyanine green retention rate at 15 min; AFP: Alpha-fetoprotein; DCP: Des-γ-carboxy prothrombin.

harmonic scalpel (UltraCision; Ethicon Endo-Surgery, Inc., Blue Ash, OH, United States), saline-associated monopolar electrocautery, and a thermofusion device (BiClamp; ERBE, Marietta, GA, United States). The resected specimen was placed in a plastic bag and extracted through a slightly enlarged periumbilical port site or additional minilaparotomy. For open laparotomy, right subcostal, upper middle, or inverted L-shaped or T-shaped incisions were made depending on tumor location. Intraoperative ultrasonography (Hitachi-Aloka Medical, Ltd., Tokyo, Japan) was performed routinely. Parenchymal transection and hemostasis were performed primarily using a CUSA, saline-associated monopolar electrocautery, and an absorbable fibrin sealant patch (Tachosil, Baxter Healthcare Corporation, Irvine, CA, United States) as necessary.

Statistical analysis

Data are presented as medians (25th-75th percentile range) for skewed distributions and as mean ± SD for normal distributions. The Pearson χ^2 analysis or Fisher exact test was used to compare categorical variables, whereas the Kruskal-Wallis, Mann-Whitney U test, or analysis of variance was used for comparisons of continuous variables. Recurrence-free survival or overall survival rates were estimated using the Kaplan-Meier method and compared using the log-rank test. A P value < 0.05 was considered statistically significant. Variables with statistical significance (P < 0.05) in the univariate analysis were subjected to multivariate analysis using a Cox forward stepwise logistic regression model. Statistical analysis was performed using StatView® software (version 5.0; SAS Institute Inc., Cary, NC, United States) and SPSS version 21.0 for Windows (IBM-SPSS Inc., Chicago, IL, United States). Interactive dot diagrams were created using MedCalc® software (version 10.2.0.0; Mariakerke, Ostend, Belgium).

RESULTS

HBV group

The clinical characteristics of all patients in the HBV group (n = 105) are shown in Table 1. The median age was 62 years, and there were more males than females. Major hepatectomy was performed in 16 patients (15.2%), and anatomical resection was

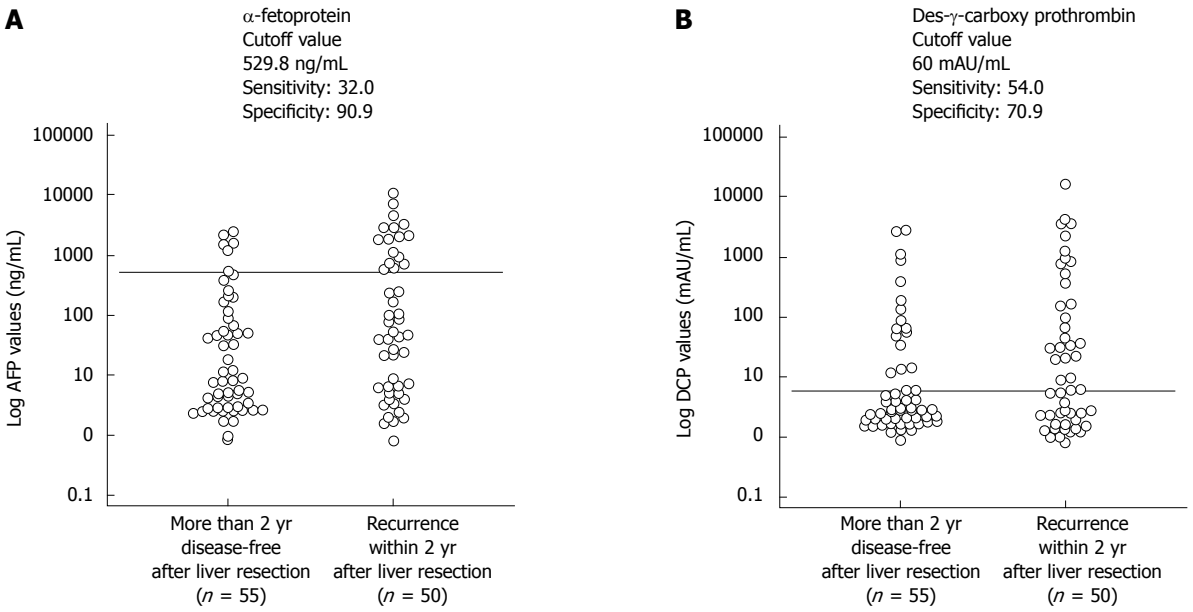

Figure 2 Interactive dot diagrams. A: Interactive dot diagrams showing α-fetoprotein levels were used to predict recurrence within 2 years after hepatectomy among hepatocellular carcinoma patients with hepatitis B infection; B: Interactive dot diagrams showing des-γ-carboxy prothrombin levels were used to predict recurrence within 2 years after hepatectomy among hepatocellular carcinoma patients with hepatitis B infection. The *horizontal line* indicates the cutoff point with the best separation (minimal false-negative and false-positive results) between the two subgroups.

performed in 55 patients (52.4%). Preoperative liver function was good in most patients, with a median $ICGR_{15}$ level of 9.6%. The median preoperative levels of AFP and DCP were 24.2 ng/mL and 38 mAU/mL, respectively. The median volume of intraoperative blood loss was 400 mL and median surgical duration was 332 min. The mean tumor size was 4.27 ± 3.21 cm; 39 patients (37.1%) had multiple tumors, and vascular invasion was observed in 34 patients (32.4%). Sixty patients (61.0%) presented with either normal liver function or chronic hepatitis. Fifty patients (47.6%) developed recurrence within 2 years after surgery.

The preoperative AFP and DCP cutoff levels for recurrence within 2 years of hepatectomy calculated using an interactive dot diagram were 529.8 ng/mL (sensitivity, 32.0%; specificity, 90.0%) and 60 mAU/mL (sensitivity, 54.0%; specificity, 70.9%), respectively (Figure 2). Table 1 depicts the characteristics of patients in three groups when these cutoff levels were used as the reference levels: a group with low AFP and DCP levels (LL group, n = 55), a group in which one of the two parameters was high and the other was low [high and low (HL) group, n = 34], and a group with high AFP and DCP levels (HH group, n = 16). The rates of anatomical resection in the LL, HL, and HH groups were significantly different (38.2%, 58.8%, and 87.5%; P = 0.002). There were no significant differences observed between groups in any preoperative liver function parameter, including aspartate transaminase levels, alanine transaminase levels, platelet count, serum ALB, serum TBIL, PT, and $ICGR_{15}$ levels. However, there were significant differences in AFP levels (P < 0.001), DCP levels (P < 0.001), volume of intraoperative blood loss (P = 0.042), surgical duration (P = 0.020), rate of intraoperative transfusion (P = 0.001), tumor size (P < 0.001), vascular invasion (P = 0.002), and histological tumor differentiation (P = 0.048). The mean disease-free survival time (MDFST) in the LL, HL, and HH groups was 64.81 ± 7.47, 36.63 ± 7.62, and 18.98 ± 6.17 mo, respectively (log-rank test, P = 0.001); the mean overall survival time (MOST) in the LL, HL, and HH groups was 85.30 ± 6.55, 59.44 ± 7.87, and 46.57 ± 11.20 mo, respectively (log-rank test, P = 0.018). Significant differences were observed between all groups on both measures (Figure 3).

Our univariate and multivariate analyses to determine the risk factors associated with tumor recurrence after hepatectomy in the patients with HBV infection (n = 105) are shown in Table 2. The multivariate analysis revealed that high levels of TBIL (P = 0.004), HH group (P = 0.031), large tumor size (P = 0.003), and the presence of vascular invasion (P = 0.002) were associated with significantly higher incidences of tumor recurrence after liver resection.

Our univariate and multivariate analyses to determine the risk factors associated with poor overall survival after hepatectomy in patients with HBV infection (n = 105) are shown in Table 3. The multivariate analysis revealed that the presence of vascular invasion (P = 0.008) was associated with significantly higher incidences of poor overall survival after liver resection.

HCV group

The clinical characteristics of all patients in the HCV group (n = 100) are shown in Table 4. The median

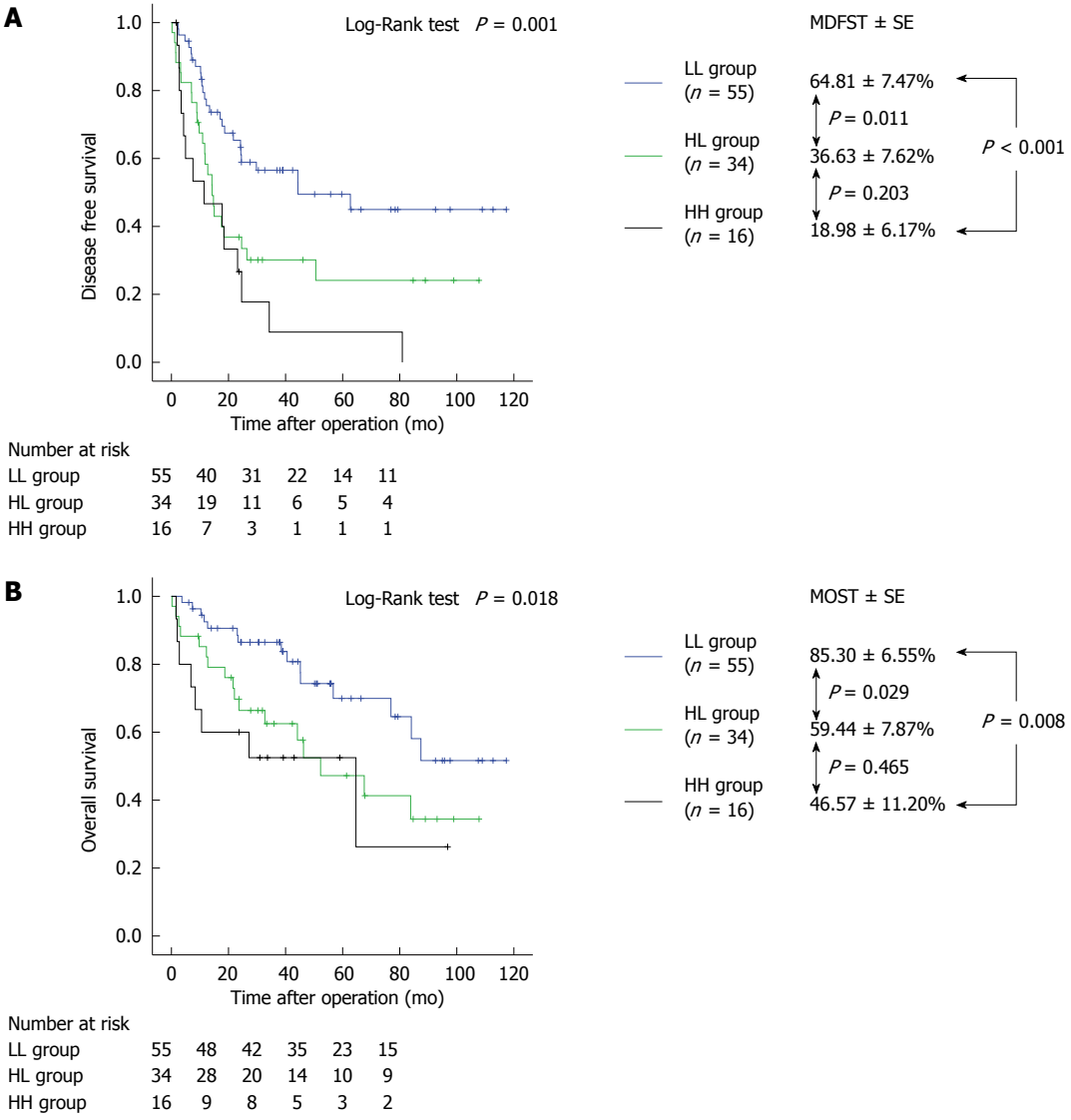

Figure 3 Disease-free survival and overall survival of hepatocellular carcinoma patients with hepatitis B viral infection. A: In the disease-free survival analysis, there were significant differences between the three groups; B: In the analysis of overall survival, there were significant differences between the three groups.

age was 71 years, and there were more males than females. Major hepatectomy of type Hr2 or higher was performed in five patients (5.0%), and anatomical resection combining subsegmentectomy of the liver, segmentectomy of the liver, and hepatic lobe resection was performed in 43 patients (43.0%). Many patients had poor preoperative liver function, and 58 patients (58.0%) had liver cirrhosis with a median $ICGR_{15}$ level of 14.2%. The median preoperative levels of AFP and DCP were 18.6 ng/mL and 45 mAU/mL, respectively. The median volume of intraoperative blood loss was 330 mL, and the median surgical duration was 304 min. The mean tumor size was 3.51 ± 2.48 cm, and multiple tumors were observed in 36 patients (36.0%). Furthermore, vascular invasion was observed in 30 patients (30.0%), and 51 patients (51.0%) developed recurrence within 2 years after hepatectomy.

The preoperative cutoff levels for recurrence within 2 years after hepatectomy, as calculated using an interactive dot diagram, were 21.0 ng/mL for AFP (sensitivity, 58.8%; specificity, 67.3%) and 67 mAU/mL for DCP (sensitivity, 60.8%; specificity, 75.5%) (Figure 4). Table 4 summarizes the characteristics of HCC patients with HCV infection in the LL group (n = 34), HL group (n = 39), and HH group (n = 27) with these cutoff levels as baselines. There were no significant differences observed in pathological background (P = 0.161), type of operation (P = 0.784), or histological differentiation (P = 0.213). There were significant differences in preoperative ALB levels (P = 0.006); however, no significant difference was observed for other laboratory variables. There were also significant differences between groups in AFP levels (P < 0.001), DCP levels (P < 0.001), volume of intraoperative blood loss (P = 0.006), surgical duration (P = 0.040), intraoperative transfusion rate (P = 0.030), tumor size (P = 0.008), and vascular invasion (P = 0.018). MDFST and MOST for the LL, HL, and HH

Table 2 Univariate and multivariate analyses of factors associated with tumor recurrence in patients with hepatitis B virus infection

Variables	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age	0.977	0.952-1.003	0.086			
Sex, male	1.077	0.512-2.264	0.843			
Preoperative laboratory values						
Aspartate transaminase	1.008	1.003-1.013	0.001[1]	1.001	0.993-1.010	0.778
Alanine transaminase	0.999	0.989-1.009	0.811			
Platelets	1.001	0.979-1.024	0.929			
Serum ALB	0.648	0.367-1.142	0.134			
PT	0.974	0.953-0.996	0.021[1]	0.976	0.946-1.007	0.124
Serum TBIL	2.305	1.346-3.947	0.002[1]	4.068	1.586-10.436	0.004[1]
ICGR15	1.033	1.008-1.059	0.011[1]	0.967	0.919-1.018	0.967
Tumor markers						
HH group	3.235	1.673-6.257	< 0.001[1]	2.464	1.086-5.587	0.031[1]
HL group	2.042	1.164-3.583	0.013[1]	1.083	0.497-2.360	0.841
Intraoperative data						
Blood loss	1.001	1.000-1.001	0.002[1]	1.000	1.000-1.001	0.416
Surgical duration	1.002	1.000-1.004	0.003[1]	1.000	0.997-1.003	0.868
Pathologic results						
Tumor size	1.148	1.068-1.234	< 0.001[1]	1.182	1.059-1.318	0.003[1]
Multiple tumors	1.363	1.152-1.612	< 0.001[1]	1.336	0.955-1.869	0.091
Vascular invasion	2.088	1.261-3.458	0.004[1]	2.624	1.408-4.892	0.002[1]

[1]Represents statistical significance. HH group: Alpha-fetoprotein values > 529.8 ng/mL and des-gamma-carboxy prothrombin values > 60 mAU/mL; HL group: Alpha-fetoprotein values > 529.8 ng/mL or des-gamma-carboxy prothrombin values > 60 mAU/mL. ALB: Albumin; PT: Prothrombin; TBIL: Total bilirubin; ICGR15: Indocyanine green retention rate at 15 min.

Table 3 Univariate and multivariate analyses of factors associated with poor overall survival in patients with hepatitis B virus infection

Variables	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age	0.996	0.964-1.028	0.797			
Sex, male	1.440	0.511-4.056	0.490			
Preoperative laboratory values						
Aspartate transaminase	1.008	1.002-1.014	0.012[1]	1.004	0.992-1.016	0.518
Alanine transaminase	0.992	0.976-1.007	0.296			
Platelets	1.015	0.994-1.035	0.158			
Serum ALB	0.469	0.234-0.939	0.033			
PT	0.975	0.950-1.000	0.051			
Serum TBIL	2.249	1.125-4.495	0.022[1]	1.584	0.447-5.613	0.476
ICGR15	1.056	1.025-1.087	< 0.001[1]	1.040	0.976-1.108	0.229
Tumor markers						
HH group	2.985	1.259-7.075	0.013[1]	2.274	0.698-7.403	0.173
HL group	2.112	1.054-4.232	0.035[1]	1.025	0.390-2.693	0.960
Intraoperative data						
Blood loss	1.001	1.001-1.001	< 0.001[1]	1.000	1.000-1.001	0.391
Surgical duration	1.004	1.001-1.008	0.005[1]	1.002	0.996-1.007	0.534
Pathologic results						
Tumor size	1.239	1.133-1.356	< 0.001[1]	1.127	0.969-1.310	0.122
Multiple tumors	1.385	1.121-1.711	0.002[1]	1.200	0.813-1.772	0.358
Vascular invasion	2.491	1.335-4.647	0.004[1]	3.173	1.352-7.447	0.008[1]

[1]Represents statistical significance. HH group: Alpha-fetoprotein values > 529.8 ng/mL and des-gamma-carboxy prothrombin values > 60 mAU/mL; HL group: Alpha-fetoprotein values > 529.8 ng/mL or des-gamma-carboxy prothrombin values > 60 mAU/mL. ALB: Albumin; PT: Prothrombin; TBIL: Total bilirubin; ICGR15: Indocyanine green retention rate at 15 min.

groups were 50.09 ± 5.90, 31.01 ± 7.21, and 14.81 ± 3.08 mo (log-rank test, $P < 0.001$), respectively, and 79.45 ± 8.30, 58.82 ± 7.56, and 32.87 ± 6.31 mo (log-rank test, $P < 0.001$), respectively, with a significant difference observed between all groups (Figure 5).

Our univariate and multivariate analyses to determine the risk factors associated with tumor recurrence after hepatectomy in the patients with HCV infection ($n = 100$) are shown in Table 5. The multivariate analysis revealed that high levels of ICGR15 ($P < 0.001$), HH group ($P < 0.001$), HL group

Table 4 Characteristics of hepatocellular carcinoma patients with hepatitis C virus infection

Variables	All patients (n = 100)	LL group (n = 34)	HL group (n = 39)	HH group (n = 27)	P value
Age (yr)	71 (62-77)	72 (64-77)	70 (62-75)	70 (65-77)	0.766
Sex, male/female	82/18	22/12	29/10	16/11	0.413
Body mass index	23.6 ± 3.6	23.6 ± 2.9	23.8 ± 3.8	23.4 ± 4.1	0.737
Open/Pure Lap/Lap-assisted	77/12/11	26/3/5	27/8/4	24/1/2	0.218
Hr					
0/S/1/2/3	57/27/11/2/3	23/8/3/0/0	21/10/5/1/2	13/9/3/1/1	0.784
Preoperative laboratory values					
Aspartate transaminase (U/L)	47 (30-64)	41 (23-56)	48 (35-67)	53 (47-71)	0.101
Alanine transaminase (U/L)	38 (26-57)	33 (21-53)	41 (28-57)	42 (29-62)	0.496
Platelets ($\times 10^4/\mu L$)	13.5 ± 6.8	13.9 ± 4.7	13.9 ± 9.2	12.5 ± 4.4	0.804
Serum ALB (g/dL)	3.76 ± 0.39	3.94 ± 0.40	3.67 ± 0.35	3.67 ± 0.38	0.006[1]
PT (%)	89.8 ± 13.4	92.5 ± 11.4	87.8 ± 15.0	89.2 ± 13.5	0.254
Serum TBIL (mg/dL)	0.78 ± 0.34	0.74 ± 0.32	0.79 ± 0.33	0.81 ± 0.39	0.777
ICGR15	14.2 (8.4-18.5)	12.1 (8.1-15.4)	14.7 (8.4-21.5)	15.6 (13.0-24.6)	0.163
AFP (ng/mL)	18.6 (5.6-134.0)	5.3 (3.8-11.0)	21.1 (6.9-99.2)	299.7 (68.1-1046.0)	< 0.001[1]
DCP (mAU/mL)	45 (21-244)	22 (15-35)	37 (23-134)	429 (124-1902)	< 0.001[1]
Intraoperative data					
Blood loss (mL)	330 (20-650)	263 (70-500)	230 (20-500)	535 (300-1270)	0.006[1]
Surgical duration (min)	304 (230-377)	303 (175-377)	296 (230-352)	345 (282-475)	0.040[1]
Blood transfusion					
RCC: yes/no (%)	12/88 (12.0%)	3/31 (8.8%)	2/37 (5.1%)	7/20 (25.9%)	0.030[1]
Pathologic results					
Tumor size (cm)	3.51 ± 2.48	2.84 ± 1.23	3.26 ± 2.31	4.72 ± 3.39	0.008[1]
Multiple tumor: yes/no (%)	36/64 (36.0%)	8/26 (23.5%)	17/22 (43.6%)	11/16 (40.7%)	0.171
Vascular invasion: yes/no (%)	30/70 (30.0%)	5/29 (14.7%)	12/27 (30.8%)	13/14 (48.1%)	0.018[1]
Remnant liver: NL/CH/LC	5/37/58	2/18/14	2027/10/2	2017/9/1	0.161
Histological tumor differentiation					
Well/Moderately/Poorly	17/63/20	7/23/4	8/24/7	2/16/9	0.213
Recurrence within 2 yr after hepatectomy					
Yes/no (%)	51/49 (51.0%)				

[1]Represents statistically significance. Data are presented as medians (25^{th}-75^{th} percentile range) for skewed distribution and mean ± SD for normal distribution. LL group: AFP values < 21.0 ng/mL and DCP values < 67 mAU/mL; HL group: AFP values < 21.0 ng/mL or DCP values < 67 mAU/mLF; HH group: AFP values < 21.0 ng/mL and DCP values < 67 mAU/mL; Open group: Open laparotomy group; Lap group: Laparoscopic hepatectomy group; Pure Lap: Pure laparoscopic hepatectomy; Lap-assisted, laparoscopy-assisted hepatectomy; Hr0: Partial hepatectomy; HrS: Sub-segmentectomy; Hr1: Sectionectomy; Hr2: Bisegmentectomy; Hr3: Trisegmentectomy. ALB: albumin; PT: prothrombin; TBIL: total bilirubin; ICGR15: indocyanine green retention rate at 15 min; AFP: Alpha-fetoprotein; DCP: Des-γ-carboxy prothrombin.

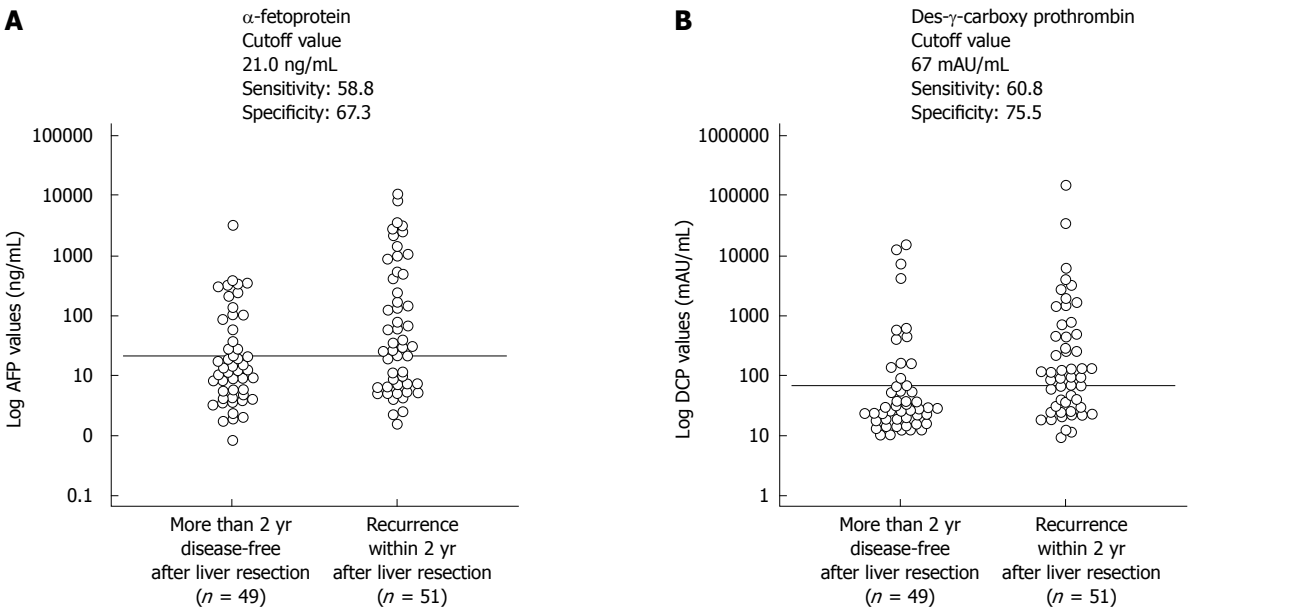

Figure 4 Interactive dot diagrams. A: Interactive dot diagrams showing α-fetoprotein levels were used to predict recurrence within 2 years after hepatectomy among hepatocellular carcinoma patients with hepatitis C infection; B: Interactive dot diagrams showing des-γ-carboxy prothrombin levels were used to predict recurrence within 2 years after hepatectomy among hepatocellular carcinoma patients with hepatitis C infection. The horizontal line indicates the cutoff point with the best separation (minimal false-negative and false-positive results) between the two subgroups.

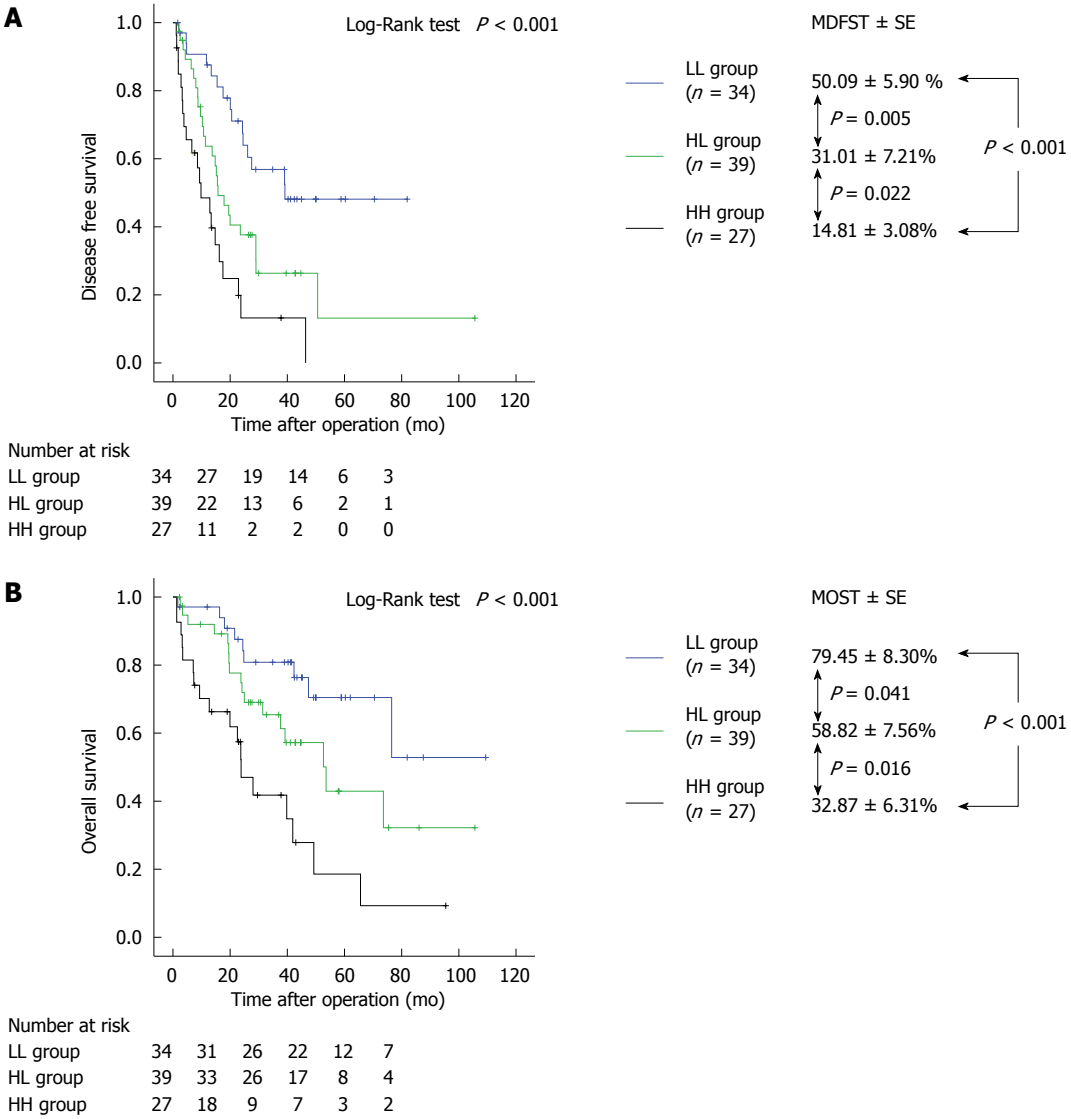

Figure 5 Disease-free survival and overall survival in hepatocellular carcinoma patients with hepatitis C viral infection. A: In the disease-free survival analysis, there were significant differences between the three groups; B: In the analysis of overall survival, there were significant differences between the three groups.

(P = 0.032), long surgical duration (P = 0.031), large tumor size (P = 0.012), and the presence of multiple tumors (P = 0.018) were associated with significantly higher incidences of tumor recurrence after liver resection.

Our univariate and multivariate analyses to determine the risk factors associated with poor overall survival after hepatectomy in patients with HCV infection (n = 100) are shown in Table 6. The multivariate analysis revealed that high levels of $ICGR_{15}$ (P = 0.047), HH group (P = 0.009), and the presence of multiple tumors (P = 0.028) were associated with significantly higher incidences of poor overall survival after liver resection.

DISCUSSION

We investigated the clinical correlations between prognosis in HCC patients who underwent initial hepatectomy and levels of the tumor markers AFP and DCP. There was a significant difference between the HBV and HCV groups in the AFP and DCP cutoff levels to predict recurrence within 2 years after hepatectomy. In the HBV group, high AFP or DCP levels were sufficient to predict poor prognosis. In contrast, a low level of either or both tumor markers was correlated with a good prognosis in the HCV group. However, when levels of both markers were high, prognosis was poor in both the HBV and HCV groups. Furthermore, HH group membership was an independent risk factor associated with tumor recurrence in both the HBV and HCV group.

It has been reported that among HCC patients who acquired HBV infection at a relatively young age (approximately 60 years), many do not have liver cirrhosis and have normal liver function[23-25]. In the HBV group in the present study (Table 1), the median patient age was 62 years, the median $ICGR_{15}$ was

Table 5 Univariate and multivariate analyses of factors associated with tumor recurrence in patients with hepatitis C virus infection

Variables	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age	1.001	0.972-1.031	0.950			
Sex, male	1.455	0.823-2.570	0.197			
Preoperative laboratory values						
Aspartate transaminase	1.005	0.999-1.011	0.134			
Alanine transaminase	1.003	0.966-1.010	0.412			
Platelets	1.015	0.983-1.049	0.363			
Serum ALB	0.569	0.297-1.092	0.090			
PT	0.999	0.980-1.019	0.943			
Serum TBIL	1.281	0.610-2.692	0.513			
ICGR15	1.049	1.017-1.081	0.002[1]	1.069	1.032-1.108	< 0.001[1]
Tumor markers						
HH group	4.427	2.238-8.760	< 0.001[1]	5.098	2.165-12.005	< 0.001[1]
HL group	2.210	1.166-4.189	0.015[1]	2.325	1.077-5.018	0.032[1]
Intraoperative data						
Blood loss	1.001	1.000-1.001	< 0.001[1]	1.000	0.999-1.000	0.292
Surgical duration	1.003	1.002-1.004	< 0.001[1]	1.002	0.998-1.006	0.129
Pathologic results						
Tumor size	1.290	1.178-1.413	< 0.001[1]	1.160	1.033-1.302	0.012[1]
Multiple tumors	1.264	1.073-1.487	0.005[1]	1.264	1.041-1.534	0.018[1]
Vascular invasion	2.454	1.479-4.071	0.001[1]	1.597	0.890-2.867	0.117

[1]Represents statistical significance. HH group: Alpha-fetoprotein values > 21.0 ng/mL and des-gamma-carboxy prothrombin values > 67 mAU/mL; HL group: Alpha-fetoprotein values > 21.0 ng/mL or des-gamma-carboxy prothrombin values > 67 mAU/mL. ALB: Albumin; PT: Prothrombin; TBIL: Total bilirubin; ICGR15: Indocyanine green retention rate at 15 min.

Table 6 Univariate and multivariate analyses of factors associated with poor overall survival in patients with hepatitis C virus infection

Variables	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Age	1.025	0.987-1.065	0.195			
Sex, male	1.328	0.668-2.642	0.418			
Preoperative laboratory values						
Aspartate transaminase	1.006	0.999-1.013	0.106			
Alanine transaminase	1.001	0.992-1.009	0.849			
Platelets	1.036	0.995-1.077	0.084			
Serum ALB	0.246	0.114-0.531	< 0.001[1]	0.611	0.219-1.706	0.347
PT	0.994	0.971-1.018	0.643			
Serum TBIL	1.206	0.523-2.780	0.660			
ICGR15	1.054	1.021-1.087	0.001[1]	1.042	1.001-1.084	0.047[1]
Tumor markers						
HH group	4.562	2.031-10.248	< 0.001[1]	4.018	1.424-11.337	0.009[1]
HL group	2.049	0.911-4.609	0.083	2.209	0.829-5.885	0.113
Intraoperative data						
Blood loss	1.001	1.000-1.001	< 0.001[1]	1.000	0.999-1.001	0.727
Surgical duration	1.003	1.002-1.004	< 0.001[1]	1.002	0.998-1.005	0.414
Pathologic results						
Tumor size	1.285	1.162-1.421	< 0.001[1]	1.081	0.946-1.235	0.255
Multiple tumors	1.204	1.021-1.420	0.027[1]	1.257	1.025-1.542	0.028[1]
Vascular invasion	2.223	1.221-4.045	0.009[1]	1.089	0.490-2.421	0.835

[1]Represents statistical significance. HH group: Alpha-fetoprotein values > 21.0 ng/mL and des-gamma-carboxy prothrombin values > 67 mAU/mL; HL group: Alpha-fetoprotein values > 21.0 ng/mL or des-gamma-carboxy prothrombin values > 67 mAU/mL. ALB: Albumin; PT: Prothrombin; TBIL: Total bilirubin; ICGR15: Indocyanine green retention rate at 15 min.

9.6%, and liver function parameters were good, with median ALB levels, PTs, and TBIL levels of 3.95 ± 0.46 g/dL, 93.3% ± 12.2%, and 0.79 ± 0.44 mg/dL, respectively. The literature finds that few HCC patients with HBV infection present with liver cirrhosis[23-25]. Similarly, in this study, relatively few patients in the HBV group had liver cirrhosis [41 patients (39.0%)], and the platelet count, which is decreased in cirrhosis, was maintained at 16.6 × 10^4/μL (Table 1). In the present study, the subjects in the HBV group were comparable to subjects in other reports to date; thus, we believe that this sample was not biased.

A study evaluating the relationship between prognosis and levels of AFP and DCP in 1447 HCC patients used AFP and DCP cutoff levels randomly set at 400 ng/mL and 100 mAU/mL, respectively. The patients with high levels of both AFP and DCP had poor prognoses, which is similar to the findings of our study. These cutoff levels were similar to the levels in our HBV group. In addition, of the 1447 patients in the abovementioned report, 1048 had HBV-induced HCC[26]. The cutoff levels commonly used to diagnose HCC are 20 ng/mL to 200 ng/mL for AFP and 40 mAU/mL to 100 mAU/mL for DCP[27-30]. The cutoff levels to predict cancer prognosis are almost twice the levels used for cancer diagnosis. Therefore, once cancer develops, treatment should be administered before the tumor marker levels increase to levels indicative of poor prognosis.

MDFST and MOST in the HBV group were significantly different from the other groups in all analyses; however, there was no significant difference in MDFST and MOST between the HH and HL groups (P = 0.203 and P = 0.465, respectively) (Figure 3). The HH group had a relatively large median tumor size of 6.24 ± 4.77 cm, and there was a high frequency of patients with vascular invasion (10/16 patients, 62.5%). Tumor size was smaller in the HL group than in the HH group, and there was less vascular invasion. However, the prevalence of poorly differentiated HCC on histological examination in the HL group was comparable to that in the HH group. Moreover, the liver function parameters, including the $ICGR_{15}$ level, ALB level, PT, and TBIL level, were worse in the HL group than in the HH group. Although the underlying mechanisms of poor liver function in the HL group remain unclear, poor liver function may explain the similar long-term prognoses found in the HH and HL groups. It is assumed that when the HBV group had high levels of either AFP or DCP, liver function parameters strongly affected prognosis.

Studies suggest that liver function decreases and liver cirrhosis progresses with age in many HCC patients with HCV infection[31,32]. In the present study, the median age in the HCV group was 71 years, and many patients had decreased liver function parameters (Table 4). Furthermore, the majority of patients (58/100, 58.0%) had liver cirrhosis (Table 4), and the characteristics of subjects in the HCV group were similar to the characteristics of subjects in other studies; thus, we believe that there was no selection bias.

In the HCV group, the AFP and DCP cutoff levels for recurrence within 2 years after hepatectomy were 21.0 ng/mL and 67 mAU/mL, respectively (Figure 4). The specificity of AFP levels to predict recurrence within 2 years after hepatectomy was relatively low (67.3%), which is similar to the results of other studies[33]. Furthermore, the DCP cutoff level was approximately the same as that for the HBV group. The lack of association between DCP cutoff level and the underlying virus may be because unlike the AFP level, the DCP level is typically not elevated in chronic hepatitis or liver cirrhosis[14]; thus, it is not affected by liver function parameters, but rather fluctuates specifically in response to tumor marker levels. The AFP cutoff level varied between the HCV and HBV groups. However, the lens culinaris agglutinin-reactive fraction of AFP (AFP-L3) has been shown to have a high specificity for a cancer diagnosis[34]. We believe that the AFP-L3 cutoff level would have been similar in the HCV and HBV groups.

MDFST and MOST in the HCV group varied by subgroup (Figure 5). This difference demonstrates that high levels of either AFP or DCP were clinically significant[31]. The analysis of the clinical characteristics (Table 4) revealed no significant differences in liver function parameters; however, tumor-related factors, including tumor size and incidence of vascular invasion, were different between the groups. This difference suggests that tumor-related characteristics strongly affected prognosis with HCV infection; therefore, we believe that prognosis can be predicted by AFP and DCP levels together with tumor-related factors.

In our study, various clinical factors were associated with tumor recurrence and overall survival; therefore, we conducted a multivariate analysis for prognosis using a Cox forward stepwise logistic regression model. In the HBV group, the independent prognostic factors associated with tumor recurrence were tumor size, vascular invasion, and TBIL (Table 2). Significant differences in tumor size and vascular invasion were found between the three groups (Table 1). Tumor recurrence prognosis among the three groups was affected by these two factors, and a significant difference in disease-free survival was found (Figure 3A). However, membership in the HH group was an independent prognostic factor associated with tumor recurrence. Therefore, high levels of both AFP and DCP may be a prognostic predictor of tumor recurrence in the HBV group. The only independent prognostic factor associated with poor overall survival was vascular invasion (Table 3), which differed significantly between the three groups (Table 1). Therefore, a significant difference in overall survival rates was found between groups (Figure 3B). The identification of AFP and DCP levels was not always useful for predicting the prognosis associated with poor overall survival in the HBV group because neither HH group membership nor HL group membership was an independent prognostic factor associated with poor overall survival (Table 3). In the HCV group, the independent prognostic factors associated with tumor recurrence were $ICGR_{15}$, surgical duration, tumor size, vascular invasion, and multiple tumors (Table 5). Surgical duration and tumor size differed significantly between the three groups (Table 4). Tumor recurrence prognosis in all three groups was affected by these two factors, yielding a significant difference between groups in disease-free survival (Figure 5A). However, membership in the HH group

and membership in the HL group were independent prognostic factors associated with tumor recurrence (Table 5), suggesting that high levels of AFP and/or DCP are prognostic predictors of tumor recurrence in the HCV group. The independent prognostic factors associated with poor overall survival were ICGR15 and multiple tumors (Table 6), neither of which differed significantly between the three groups (Table 4). A prognosis of poor overall survival in all three groups was not affected by ICGR15 or multiple tumors. Membership in the HH group was an independent prognostic factor associated with poor overall survival (Table 6), indicating that high levels of both AFP and DCP are a prognostic predictor of poor overall survival in the HCV group.

In conclusion, among HCC patients treated in our department, the AFP cutoff level for recurrence within 2 years after surgery was 21.0 ng/mL in the HCV group and 529.8 ng/mL in the HBV group. Furthermore, patients in the HBV group with high levels of either AFP or DCP had poor prognoses, as did patients with high levels of both AFP and DCP. In contrast, poor prognoses were found in patients in the HCV group only when both levels were high. High AFP and DCP levels were an independent risk factor for tumor recurrence in both the HBV and HCV groups. We believe that to predict prognosis, preoperative levels of tumor markers should be distinguished and assessed according to the type of viral hepatitis.

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COMMENTS

Background

There is no consensus regarding preoperative α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP) cutoff levels to predict survival and recurrence after hepatectomy. Furthermore, the prognostic characteristics of these tumor markers according to hepatitis type remain unclear. Therefore, the aim of the present study was to compare these preoperative tumor marker levels and prognosis after hepatectomy as well as to clarify the characteristics of these tumor marker levels according to hepatitis type.

Research frontiers

Chronic hepatitis caused by viral hepatitis often progresses to cirrhosis and hepatocellular carcinoma. The oncogenic mechanisms differ between hepatitis B and C viral infections, and these mechanisms should be taken into consideration when evaluating prognosis and establishing treatment regimens. In this study, the authors demonstrate that the relationship between preoperative tumor marker levels and prognosis varied by the type of viral hepatitis.

Innovations and breakthroughs

Although associations between preoperative tumor markers and postoperative hepatocellular carcinoma prognosis have been reported, this is the first study to report that the AFP cutoff level for recurrence within 2 years after surgery was 21.0 ng/mL in hepatitis C virus (HCV) patients compared with a higher level of 529.8 ng/mL in hepatitis B virus (HBV) patients. Furthermore, patients in the HBV group with high levels of either AFP or DCP had poor prognoses, as did those patients with high levels of both tumor markers. In contrast, in patients in the HCV group, poor prognoses were found only when levels of both AFP and DCP were high.

Applications

In the HBV group, prognosis was poor when either AFP or DCP levels were high. In the HCV group, prognosis was good when either or both levels were low; however, prognosis was poor when both levels were high.

Terminology

AFP is a glycoprotein produced by liver cells and in the yolk sac during the fetal stage with an albumin-like structure, a half-life of 4-6 d and a molecular weight of 65 kDa. AFP production in the liver is increased in hepatocellular cancer as well as in chronic hepatitis and cirrhosis; therefore, AFP is considered to have low specificity for the diagnosis of cancer. Prothrombin is formed after the γ-carboxylation of vitamin K-dependent propeptides, and DCP is produced as a result of an acquired posttranslational defect in the vitamin K-dependent carboxylase system. DCP has a molecular weight of 72 kDa and a half-life of 40-72 h. DCP production does not increase in chronic hepatitis or cirrhosis, and it is considered to have high specificity for the diagnosis of cancer. However, DCP has no prognostic value in cases with vitamin K deficiency or vitamin K function inhibition.

Peer-review

Nice paper. Many patients included but a regression model is needed to confirm data. If they would do these statistical analysis, paper is interesting enough to be published.

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ORIGINAL ARTICLE

Retrospective Study

Biliary drainage strategy of unresectable malignant hilar strictures by computed tomography volumetry

Ei Takahashi, Mitsuharu Fukasawa, Tadashi Sato, Shinichi Takano, Makoto Kadokura, Hiroko Shindo, Yudai Yokota, Nobuyuki Enomoto

Ei Takahashi, Mitsuharu Fukasawa, Tadashi Sato, Shinichi Takano, Makoto Kadokura, Hiroko Shindo, Yudai Yokota, Nobuyuki Enomoto, First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, Yamanashi 409-3898, Japan

Author contributions: Takahashi E analyzed and interpreted data, drafted the article, and performed biliary stenting; Fukasawa M conceived and designed the study, drafted the article, and performed biliary stenting; Sato T drafted the article and critically revised it for important intellectual content; Takano S, Kadokura M, Shindo H and Yokota Y performed biliary stenting and collected data; Enomoto N critically revised the manuscript for important intellectual content.

Ethics approval: The study was reviewed and approved by the University of Yamanashi Institutional Review Board.

Conflict-of-interest: None of the authors have any conflicts of interest.

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Correspondence to: Mitsuharu Fukasawa, MD, PhD, First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan. fmitsu@yamanashi.ac.jp
Telephone: +81-5-52739584
Fax: +81-5-52736748

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Abstract

AIM: To identify criteria for predicting successful drainage of unresectable malignant hilar biliary strictures (UMHBS) because no ideal strategy currently exists.

METHODS: We examined 78 patients with UMHBS who underwent biliary drainage. Drainage was considered effective when the serum bilirubin level decreased by ≥ 50% from the value before stent placement within 2 wk after drainage, without additional intervention. Complications that occurred within 7 d after stent placement were considered as early complications. Before drainage, the liver volume of each section (lateral and medial sections of the left liver and anterior and posterior sections of the right liver) was measured using computed tomography (CT) volumetry. Drained liver volume was calculated based on the volume of each liver section and the type of bile duct stricture (according to the Bismuth classification). Tumor volume, which was calculated by using CT volumetry, was excluded from the volume of each section. Receiver operating characteristic (ROC) analysis was performed to identify the optimal cutoff values for drained liver volume. In addition, factors associated with the effectiveness of drainage and early complications were evaluated.

RESULTS: Multivariate analysis showed that drained liver volume [odds ratio (OR) = 2.92, 95%CI: 1.648-5.197; $P < 0.001$] and impaired liver function (with decompensated liver cirrhosis) (OR = 0.06, 95%CI: 0.009-0.426; $P = 0.005$) were independent factors contributing to the effectiveness of drainage. ROC analysis for effective drainage showed cutoff values of 33% of liver volume for patients with preserved liver function (with normal liver or compensated liver

cirrhosis) and 50% for patients with impaired liver function (with decompensated liver cirrhosis). The sensitivity and specificity of these cutoff values were 82% and 80% for preserved liver function, and 100% and 67% for impaired liver function, respectively. Among patients who met these criteria, the rate of effective drainage among those with preserved liver function and impaired liver function was 90% and 80%, respectively. The rates of effective drainage in both groups were significantly higher than in those who did not fulfill these criteria ($P < 0.001$ and $P = 0.02$, respectively). Drainage-associated cholangitis occurred in 9 patients (12%). A smaller drained liver volume was associated with drainage-associated cholangitis ($P < 0.01$).

CONCLUSION: Liver volume drainage ⩾ 33% in patients with preserved liver function and ⩾ 50% in patients with impaired liver function correlates with effective biliary drainage in UMHBS.

Key words: Biliary drainage; Computed tomography volumetry; Hilar biliary stricture; Cholangiocarcinoma; Liver function; Cholangitis

Core tip: An ideal biliary drainage strategy for unresectable malignant hilar biliary strictures (UMHBS) has not been defined. The aim of our study was to identify useful criteria for predicting successful drainage of UMHBS. In the present study, multivariate analysis revealed that liver function and drained liver volume calculated by computed tomography volumetry (CTV) are independent factors of drainage effectiveness for UMHBS. Receiver operating characteristic analysis showed cutoff values of 33% of liver volume for patients with preserved liver function and 50% for patients with impaired liver function. Before attempting biliary drainage procedures, establishing an appropriate drainage strategy using CTV is important.

Takahashi E, Fukasawa M, Sato T, Takano S, Kadokura M, Shindo H, Yokota Y, Enomoto N. Biliary drainage strategy of unresectable malignant hilar strictures by computed tomography volumetry. *World J Gastroenterol* 2015; 21(16): 4946-4953 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4946.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4946

INTRODUCTION

Effective treatment of obstructive jaundice is essential for improving the quality of life of patients with unresectable malignant hilar biliary strictures (UMHBS). These strictures may be caused by malignancies of the bile ducts, pancreas, or liver (primary or metastatic) or by metastases to lymph nodes around the bile ducts. In biliary obstruction in the hepatic hilum, effective drainage is difficult to achieve because of the anatomical complexity of the bile ducts. Various procedures of biliary drainage of hilar biliary obstruction have been performed: percutaneous or endoscopic routes[1-5], plastic or metallic stents[6-11], and unilateral or bilateral hepatic duct drainage[11-21]. However, no consensus has been reached on the optimal drainage strategy for treating biliary obstruction.

In recent years, advances in diagnostic imaging, such as multi-detector computed tomography (CT) and magnetic resonance cholangiopancreatography, have facilitated improved preoperative identification of an aberrant hepatic duct and determination of hilar tumor progression. In this study, we estimated the volumes of the lateral and medial sections of the left hepatic liver and the anterior and posterior sections of the right liver by using CT volumetry in patients who required drainage for UMHBS. We then measured the drained liver volume after stenting according to the type of bile duct stricture (bismuth classification) and assessed the effects of drainage and early complications related to drainage procedures.

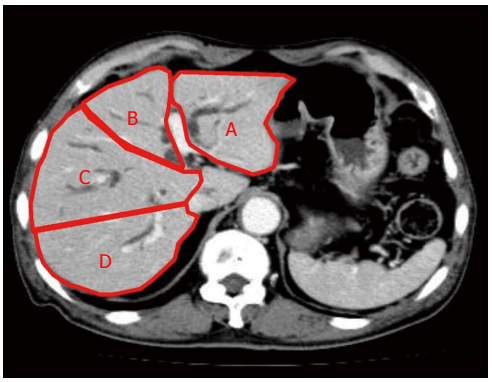

Figure 1 Computed tomography volumetric identification of liver sections. Lateral section (A) and medial section (B) of the left liver, and anterior section (C) and posterior section (D) of the right liver.

MATERIALS AND METHODS

We retrospectively reviewed data on 78 patients who underwent biliary drainage procedures for UMHBS between March 2004 and April 2013. The inclusion criteria were as follows: obstructive jaundice caused by hilar malignancy (Bismuth type Ⅱ or higher); abdominal CT scan performed within 2 wk before drainage; and clinical biochemical tests performed before and 14 d after stent placement. The exclusion criteria were as follows: benign stenosis; Bismuth type Ⅰ obstruction; history of hepatectomy; or the presence of UMHBS without jaundice before stent placement, for which a stent was prophylactically placed. The presence of distant metastasis, locally far-advanced tumors, and/or poor liver function would preclude resection based on the consensus of

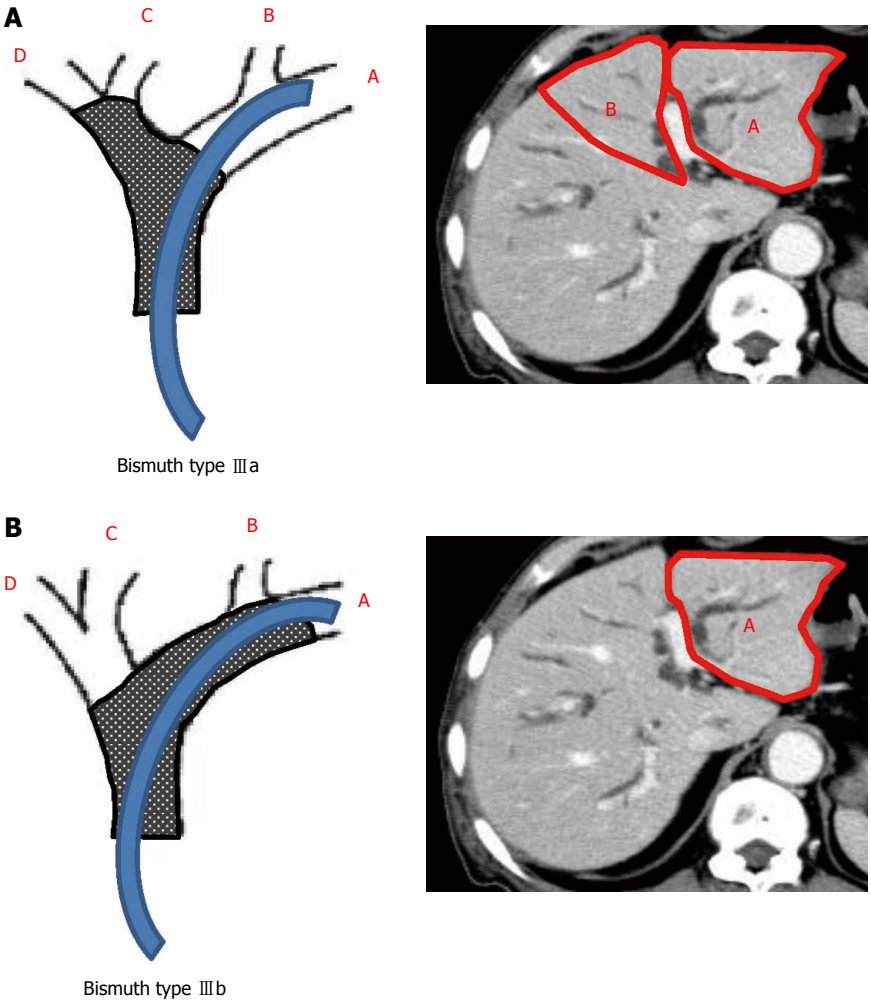

Figure 2 Calculation of drained liver volume according to the Bismuth classification. The stent is inserted into the left hepatic duct. Drained volume is calculated as a sum of A + B in Bismuth type ⅢaB (A) and A in Bismuth type ⅢbB (B).

gastroenterologists and surgeons. Figure 1 shows a CT volumetric image of a patient with UMHBS.

The 4 sections of the liver were defined according to the distribution of the portal vein branches: lateral and medial sections of the left liver, and anterior and posterior sections of the right liver. The drained liver volume was evaluated as follows. The area of each section was calculated by manual tracing using CT scans (axial view) with a 5 mm slice thickness. The volume of each section was calculated as the summed area of the comprising sections. Thereafter, drained liver volume was calculated based on the volume of each liver section and the type of bile duct stricture (according to the Bismuth classification). Figure 2A and B show the valid drainage calculation method. Tumor volume, which was calculated by using CT volumetry, was excluded from the volume of each section. Drainage was considered effective when the serum bilirubin level decreased by ⩾ 50% of the value before stent placement within 2 wk after drainage, without additional intervention. Complications that occurred within 7 d after stent placement were considered as the early complications.

Receiver operating characteristic (ROC) analysis was performed to determine the cutoff value for drained liver volume in the group with preserved liver function (with normal liver or compensated liver cirrhosis) and the group with impaired liver function (with decompensated liver cirrhosis). Patients with hepatic encephalopathy and/or cytologically negative ascites in addition to chronic liver disease were considered to have decompensated liver cirrhosis.

Statistical analysis

The χ^2 test or Fisher's exact test of the contingency table was used for univariate analysis of the categorical data. Student's *t*-test was used for analysis of the quantitative data. Multivariate analysis of the factors contributing to the initial drainage effect was performed using multiple logistic regression analysis. A statistically significant difference was defined as $P < 0.05$. The statistical methods of this study were reviewed by Dr. Kohta Suzuki from the Department of Health Sciences, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi.

Table 1 Clinical features of 78 patients with malignant hilar strictures *n* (%)

Characteristic	
Age (yr), mean ± SD	74.8 ± 10.3
Gender, male/female	53/25
Etiology	
Cholangiocarcinoma	50 (64)
Hepatocellular carcinoma	10 (13)
Gallbladder carcinoma	9 (12)
Liver metastasis	5 (6)
Lymph node metastasis	4 (5)
Bismuth type (Ⅱ/Ⅲa/Ⅲb/Ⅳ)	11/24/7/36
Total bilirubin (mg/dL), median (range)	9.0 (3.0-31.0)
Cholangitis	13 (17)
Liver function	
Preserved liver function (without decompensated liver cirrhosis)	64 (82)
Impaired liver function (with decompensated liver cirrhosis)	14 (18)
Drainage method (MS/PS/ENBD/PTBD)	11/38/21/8
Drainage areas	
Unilateral/bilateral	72/6
Drained liver volume (%), mean ± SD	44.6 ± 18.6

MS: Metal stent; PS: Plastic stent; ENBD: Endoscopic nasobiliary drainage; PTBD: Percutaneous transhepatic biliary drainage.

Table 2 Predictors of initial drainage effectiveness by univariate analysis *n* (%)

	Effective (*n* = 49)	Ineffective (*n* = 29)	*P* value
Age (yr), mean ± SD	74.0 ± 11.6	76.0 ± 7.5	0.39
Gender, male/female	32/17	21/8	0.78
Etiology, cholangiocarcinoma	32 (65.3)	18 (62.1)	0.81
Bismuth type Ⅳ	17 (34.6)	19 (65.5)	0.02
Total bilirubin (mg/dL), mean ± SD	9.9 ± 6.3	10.0 ± 4.7	0.93
Cholangitis	8 (16.3)	5 (17.2)	0.91
Impaired liver function	5 (10.2)	9 (31.0)	0.03
Drainage method, endoscopic drainage	43 (87.8)	27 (93.1)	0.71
Type of stent, metal stent	7 (14.3)	4 (13.8)	1.00
Drainage areas			
Unilateral/bilateral	43/6	29/0	0.16
Drained liver volume (%), mean ± SD	51.1 ± 18.6	33.5 ± 12.5	< 0.01

Table 3 Predictors of initial drainage effectiveness by multivariate analysis

Factors	OR	95%CI	*P* value
Bismuth type Ⅳ	0.92	0.245-3.418	0.896
Impaired liver function	0.06	0.009-0.426	0.005
Drained liver volume	2.92	1.648-5.197	< 0.001

RESULTS

Table 1 shows patient characteristics and morphological data. The subject cohort had a mean age of 74.8 ± 10.3 years (53 men and 25 women) and included 50 patients (64%) with cholangiocarcinoma, 10 (13%) with hepatocellular carcinoma, 9 (12%) with gallbladder carcinoma, 5 (6%) with metastatic liver carcinoma, and 4 (5%) with lymph node metastasis. According to Bismuth classification, 11 patients were classified as type Ⅱ, 24 as type Ⅲa, 7 as type Ⅲb, and 36 as type Ⅳ. The median total bilirubin level was 9.0 mg/dL (3.0-31.0 mg/dL). Sixty-four patients (82%) had preserved liver function (without decompensated liver cirrhosis), and 14 (18%) had impaired liver function (with decompensated liver cirrhosis). With regard to the drainage procedure, endoscopic biliary stenting (metal stent/plastic stent), endoscopic nasobiliary drainage, and percutaneous transhepatic biliary drainage were performed in 49 (11/38), 21, and 8 patients, respectively. Unilateral and bilateral drainage were performed in 72 patients (92%) and 6 patients (8%), respectively. The average drained liver volume was 44.6% ± 18.6%.

Drainage procedures were successfully performed in 78 patients (procedure success rate, 100%). Effective drainage was achieved in 49 of the patients (63%). Of the 21 patients who underwent additional drainage because of ineffective initial drainage, successful drainage was achieved in 17. Thus, eventually, 66 patients (85%) had effective drainage. Univariate analysis for each factor potentially influencing the effectiveness of initial drainage showed that there were significant differences in Bismuth type Ⅳ (P = 0.02), impaired liver function (P = 0.03), and drained liver volume (%) (P < 0.01). There was no significant difference between unilateral and bilateral drainage (Table 2). Multivariate analysis (multiple logistic analysis) of the 3 factors with significant differences in univariate analysis indicated that the independent factors contributing to the effectiveness of drainage were impaired liver function (OR = 0.06, 95%CI: 0.009-0.426; P = 0.005) and drained liver volume (OR = 2.92, 95%CI: 1.648-5.197; P < 0.001) (Table 3).

In ROC analysis of the drained liver volume required for effective drainage, the area under the curve with preserved liver function and with impaired liver function was 0.83 and 0.93, respectively (Figure 3). The optimal cutoff (%), 33% of liver volume for subjects with preserved liver function and 50% of liver volume for subjects with impaired liver function, was calculated by determining the smallest distance between the ROC curve and the upper left corner of the graph. The sensitivity and specificity of these cutoff values were 82% and 80% for preserved liver function, and 100% and 67% for impaired liver function, respectively. When the cutoff value was set at 33% in patients with preserved liver function, the rate of effective drainage was 90% (≥ 33% of liver volume) and 33% (< 33% of liver volume) (P < 0.001) (Figure 4A). When the cutoff value was set at 50% in patients with impaired liver function, the rate of effective drainage was 80% (≥ 50% of liver volume) and 11% (< 50% of liver volume; P = 0.02) (Figure 4B).

Early complications occurring within 7 d after stent placement were found in 14 patients (18%) and included pancreatitis in 5 (6%) and drainage-

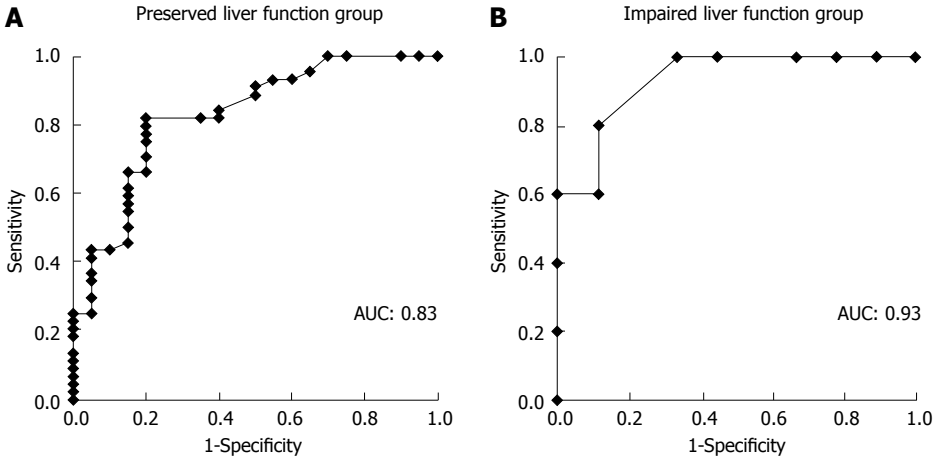

Figure 3 Area under the receiver operating characteristic curve for predicting effective drainage. Area under the curve in subjects with preserved liver function (with normal liver or compensated liver cirrhosis) (A) and with impaired liver function (with decompensated liver cirrhosis) (B) is 0.83 and 0.93, respectively. AUC: Area under the curve.

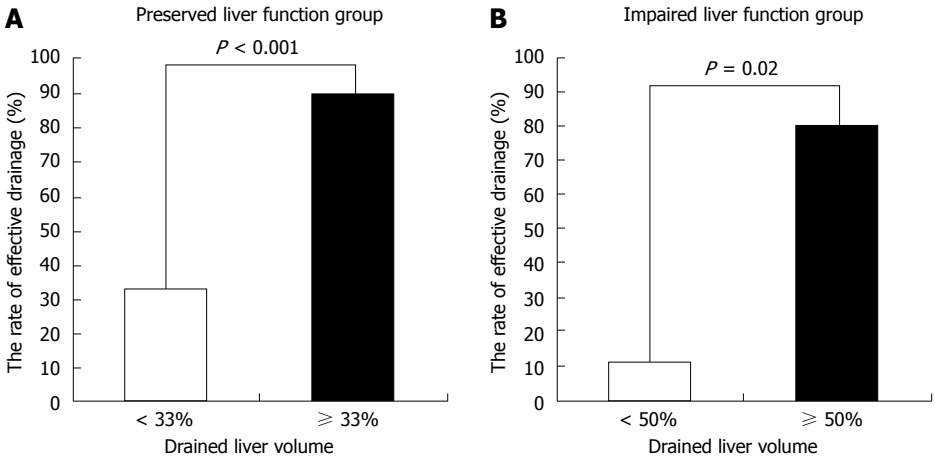

Figure 4 Rate of effective drainage and drained liver volume. The rate is significantly higher in patients with drainage ⩾ 33% of liver volume in subjects with preserved liver function (A) and with drainage ⩾ 50% of liver volume in subjects with impaired liver function (B).

associated cholangitis in 9 (12%). All pancreatitis episodes were mild and resolved with conservative management alone. Among the 9 patients with cholangitis, 5 patients required additional stenting and the remaining 4 patients were managed with antibiotics alone.

Factors associated with cholangitis are shown in Table 4. Univariate analysis for each factor associated with cholangitis showed that there was a significant difference in drained liver volume. Smaller drained liver volume was associated with drainage-associated cholangitis ($P < 0.01$). In the preserved liver function group, the incidence of cholangitis was 25% in patients with < 33% of liver volume *vs* 2.5% in patients with ⩾ 33% of liver volume ($P < 0.01$) (Figure 5A). In the impaired liver function group, the incidence of cholangitis was 22% in patients with < 50% of liver volume *vs* 0% in patients with ⩾ 50% of liver volume ($P = 0.51$) (Figure 5B).

DISCUSSION

To establish a more effective strategy for biliary drainage, a number of studies concerning the drainage route, the number, size, and material of the stent have been reported[1-23]. In recent years, advances in diagnostic imaging have provided detailed information about bile duct strictures and liver volume. The surgical indications and extent of resection for hepatectomy are determined based on the results of preoperative liver function and remnant liver volume[24-26]. However, few research studies are available on the significance of individual liver function and liver volume in biliary drainage.

In the present study, multivariate analysis revealed that liver function and drained liver volume were independent factors of drainage effectiveness for UMHBS. Vienne *et al*[27] reported that effective drainage could be attained by draining ⩾ 50% of the liver

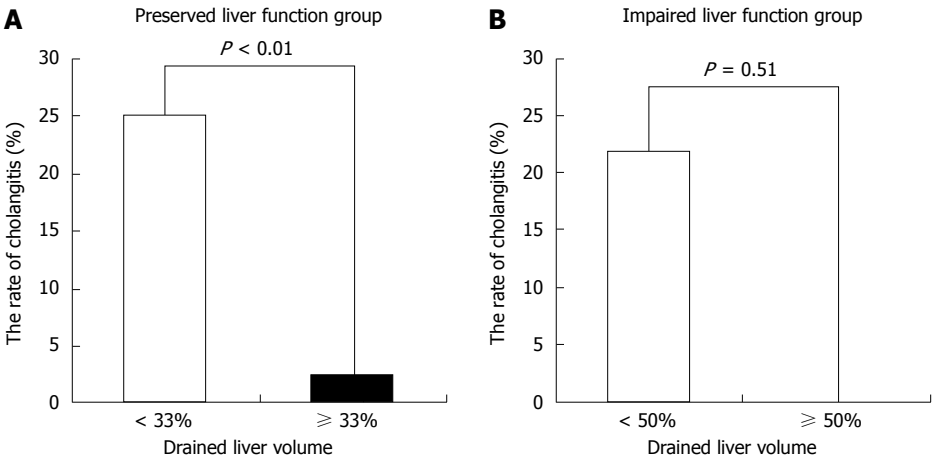

Figure 5 Rate of drainage-associated cholangitis and drained liver volume. There is significant difference between the rate of cholangitis and drained liver volume in subjects with preserved liver function (A), but no significant difference in those with impaired liver function (B).

Table 4 Predictors of drainage-associated cholangitis by univariate analysis *n* (%)

	Cholangitis *n* = 9	Non-cholangitis *n* = 69	*P* value
Age (yr), mean ± SD	76.9 ± 8.3	74.5 ± 10.5	0.51
Gender, male/female	5/4	48/21	0.46
Etiology, cholangiocarcinoma	6 (66.7)	44 (63.8)	1.00
Bismuth type Ⅳ	6 (66.7)	30 (43.5)	0.29
Total bilirubin (mg/dL)	8.3 ± 3.5	10.6 ± 6.1	0.12
Impaired liver function	2 (22.2)	7 (10.1)	0.66
Drainage method, endoscopic drainage	7 (77.8)	63 (91.3)	0.23
Type of stent, metal stent	1 (11.1)	10 (14.5)	1.00
Drainage areas			
Unilateral/bilateral	9/0	63/6	1.00
Drained liver volume (%), mean ± SD	29.3 ± 4.4	46.6 ± 18.9	< 0.01

volume based on examination of bile duct stricture type and volume of each section. The required drained liver volume is expected to be variable depending on liver function; however, no previous reports are available on the role of liver function in determining the drainage method. Our study revealed that effective drainage could be expected by draining ⩾ 33% of the liver volume in those with preserved liver function and ⩾ 50% of the liver volume in those with impaired liver function. The overall drainage response rate in this study was 63%, but the response rate was 89% for the subjects who met the abovementioned criteria. These subjects had a better response rate than those in earlier studies (49%-87%)[3,13-19]. In addition, of the 29 patients who did not respond to the initial drainage, 24 (83%) did not meet the above criteria. Inadequate drained volume is a possible reason for drainage failure. This study is the first report to determine the drainage method of UMHBS by using both drained liver volume and liver function. When unilateral drainage achieves the required drained liver volume, which occurred in 83% of the subjects, unilateral drainage is sufficient. Bilateral drainage should be considered only when the estimated drained liver volume by unilateral stenting does not reach the abovementioned criteria.

Cholangitis is an important complication after stenting for drainage of hilar biliary strictures. Contrast injection into the undrained sector and stent placement within a small area were risk factors for cholangitis[5,6,13,16,27], which may be attributed to the decreased bile excretory function in an atrophied area[27]. In our series, 3 of 9 patients (33%) who underwent cholangiography of the undrained section developed cholangitis (data not shown). In addition, our study found that drainage of a small area was a risk factor contributing to post-drainage cholangitis. This finding supports those of previous studies. Although there was no statistical difference in cholangitis in impaired liver function because of a lack of power, cholangitis was not observed in patients with the obtained cutoff values or more.

Our study has 2 limitations. First, because it was a retrospective study, the drainage procedures were not uniform; the procedures were chosen by the patient's attending physician, which might have led to bias. Second, because of the sample size, statistical subgroup analyses were not possible (*e.g.*, analysis by the type of disease and Bismuth classification). Our observations should be confirmed in a multicenter prospective study with a large number of patients.

In conclusion, liver function and drained liver volume are important factors in the drainage of unresectable malignant hilar biliary strictures. Assembling an appropriate drainage strategy using CT volumetry before ERCP is important.

ACKNOWLEDGMENTS

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COMMENTS

Background

The effective treatment of obstructive jaundice is essential for improving the quality of life of patients with unresectable malignant hilar biliary strictures (UMHBS). In cases of biliary obstruction in the hepatic hilum, effective drainage is difficult to achieve because of the anatomical complexity of the bile ducts. Various procedures of biliary drainage for hilar biliary obstruction have been performed, involving percutaneous or endoscopic routes, plastic or metallic stents, and unilateral or bilateral hepatic duct drainage. However, no consensus has been reached on the optimal drainage strategy for treating biliary obstruction.

Research frontiers

The appropriate treatment for patients with UMHBS has been attempted through the development of stents of various shapes and materials. Computed tomography (CT) volumetry enables the accurate estimation of drained liver volume.

Innovations and breakthroughs

In the present study, multivariate analysis revealed that liver function and drained liver volume calculated by computed tomography volumetry are independent factors of drainage effectiveness for UMHBS. Receiver operating characteristics analysis for effective drainage showed cutoff values of 33% for the liver volume of patients with preserved liver function (without decompensated liver cirrhosis) and 50% for the liver volume of patients with impaired liver function (with decompensated liver cirrhosis). This study is the first report to assess the drainage method of UMHBS by using both drained liver volume calculated by using CT volumetry and liver function.

Applications

By using this cutoff value, appropriate drainage may be possible for individual cases.

Terminology

CT volumetry involves the measurement of volume using CT.

Peer-review

To estimate whether the accurate drained liver volume using CT volumetry is superior as an objective evaluation. To choose an appropriate drainage method for individual cases by using the cutoff determined in the present study before stating that biliary drainage procedures are useful.

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ORIGINAL ARTICLE

Retrospective Study

Characteristics of gastric cancer in peptic ulcer patients with *Helicobacter pylori* infection

Jae Jin Hwang, Dong Ho Lee, Ae-Ra Lee, Hyuk Yoon, Cheol Min Shin, Young Soo Park, Nayoung Kim

Jae Jin Hwang, Dong Ho Lee, Ae-Ra Lee, Hyuk Yoon, Cheol Min Shin, Young Soo Park, Nayoung Kim, Department of Internal Medicine, Seoul National University Bundang Hospital, Seongnam, Gyeonggi-do 463-707, South Korea

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Ethics approval: The study was reviewed and approved by the Seoul National University Bundang Hospital Institutional Review Board.

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Data sharing: No additional data are available.

Correspondence to: Dong Ho Lee, MD, Department of Internal Medicine, Seoul National University Bundang Hospital, 300 Gumi-dong, Bundang-gu, Seongnam, Gyeonggi-do 463-707, South Korea. dhljohn@yahoo.co.kr
Telephone: + 82-31-7877006
Fax: + 82-31-7874051

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Abstract

AIM: To evaluate the incidence and clinical characteristics of gastric cancer (GC) in peptic ulcer patients with *Helicobacter pylori* (*H. pylori*) infection.

METHODS: Between January 2003 and December 2013, the medical records of patients diagnosed with GC were retrospectively reviewed. Those with previous gastric ulcer (GU) and *H. pylori* infection were assigned to the HpGU-GC group (n = 86) and those with previous duodenal ulcer (DU) disease and *H. pylori* infection were assigned to the HpDU-GC group (n = 35). The incidence rates of GC in the HpGU-GC and HpDU-GC groups were analyzed. Data on demographics (age, gender, peptic ulcer complications and cancer treatment), GC clinical characteristics [location, pathological diagnosis, differentiation, T stage, Lauren's classification, atrophy of surrounding mucosa and intestinal metaplasia (IM)], outcome of eradication therapy for *H. pylori* infection, esophagogastroduodenoscopy number and the duration until GC onset were reviewed. Univariate and multivariate analyses were performed to identify factors influencing GC development. The relative risk of GC was evaluated using a Cox proportional hazards model.

RESULTS: The incidence rates of GC were 3.60% (86/2387) in the HpGU-GC group and 1.66% (35/2098) in the HpDU-GC group. The annual incidence was 0.41% in the HpGU-GC group and 0.11% in the HpDU-GC group. The rates of moderate-to-severe atrophy of the surrounding mucosa and IM were higher in the HpGU-GC group than in the HpDU-GC group (86% *vs* 34.3%, respectively, and 61.6% *vs* 14.3%, respectively, $P < 0.05$). In the univariate analysis, atrophy of surrounding mucosa, IM and eradication therapy for *H. pylori* infection were significantly associated with the development of GC ($P < 0.05$). There was no significant difference in the prognosis of GC patients between the HpGU-GC and HpDU-GC groups ($P = 0.347$). The relative risk of GC development in the HpGU-GC group compared to that of the HpDU-GC group,

after correction for age and gender, was 1.71 (95%CI: 1.09-2.70; P = 0.02).

CONCLUSION: GU patients with *H. pylori* infection had higher GC incidence rates and relative risks. Atrophy of surrounding mucosa, IM and eradication therapy were associated with GC.

Key words: Gastric cancer; Gastric ulcer; Duodenal ulcer; *Helicobacter pylori*; Eradication therapy

Core tip: This is the first study to investigate gastric cancer (GC) incidence in peptic ulcer patients with *Helicobacter pylori* (*H. pylori*) infection and to compare GC clinical characteristics between patients with gastric ulcer (GU) and duodenal ulcer (DU) disease. The GC incidence rate and relative risk in GU patients with *H. pylori* infection were higher than in DU patients. The *H. pylori* eradication rate was lower in GU than in DU patients, although the success rate of therapy was lower than the failure rate in both groups. Atrophy of surrounding mucosa, intestinal metaplasia and *H. pylori* eradication therapy were significantly associated with GC.

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INTRODUCTION

Following the establishment of the association between *Helicobacter pylori* (*H. pylori*) and chronic gastritis in 1983 by Warren and Marshall[1], this association has been implicated in numerous gastrointestinal diseases. This spiral-shaped, gram-negative bacterium remains the most common source of chronic bacterial infection in humans worldwide. In 1994, the International Agency for Research on Cancer classified *H. pylori* as a group 1 carcinogen[2], a definite cause of cancer in humans. This classification was based on epidemiological studies which demonstrated that individuals infected with *H. pylori* are at an increased risk of distal gastric adenocarcinoma[3]. Gastric cancer (GC) is the second most common cause of cancer-related deaths in the world[4]. *H. pylori* infection has also been implicated in peptic ulcer diseases[5,6]. Therefore, it is possible that patients with peptic ulcers and *H. pylori* infection have a high risk of GC development. However, a number of studies have shown that while patients with gastric ulcer (GU) disease have a high risk of GC, those with duodenal ulcer (DU) disease do not[7-10]. Recently, this paradoxical phenomenon has been described in several studies[7-10].

Post-*H. pylori* infection clinical prognoses are associated with increased acid secretion levels and inflammation extent and severity[11]. DU disease is typically associated with antral-predominant gastritis that leads to normal or increased acid secretion[12,13]. In contrast, GU disease is associated with corpus-predominant gastritis, which provides information on the extent and severity of gastritis, atrophy and acid secretion. GC is associated with pangastritis, which ultimately results in progression from normal gastric mucosa to intestinal metaplasia (IM) and little to no acid secretion[11]. The incidence of GC increases with the extent of gastritis and the severity[13-15], such that GU disease and GC form one axis (*e.g.*, atrophic pangastritis) and DU disease forms a second axis (antral-predominant or corpus-sparing gastritis). Thus, GU disease and GC can evolve from DU disease but the opposite cannot occur. A prospective Japanese study that followed 275 DU patients found that while none of the DU patients developed GC, 3.4% of 297 GU patients did develop GC[8]. A recent retrospective study of 37 patients with both DU disease and GC reported clinical and pathological features relevant to both diseases[16]. However, while there have been many studies on GC development in either patients with *H. pylori* infection or DU disease, there have been no studies on GC development in peptic ulcer (either GU or DU) patients with *H. pylori* infection.

The aim of this study was to investigate the incidence of GC development in peptic ulcer patients with *H. pylori* infection and to compare the clinical characteristics of GC between GU and DU patients with *H. pylori* infection.

MATERIALS AND METHODS

Patient selection

This study was conducted at Seoul National University Bundang Hospital between January 2003 and December 2013. The medical records of patients newly diagnosed with GC were retrospectively reviewed. The patients selected for the study met the following inclusion criteria: (1) age older than 18 years; (2) a previous diagnosis of peptic ulcer (GU or DU) disease by esophagogastroduodenoscopy (EGD); and (3) a diagnosis of *H. pylori* infection by EGD concomitant with diagnosis of peptic ulcer disease. The exclusion criteria were the following: (1) age younger than 18 years; (2) previous endoscopic submucosal dissection (ESD) or gastric surgery for GC; (3) a previous history of *H. pylori* eradication; (4) a history of medication with proton pump inhibitors (PPIs) in the 4 wk preceding the EGD; and (5) a diagnosis of GC within 1 year of study enrollment. The patients participating in the study were advised to undergo EGD every year in order to confirm the occurrence of recurrent peptic ulcer disease and the development of GC.

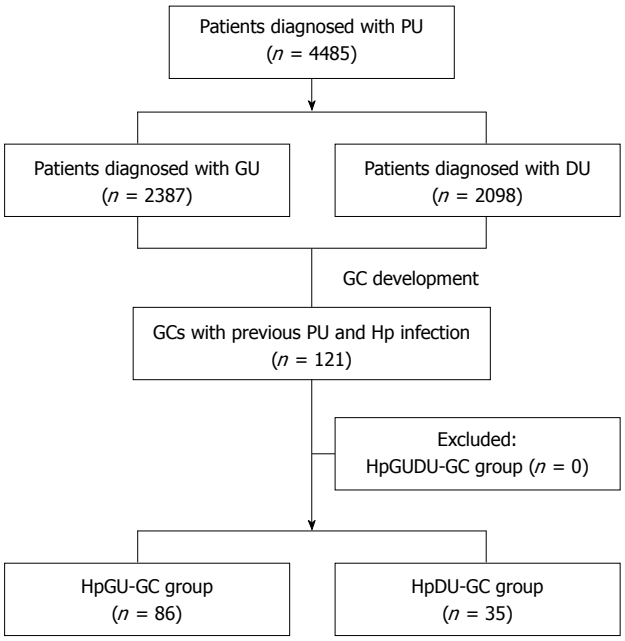

Figure 1 A schematic diagram of the study. PU: Peptic ulcer; GU: Gastric ulcer; DU: Duodenal ulcer; GC: Gastric cancer; Hp: *Helicobacter pylori*; HpGU-GC: GCs with previous GU and *H. pylori infection*; HpDU-GC: GCs with previous DU and *H. pylori* infection; HpGUDU-GC: GCs with previous both GU and DU and *H. pylori* infection.

Esophagogastroduodenoscopy and H. pylori infection

EGD was performed annually on the enrolled patients. A histopathological exam was performed simultaneously *via* endoscopic biopsy. The presence of *H. pylori* infection was defined by at least one of the following criteria: (1) a positive ^{13}C-urea breath test; (2) histological evidence of *H. pylori* by modified Giemsa staining in the lesser and greater curvatures of the body and antrum; and (3) a positive rapid urease test (CLOtest; Delta West, Bentley, Australia) by gastric mucosal biopsy from the lesser curvature of the body and antrum. All of the patients with *H. pylori* infection received standard first-line triple therapy [1 g amoxicillin twice a day (b.i.d), 500 mg clarithromycin b.i.d. and 20 mg rabeprazole (or 40 mg esomeprazole) b.i.d. for 7 d]. Patients that failed first-line triple therapy received rescue therapy until the eradication treatment was successful.

Histopathological evaluation of GC

Pathological stage determinations were made using the American Joint Committee on Cancer TNM classification system and the 6th edition of the International Union Against Cancer[17]. The histological types and tumor differentiation were identified using Lauren's classification[18] and the World Health Organization criteria[19], respectively. The cancer location was determined according to the Japanese Classification of Gastric Cancer[20].

Study protocol

The enrolled patients were classified into two groups. Patients who were newly diagnosed with GC and those who were previously diagnosed with GU and *H. pylori* infection were assigned to the HpGU-GC group and patients who were newly diagnosed with GC and those diagnosed previously with DU and *H. pylori* infection were assigned to the HpDU-GC group. Patients with GU and DU disease and *H. pylori* infection (the HpGUDU-GC group) were excluded from the analysis because none of them developed GC. Data on demographics (age, gender, peptic ulcer complications and cancer treatment), GC clinical characteristics (location, pathological diagnosis, differentiation, T stage, Lauren's classification, atrophy of surrounding mucosa and IM), outcome of eradication therapy for *H. pylori* infection, EGD number and the duration until GC onset were recorded. The study protocol was approved by the Ethics Committee of Seoul National University Bundang Hospital (IRB number: B-1408/262-108).

Statistical analysis

The statistical analysis was performed using the Predictive Analytics Software 20.0 version for Windows package (SPSS Inc., IBM, Chicago, IL, United States). The mean ± SD for the quantitative variables were calculated. The student's *t*-test was used to evaluate continuous variables and the chi square and Fisher's exact tests were utilized to assess non-continuous variables. Additionally, univariate and multivariate analyses were performed to evaluate independent factors that determine GC development. A Cox's proportional hazards model was used to calculate the relative risk (corrected for age and gender) for each group. A *P* value of less than 0.05 was defined as clinically significant.

RESULTS

Patient characteristics

A schematic diagram of the study is shown in Figure 1. Between 2003 and 2013, 4485 patients were diagnosed with peptic ulcer disease. Of these patients, 2387 had GU and 2098 patients had DU disease. A total 121 of the patients were newly diagnosed with GC and previously with a peptic ulcer (GU or DU) with *H. pylori* infection. No patient previously diagnosed with GU and DU disease as well as *H. pylori* infection was newly diagnosed with GC. Of the 121 patients newly diagnosed with GC, 86 were from the HpGU-GC group and 35 were from the HpDU-GC group. The baseline characteristics of the enrolled patients are provided in Table 1. The average ages of the HpGU-GC and HpDU-GC groups were 62.2 ± 10.1 and 62.5 ± 13.2 years, respectively (*P* = 0.412). There were no statistically significant differences in gender distribution or peptic ulcer complications (Table 1). Three patients experienced bleeding, a complication of peptic ulcers, although it spontaneously stopped without endoscopic hemostatic therapy and the patients were treated medically with PPIs. In terms of GC treatment, the rates of ESD and surgery were 58.1% (50/86) and

Table 1 Baseline characteristics of the patients *n* (%)

	HpGU-GC group (*n* = 86)	HpDU-GC group (*n* = 35)	*P* value
Age (yr), mean ± SD	62.2 ± 10.1	62.5 ± 13.2	0.412
Gender			0.935
Male	68 (79.1)	28 (80.0)	
Female	18 (20.9)	7 (20.0)	
Complication of peptic ulcer	3 (3.5)	0 (0.0)	0.463
Treatment of cancer			0.728
ESD	50 (58.1)	18 (51.4)	
Surgery	36 (41.9)	17 (48.6)	

Hp: *Helicobacter pylori*; GU: Gastric ulcer; DU: Duodenal ulcer; GC: Gastric cancer; ESD: Endoscopic submucosal dissection.

41.9% (36/86), respectively, in the HpGU-GC group and 51.4% (18/35) and 48.6% (17/35), respectively, in the HpDU-GC group. The inter-group differences, however, were not statistically significant (Table 1).

Development of GC

In the HpGU-GC group, 86 patients (3.6%) developed GC during the follow-up period, whereas in the HpDU-GC group, 35 (1.66%) developed GC. The annual incidence was 0.41% in the HpGU-GC group and 0.11% in the HpDU-GC group. The GC characteristics are listed in Table 2. The rate of early GC was higher than that of advanced gastric cancer in both groups (HpGU-GC: 88.4% *vs* 11.6%; HpDU-GC: 80.0% *vs* 20.0%). The most common location of GC in the HpGU-GC group was the middle portion (52.3%) and the lower portion (65.7%) in the HpDU-GC group. Adenocarcinoma and intestinal type were, by World Health Organization criteria and Lauren's classification, the most common pathological features of GC in both groups, although the differences were not statistically significant. Pathologically, well-differentiated GCs were more common in the HpGU-GC (48.8%) and HpDU-GC (40.0%) groups but this was not statistically significant (Table 2). In both groups, the GC involvement was within the lamina propria (defined as the T1a stage), which was statistically significant ($P = 0.007$). The rate of moderate-to-severe atrophy of surrounding mucosa was 86% in the HpGU-GC group and 34.3% in the HpDU-GC group ($P = 0.041$). The rate of moderate-to-severe IM was 61.6% in the HpGU-GC group and 14.3% in the HpDU-GC group ($P = 0.037$). Notably, both of these rates were significantly higher in the HpGU-GC group than in the HpDU-GC group ($P < 0.05$). The eradication rate of *H. pylori* infection was 40.6% in the HpGU-GC group and 48.6% in the HpDU-GC group. The *H. pylori*-eradication rate in the HpDU-GC group was significantly higher than in the HpGU-GC group. However, the success rate of eradication therapy was lower than the failure rate in both groups ($P = 0.039$). The mean EGD from peptic ulcer diagnosis to GC development was 5.5 ± 3.2 in the HpGU-GC group and 4.9 ± 3.5 in the HpDU-GC group ($P = 0.076$). The mean time from peptic ulcer diagnosis to GC development was 3.5 ± 2.4 years in the HpGU-GC group and 3.1 ± 2.7 years in the HpDU-GC group ($P = 0.09$). In the univariate analysis, atrophy of surrounding mucosa, IM and eradication therapy for *H. pylori* infection were significantly associated with development of GC ($P < 0.05$; Table 2).

Table 2 Characteristics of the developed gastric cancer during follow-up *n* (%)

	HpGU-GC (*n* = 86)	HpDU-GC (*n* = 35)	*P* value	Univariate analysis
Annual incidence (yr)	0.41%	0.11%		
Type of cancer			0.534	-
Early gastric cancer	76 (88.4)	28 (80.0)		
Advanced gastric cancer	10 (11.6)	7 (20.0)		
Location of cancer			0.341	-
Upper	1 (1.2)	0 (0.0)		
Middle	45 (52.3)	12 (34.3)		
Lower	40 (46.5)	23 (65.7)		
Diagnosis of cancer			0.515	-
Adenocarcinoma	77 (89.5)	28 (80.0)		
Signet ring cell carcinoma	7 (8.1)	5 (14.2)		
Mixed carcinoma	2 (2.4)	2 (5.8)		
Differentiation of cancer			0.134	-
Well-differentiated	42 (48.8)	14 (40.0)		
Moderate-differentiated	31 (36.0)	11 (31.4)		
Poor-differentiated	13 (15.2)	10 (28.6)		
T-stage of cancer			0.007	-
T1a	59 (68.6)	16 (45.7)		
T1b	16 (18.6)	12 (34.2)		
T2	10 (11.6)	3 (8.7)		
T3	1 (1.2)	0 (0.0)		
T4	0 (0.0)	4 (11.4)		
Lauren's classification			0.083	-
Intestinal type	74 (86.0)	23 (65.7)		
Diffuse type	10 (11.6)	12 (34.3)		
Mixed type	2 (2.4)	0 (0.0)		
Atrophy of surrounding mucosa			0.041	0.038
Non-mild	12 (14.0)	23 (65.7)		
Moderate-severe	74 (86.0)	12 (34.3)		
Intestinal metaplasia			0.037	0.032
Non-mild	33 (38.4)	30 (85.7)		
Moderate-severe	53 (61.6)	5 (14.3)		
H. pylori eradication			0.039	0.041
Success	35 (40.6)	17 (48.6)		
Failure	51 (59.4)	18 (51.4)		
Mean number of endoscopy until GC onset, mean ± SD	5.5 ± 3.2	4.9 ± 3.5	0.076	-
Mean time until GC onset (yr)	3.5 ± 2.4	3.1 ± 2.7	0.09	-

Hp: *Helicobacter pylori*; GU: Gastric ulcer; DU: Duodenal ulcer; GC: Gastric cancer; Mixed carcinoma: Adenocarcinoma and signet ring cell carcinoma; Mixed type: Intestinal and diffuse type.

Prognosis of GC

Table 3 shows the GC prognoses for the two groups. The number of patients that had a recurrence of GC after ESD or surgery during the follow-up period was 6 (7.0%) in the HpGU-GC group and 2 (5.7%) in the HpDU-GC group, although this difference was not statistically significant. The end-of-study survival

Table 3 Prognosis of the developed gastric cancer *n* (%)

	HpGU-GC group (*n* = 86)	HpDU-GC group (*n* = 35)	*P* value
Recurrence of cancer	6 (7.0)	2 (5.7)	0.965
Prognosis of cancer			0.347
Alive	69 (80.2)	28 (80.0)	
Death	15 (17.5)	6 (17.2)	
Unknown	2 (2.3)	1 (2.8)	

Hp: *Helicobacter pylori*; GU: Gastric ulcer; DU: Duodenal ulcer; GC: Gastric cancer.

rate was 80.2% in the HpGU-GC group and 80.0% in the HpDU-GC group but the difference was not statistically significant (Table 3). The relative risk of GC development of the HpGU-GC group compared to that of the HpDU-GC group, as corrected for age and gender, was 1.71 (95%CI: 1.09-2.70, *P* = 0.02) according to a Cox proportional hazards model (Table 4).

DISCUSSION

Patients with *H. pylori* infection have a high risk of GC[2,3]. In fact, a recent review indicated that 2 million cases of cancer each year worldwide could be attributed to *H. pylori*, a key infectious agent leading to GC[21]. The EUROGAST study on diverse populations found a 6-fold increase in the risk of gastric adenocarcinoma development for patients with *H. pylori* infection compared to patients that were not infected[22]. There is a still much greater risk of adenocarcinoma in *H. pylori*-infected individuals younger than 30 years of age[23]. *H. pylori* infection has been associated with increases in both intestinal and diffuse types of gastric adenocarcinoma[23,24]. However, there appears to be a difference in the locations of gastric adenocarcinoma in *H. pylori*-infected patients. Distal gastric adenocarcinoma is much more likely to develop in *H. pylori*-infected patients than gastroesophageal junction adenocarcinoma[25]. In any case, despite the well-established and clear association between persistent *H. pylori* infection and gastric adenocarcinoma, only a small percentage of infected individuals will develop malignancies. This is likely due to a myriad of external and environmental factors that are believed to affect the disease course and progression. Factors that promote malignancy development include certain dietary influences such as high salt intake, red and processed meat consumption, and nitrosamines, while factors that can reduce the risk of malignancy include diets that are high in fresh foods and vegetables[26,27].

Hansson *et al*[7] investigated the risk of GC among patients with peptic ulcer disease in a Swedish population-based study. The authors reported that GC risk leveled off and stabilized after the first 3 and 2 years of GU and DU, respectively. GU patients had a relative risk of 1.8 throughout the follow-up period, whereas the relative risk for DU patients was only

Table 4 Relative risk of gastric cancer development adjusted by Cox's proportional hazard model

	Relative risk	95%CI	*P* value
Group			
HpDU-GC	1.00	-	-
HpGU-GC	1.71	1.09-2.70	0.02

Hp: *Helicobacter pylori*; GU: Gastric ulcer; DU: Duodenal ulcer; GC: Gastric cancer.

0.6. Female patients and patients who were younger than 50 years old were found to have a higher risk of GC than an age- and gender-matched background population. In the present study, the relative risk of the HpGU-GC group compared to that of the HpDU-GC group (as adjusted for age and gender using a Cox proportional hazards model) was 1.71 (95%CI: 1.09-2.70, *P* = 0.02), without inclusion of a reference to the success of *H. pylori* eradication therapy. This rate was very similar to the value of 1.8 reported for GU patients in the study by Hansson *et al*[7]. Patient age and gender were not associated with GC development.

Uemura *et al*[8] evaluated a large Japanese cohort with *H. pylori* infection over the course of a 5-year follow-up period and reported that even although all of the DU patients had *H. pylori* infection, none developed GC. On the other hand, the rate of *H. pylori* infection was 3.4% in GU patients but GC occurs in 5% of GU patients with *H. pylori* infection. These inconsistent results may be explained by the lower rate of mucosal atrophy in DU compared to GU patients.

In the present study, 86/2387 GU patients with *H. pylori* infection developed GC, representing an annual incidence rate of 0.41%. In contrast, only 35/2098 DU patients with *H. pylori* infection developed GC, corresponding to an annual incidence rate of 0.11%. This is markedly lower than that of GU patients and is consistent with a study by Uemura *et al*[8]. The most common site involved in both groups was the lamina propria (defined as stage T1a). This may reflect the increased possibility of early diagnosis of GC with regular EGD. The incidence rate and relative risk of GC development in GU patients with *H. pylori* infection were significantly higher than in DU patients with *H. pylori* infection. The *H. pylori* eradication rate in GU patients was significantly lower than in DU patients, although the success rate of eradication therapy was lower than the failure rate in both patient groups. This finding might reflect the possibility of an association between the high incidence rate, relative risk of GC development and low *H. pylori* eradication rate in GU patients with *H. pylori* infection.

One of the most important factors affecting variable patient outcomes is the extent of gastritis. GU and GC are associated with atrophic pangastritis and DU is associated with non-atrophic antral-predominant gastritis[13-15]. In the present study, the rates of

moderate-to-severe atrophy of the surrounding mucosa and IM were significantly higher in the HpGU-GC group than in the HpDU-GC group. These results are similar to those of a previous study which showed that the severity and extent of gastritis are important factors that determine disease prognosis after *H. pylori* infection[28]. Because intestinal crypts replace the specialized glands of atrophic gastritis in IM, it was thought that atrophic gastritis and IM were the same entity[29].

Recent studies have indicated that gastric carcinogenesis consists of multiple processes. IM, in accordance with the updated Sydney system, is considered to be an important step[30]. It is more closely associated with GC than atrophic gastritis as a premalignant lesion. The odds ratios reported for GC in superficial gastritis and atrophic gastritis according to IM severity are 29.3 and 17.4, respectively[31]. In another study, the annual incidence of GC was determined to be higher in patients with IM (0.25%) than in patients with atrophic gastritis (0.1%)[32]. Results such as these might constitute evidence supporting the multiple processes of gastric carcinogenesis identified by Correa[33]. Indeed, IM can be an important predictor of GC risk[34].

In a study that compared the histopathological features of GC patients with those of a healthy control population, the frequency of IM was higher in GC patients than in the healthy control population, even although the severity and extent of atrophic gastritis were similar in both groups[35]. These results suggest that for a population in which progression from atrophic mucosa to IM is possible, there is a higher risk of GC[35]. In the present study, the rates of moderate-to-severe atrophy of surrounding mucosa and IM were significantly higher in the HpGU-GC group than in the HpDU-GC group. Furthermore, atrophy of the surrounding mucosa and IM were significantly associated with GC development in a univariate analysis. These results were similar to those from previous studies[30-35], regardless of the success of *H. pylori* eradication therapy.

To our knowledge, this is the first study to have investigated the incidence of GC development in peptic ulcer patients with *H. pylori* infection and to have compared the clinical characteristics of GC between GU and DU patients. However, there are several limitations. Firstly, because this was a retrospective study based on patients with peptic ulcer diseases and *H. pylori* infection, we could not investigate the causes of failures in *H. pylori* eradication therapy, histories of drug intake (non-steroidal anti-inflammatory drug or aspirin) and the proportion of patients with *H. pylori* infection at the time of diagnosis with GU and DU disease. Secondly, we could not evaluate the association of the incidence of developing GC in both GU and DU patients and the success or failure *H. pylori* eradication. Selection bias may also exist. Thirdly, relevant genetic factors were not investigated. Fourthly, we did not analyze the relative risk of patients with peptic ulcer disease without *H. pylori* infection.

In summary, the incidence rate and relative risk of GC development in GU patients with *H. pylori* infection were significantly higher than in DU patients with *H. pylori* infection. The *H. pylori* eradication rate in GU patients was significantly lower than in DU patients, although the success rate of eradication therapy was lower than the failure rate in both groups of patients. Atrophy of the surrounding mucosa, IM and eradication therapy for *H. pylori* infection were significantly associated with GC development.

COMMENTS

Background

It is well established that *Helicobacter pylori* (*H. pylori*) infection is a strong risk factor for gastric cancer (GC). *H. pylori* infection has also been implicated in peptic ulcer diseases. There is an urgent need to elucidate the relationship between GC, peptic ulcers and *H. pylori* infection.

Research frontiers

There is controversy as to whether gastric and duodenal ulcers (DUs) have similar effects on GC development. Several animal and human studies have suggested that *H. pylori* infection is a risk factor for GC development.

Innovations and breakthroughs

This is the first study to investigate the incidence of GC development in peptic ulcer patients with *H. pylori* infection and to compare the clinical characteristics of GC between gastric ulcer (GU) and DU patients. The incidence rate and relative risk of GC development in GU patients with *H. pylori* infection were significantly higher than in DU patients with *H. pylori* infection. The *H. pylori* eradication rate in GU patients was significantly lower than in DU patients, although the success rate of eradication therapy was lower than the failure rate in both groups of patients. Atrophy of surrounding mucosa, IM and eradication therapy for *H. pylori* infection were significantly associated with GC development.

Applications

This study urges clinicians to confirm *H. pylori* infection and to start eradication therapy to prevent GC development in patients with peptic ulcers.

Terminology

H. pylori is a bacterium found in the stomach. It is linked to the development of gastritis, peptic ulcers and stomach cancer. To prevent recurrence in patients with these diseases, it is necessary to eradicate bacterial infections with *H. pylori*.

Peer-review

This study evaluated the incidence of GC development in patients with peptic GU and/or DU disease that were positive for *H. pylori* infection. The incidence rate and relative risk of GC development in patients with GUs were significantly higher than in patients with DUs. The findings are reasonable and make sense.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic value of neutrophil distribution in cholangiocarcinoma

Zhi-Yuan Mao, Guang-Qing Zhu, Mei Xiong, Li Ren, Li Bai

Zhi-Yuan Mao, Guang-Qing Zhu, Department of Oncology, Air Force General Hospital of PLA, Beijing 100142, China
Mei Xiong, Department of Blood Transfusion, Air Force General Hospital of PLA, Beijing 100142, China
Li Ren, Department of Pathology, Air Force General Hospital of PLA, Beijing 100142, China
Li Bai, Department of Oncology, Chinese PLA General Hospital, Beijing 100853, China
Author contributions: Mao ZY contributed to the study conception and design, experimental research and data analysis, and manuscript preparation; Zhu GQ performed the clinical studies; Xiong M contributed to the literature search and data acquisition; Ren L performed the statistical analysis; Bai L revised the manuscript.

Correspondence to: Dr. Zhi-Yuan Mao, Department of Oncology, Air Force General Hospital of PLA, No. 30 Fucheng Road, Haidian District, Beijing 100142, China. maozhiyuan666@163.com
Telephone: +86-10-66928571
Fax: +86-10-66928571
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Abstract

AIM: To explore the relationship of clinicopathological features and the distribution of neutrophils in the tumor microenvironment with the prognosis of cholangiocarcinoma.

METHODS: Two hundred and fifty-four formalin-fixed and paraffin embedded tissue blocks were analyzed, including tissues from cholangiocarcinoma (n = 254), and tumor adjacent tissues (n = 238). Tissue sections were stained for CD15 using immunohistochemical staining. CD15 expression was detected to identify the distribution of neutrophils in the local tumor microenvironment. The neutrophil density of the tumor tissues and the adjacent tumor tissues was detected to reflect their inflammatory status. Clinical data and follow-up information of cholangiocarcinoma patients who underwent surgery from January 2004 to December 2010 were analyzed retrospectively. The relationship between clinicopathological features and the distribution of neutrophils with prognosis of the patients were analyzed.

RESULTS: The positive expression level of CD15 was only significantly related to the TNM stage. CD15 expression was higher in tumor tissues than in adjacent tissues (73.6% *vs* 54.6%), with significant differences. Patients with high expression of CD15 had significantly shorter overall survival (OS) than those with low expression of CD15 (median overall survival time 39.77 mo *vs* 16.87 mo, P = 0.008). Patients with high CD15 expression had significantly shorter disease free survival time (DFS) than those with low expression of CD15 (median DFS 38.27 mo *vs* 16.83 mo, P = 0.029). COX multivariate analysis indicated that high CD15 expression in tumor tissues was an independent risk factor for predicting OS for patients with cholangiocarcinoma [P = 0.012, relative risk (RR) = 1.601], but it was not an independent risk factor for predicting DFS (P = 0.073, RR = 1.462).

CONCLUSION: Patients with high CD15 expression in cancer tissues had shorter DFS and OS. High expression of CD15 is an independent risk factor for OS.

Key words: Cholangiocarcinoma; Surgery; Neutrophils; CD15; Prognosis; Tumor microenvironment

Core tip: Inflammation is the 7th important feature to promote tumor development. However, whether inflammation promotes tumor growth in cholangiocarcinoma remains unclear. This study analyzed the inflammation in the tumor microenvironment of the cholangiocarcinoma tissues, and further elaborated the mechanisms of inflammation in cholangiocarcinoma occurrence, development and metastasis, and provided new insights into the treatment of cholangiocarcinoma.

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INTRODUCTION

Cholangiocarcinoma originates from epithelial tumors. Diagnosis is often delayed, and prognosis is poor. Clinicians and scientists around the world are committed to determining the etiology of cholangiocarcinoma. Most cholangiocarcinomas are primary. Research from the United States and Europe[1-4], found that hepatitis C is the most important risk factor for cholangiocarcinoma (intrahepatic cholangiocarcinoma in particular), but research from South Korea and China[5-7] found that hepatitis B is a risk factor in intrahepatic cholangiocarcinoma. A study from Japan confirmed the results from Europe and the United States[8]. These studies also confirmed that cirrhosis is a risk factor of cholangiocarcinoma. Primary sclerosing cholangitis may develop into cholangiocarcinoma (especially hilar cholangiocarcinoma), which is characterized by chronic inflammation complicated with liver damage and, possibly, the proliferation of progenitor cells. The incidence rate of cholangiocarcinoma among these patients is 5%-10%[9-12]. Approximately 50% of the patients with primary sclerosing cholangitis were later diagnosed with cholangiocarcinoma within 24 mo[9-13]. The average age of these patients is about 40 years old, while the average age of the general population who develop cholangiocarcinoma is about 70 years old[4,14]. Although there are many risk factors that might promote the development of primary sclerosing cholangitis into cholangiocarcinoma, they are not sufficient to guide risk stratification for disease surveillance[11,15,16]. A full understanding of the biological characteristics of cholangiocarcinoma and important factors that may affect treatment strategies, and implementation of standardized and individualized treatment are the keys to the treatment of cholangiocarcinoma. The relationship between inflammation and cancer development has been the concern of many scholars, and this relationship is also the focus of the present study. In 2011, Hanahan *et al*[17] in a review published in Cell proposed ten important features of malignant tumors, including self-sufficiency in growth signals, insensitivity to antigrowth signals, resisting cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, tumor promotion inflammation, avoiding immune destruction, deregulating cellular energetics, genome instability and mutation. Among these, the tumor-promoting inflammation represents the seventh most important feature. In the course of infection or injury repair, a large number of inflammatory cells *in vivo* aggregate and release large amounts of cytokines, chemokines, growth factors and cytotoxic mediators, forming a local inflammatory microenvironment. In the process of tumor formation, development and metastasis, cell death and appreciation would occur in a large number of cells, which would produce a large amount of inflammatory mediators. Tumor cells can also produce the factors released through accumulation of inflammatory cells, and these factors can lead to the formation of blood vessels and inflammatory responses. The persistent interactions of inflammatory mediators and cytokines in inflammatory cells lead to the long-term presence of a tumor inflammatory microenvironment, thereby promoting tumorigenesis, accelerating tumor development, and playing an important role in the various stages of tumor metastasis. Neutrophils are major inflammatory cells in the tumor microenvironment. CD15 is one of the markers of mature granulocytes. We used CD15 staining to evaluate the distribution of neutrophils in tumor microenvironment, and to explore the relationship of clinicopathological features and the neutrophil distribution in the tumor microenvironment with prognosis.

MATERIALS AND METHODS

Tumor tissues

Two hundred and ninety one resected specimens from patients who underwent operations for cholangiocarcinoma from January 2004 to December 2010 at the Department of General Surgery, General Hospital of Air Force, China, were analyzed retrospectively. Clinical data (including age, gender and tumor pathological features) were collected. The patients had a clear diagnosis of cholangiocarcinoma and had neither received radiotherapy or chemotherapy before surgery, nor preoperative liver transplantation. Those with a pathological diagnosis of sarcoma, mixed cell carcinoma or hepatocellular carcinoma were excluded. After surgery, all tissue samples were fixed in 4% formalin for 24 h, and then embedded in paraffin for hematoxylin and eosin (HE) and immunohistochemical

Table 1 Relationship between CD15 expression and clinicopathological characteristics in tumor tissues *n* (%)

Clinicopathological parameter	Group	*n*	CD15 expression in tumor tissues			
			High	Low	χ^2	*P* value
Age (yr)	< 60	119	92 (77.3)	27 (22.7)	1.569	0.210
	≥ 60	135	95 (70.4)	40 (29.6)		
Gender	Male	170	128 (75.3)	42 (24.7)	0.740	0.390
	Female	84	59 (70.2)	25 (29.8)		
Differentiation	Well differentiated	17	11 (64.7)	6 (35.3)	1.093	0.579
	Moderately differentiated	140	102 (72.9)	38 (27.1)		
	Poorly differentiated	97	74 (76.3)	23 (23.7)		
TNM stage	Ⅰ	136	92 (67.6)	44 (32.4)	8.589	0.032
	Ⅱ	71	54 (76.1)	17 (23.9)		
	Ⅲ	34	31 (91.2)	3 (8.8)		
	Ⅳ	13	10 (76.9)	3 (23.1)		
Lymphatic metastasis	Metastasis	39	33 (84.6)	6 (15.4)	2.867	0.090
	No metastasis	215	154 (71.6)	61 (28.4)		
Neural invasion	Yes	82	56 (68.3)	26 (31.7)	1.771	0.183
	No	172	131 (76.2)	41 (23.8)		
Vascular invasion	Yes	14	10 (71.4)	4 (28.6)	0.037	0.765
	No	240	177 (73.8)	63 (26.2)		
Infiltration	Non- infiltrative	148	106 (71.6)	42 (28.4)	0.731	0.393
	Infiltrative	106	81 (76.4)	25 (23.6)		
Size	< 3 cm	138	95 (68.8)	43 (31.2)	3.557	0.059
	⩾ 3 cm	116	92 (79.3)	24 (20.7)		
Edge	Positive	63	46 (73.0)	17 (27.0)	0.016	0.900
	Negative	191	141 (73.8)	50 (26.2)		
Position	Intrahepatic	69	52 (75.4)	17 (24.6)	0.148	0.701
	Extrahepatic	185	135 (73.0)	50 (27.0)		
Surgical approach	Radical	243	179 (73.7)	64 (26.3)	0.000	1.000
	Palliative	11	8 (72.7)	3 (27.3)		

TNM stage: Tumor node metastasis stage.

(IHC) staining. Sections were cut at 4 μm. HE-stained samples were individually examined microscopically by two independent pathologists (Ren L and Mao ZY). The clinicopathological characteristics of the tumors are shown in Table 1.

IHC for CD15

Mouse anti-CD15 Monoclonal Antibody (ZM-0037, Working Solution, Zhongshan Golden Bridge Biotechnology Inc., Beijing, China) was used and heat-induced epitope retrieval (pressure cooker preheat for 5 min in citrate buffer, pH = 6) was used before to CD15 staining. Primary antibodies were then added and incubated for 2 h at 37 ℃. Slides were processed on an immunostainer (LabVision Autostainer 360, Fujian, China). The primary antibody was replaced by phosphate-buffered saline as a negative control to assess the specificity of the antibodies. Hematoxylin-counterstained sections were mounted in aqueous mounting medium and observed under a light microscope.

The IHC staining (percentage of stained cells × staining intensity) for CD15 was scored for each case after semi-quantitative evaluation by two independent pathologists (Ren L and Mao ZY). The percentage of stained cells observed in every 100 positive cells was used as the percentage of positive cells. Staining intensity was scored as negative (0), weak (1), moderate (2), or strong (3). The final score of the specimen area were: percentage of positive cells × staining intensity × 100. The final score for each antibody in immunohistochemical staining ranged from 0 to 300 points. With the median score as a borderline, antibodies were assigned to a high expression group and a low expression group, and the median value was assigned to the high expression group.

Statistical analysis

SPSS19.0 statistical software was used for statistical analysis. A χ^2 test was used to compare rates, and Spearman's test was used for correlation analysis. Kaplan-Meier survival curves were used to calculate survival, the log-rank test was used for univariate analysis, and COX regression was adopted for multivariate survival analysis. A *P* value < 0.05 was considered statistically significant.

RESULTS

Expression of CD15 in tumor tissues and adjacent tissues

The expression of CD15 in the tumor tissues and adjacent tissues is presented in Table 2. CD15 staining in membrane and perinuclear granules is shown in Figure 1. The CD15 expression rate in tumor tissues was 73.6% (187/254), and was 54.6% in adjacent

Table 2 CD15 expression in tumor tissues and adjacent tissues

Group	*n*[1]	High CD15 expression	Low CD15 expression
Tumor tissues	254	187	67
Adjacent tissues	238	130	108

[1]The number of cases is different from the previous because of off-chip effects during the dyeing process or no tumor tissue.

tissues (130/238). CD15 expression in tumor tissues was higher than in adjacent tissues (P = 0.000).

Correlations between CD15 expression levels and clinicopathological characteristics of tumors

The correlations between CD15 expression and clinicopathological features are shown in Table 3. The positive expression rate of CD15 was significantly related to the TNM stage (Figure 2).

Correlation between CD15 expression in tumor tissues and prognosis

Patients with high expression of CD15 had significantly shorter overall survival (OS) than those with low expression, and the difference was statistically significant (P = 0.008). Patients with high CD15 expression had significantly shorter disease free survival time (DFS) than those with low CD15 expression (P = 0.029). Based on COX multivariate analysis, high CD15 expression in cancer tissues was an independent risk factor in predicting OS for patients with cholangiocarcinoma [P = 0.012, relative risk (RR) =1.601]. Univariate analysis showed that patients with high CD15 expression in tumor tissues had significantly shorter progression-free survival and overall survival than those with low expression, and the difference was statistically significant (median DFS: 38.37 mo *vs* 16.83 mo; median OS: 39.77 mo *vs* 16.87 mo, P = 0.029 and 0.008, respectively), as shown in Figures 3 and 4.

Multivariate analysis in CD15 expression and prognosis of patients with cholangiocarcinoma

The clinical and pathological features of patients with cholangiocarcinoma were analyzed using univariate analysis, and the statistically significant results obtained and CD15 levels were then included in COX multivariate analysis to observe the effects of the indicators on prognosis. As shown in Table 3, high CD15 expression in tumor tissues is an independent risk factor for OS of the patients with cholangiocarcinoma, and patients with high CD15 expression in tumor tissues had a 1.601 times higher risk of death compared with patients with low CD15 expression.

DISCUSSION

Although inflammation is a risk factor for the onset of cholangiocarcinoma, the mechanism is not fully understood. Many experiments found that the relationship between a tumor and inflammation can be studied based on the local inflammatory tumor microenvironment, and a systemic inflammatory response. For example, C-reactive protein and IL-6 are considered to reflect the level of markers of systemic inflammation. Increased levels can predict the prognosis of various tumors, and the higher the level of the inflammation, the worse the prognosis[18,19]. Coincident with our results, Jensen *et al*[20] reported that the presence of intratumoral neutrophils was an independent prognostic factor for poor OS following nephrectomy in localized clear cell tumors, such as renal cell carcinoma. Li *et al*[21] confirmed recently that intratumoral neutrophils were a poor prognostic factor for hepatocellular carcinoma following resection. Furthermore, elevated neutrophil and monocyte counts in peripheral blood are associated with poor survival in patients with metastatic melanoma[22,23], small-cell lung carcinoma[24], gastric cancer[25-27] and other tumors[28]. All these studies indicated that patients with tumors infiltrating neutrophils have a poor prognosis, which may offer some help in a preliminary clinical prognosis of cancer patients, and provide clues for treatment.

CD15 is a specific marker of neutrophils[29]. The white blood cell count is an important index in the detection of inflammation *in vivo*. Inflammation can promote tumor angiogenesis, growth and invasion[30-32]. Neutrophils account for 50%-70% of total circulating leukocytes[33,34]. Neutrophils are the most abundant white blood cells, which help the body against invading microorganisms. Therefore, the number of neutrophils is an important inflammatory marker. Currently the neutrophil-to-lymphocyte ratio is studied extensively, and the ratio is used to assess the overall survival of cancer patients as a simple and effective indicator. A higher ratio indicates a poor overall survival, and a risk of early relapse[35-37]; thus, an increased neutrophil count and CD15 expression. This study analyzed the effects of CD15 expression in local inflammatory status on DFS and OS, and found that CD15 expression in cancer tissues was significantly higher than in the adjacent tissues. By univariate analysis, CD15 expression in cancer tissues affected significantly the DFS and OS, and high CD15 expression in cancer tissues predicted higher risk of tumor recurrence and mortality. CD15 is a primary marker for neutrophils, high CD15 expression indicates increased neutrophil count and reduced lymphocyte count. The release of large amounts of cytokines and other inflammatory cytokines may contribute to an increase in tumor metastasis and tumor angiogenesis[38-43]. Therefore, the CD15 expression in cancer tissues suggested poor prognosis in this study, which is consistent with the findings mentioned above. CD15 positive neutrophils may predict poor prognosis, especially for the prediction of OS, but not for DFS. However, prediction of OS could help in the treatment of postoperative

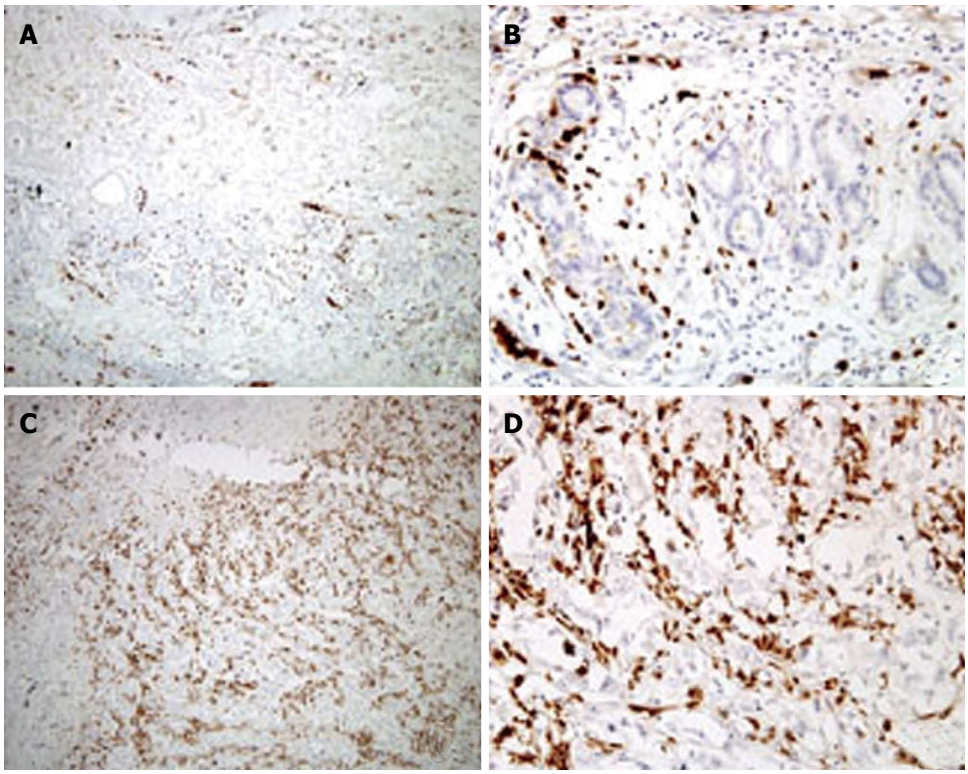

Figure 1 Immunophenotypes of the investigated antigens in tumor tissues. CD15 staining in membrane and perinuclear granules. A: Low CD15 expression, magnification × 100; B: Low CD15 expression, magnification × 400; C: High CD15 expression, magnification × 100; D: High CD15 expression, magnification × 400.

Table 3 Multivariate survival analysis of CD15 expression in cancer tissues and clinicopathological features

Parameters	Risk of recurrence				Risk of death			
	P value	Exp(B)	95%CI for Exp(B)		*P* value	Exp(B)	95%CI for Exp(B)	
			Lower	Upper			Lower	Upper
Differentiation	0.004	1.545	1.148	2.079	0.007	1.43	1.103	1.853
TNM stage	0.001	1.535	1.185	1.989	0.013	1.324	1.061	1.652
Lymphatic metastasis	0.854	0.952	0.562	1.611	0.114	1.427	0.918	2.217
Neural invasion	0.532	1.134	0.764	1.684	0.827	1.039	0.736	1.468
Vascular invasion	0.227	0.594	0.255	1.382	0.965	0.986	0.523	1.859
Infiltration	0.742	0.928	0.595	1.448	0.585	1.112	0.759	1.63
Size	0.943	1.014	0.692	1.487	0.758	0.949	0.679	1.326
Edge	0.014	1.014	0.692	1.487	0.007	1.641	1.148	2.347
Position	0.551	0.859	0.521	1.416	0.466	0.849	0.546	1.319
Surgical approach	0.575	1.258	0.564	2.804	0.161	1.62	0.826	3.178
CD15	0.073	1.462	0.965	2.215	0.012	1.601	1.107	2.314

TNM: Tumor node metastasis.

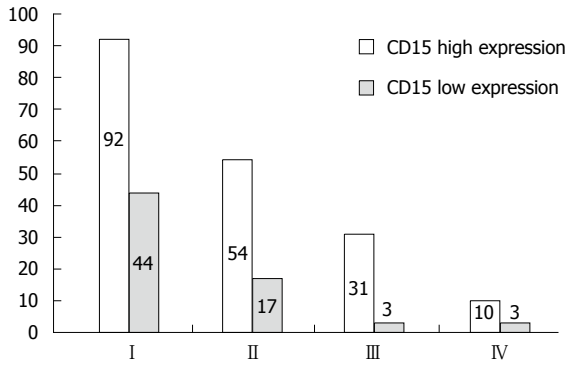

Figure 2 Histogram of the correlation between CD15 expression in tumor tissues and tumor node metastasis staging. TNM: Tumor node metastasis.

cholangiocarcinoma, and CD15 immunohistochemical staining results could predict the survival of patients earlier, such that intervention can be started earlier, which would extend the survival of patients.

This study used inflammation-related prognostic indicators to predict the prognosis of patients with cholangiocarcinoma. The local inflammatory status in the tumor microenvironment was evaluated by CD15 immunohistochemical staining. The results were scored to detect the distribution of cholangiocarcinoma inflammatory cells in the tumor microenvironment and to analyze the correlation between the distribution of cholangiocarcinoma inflammatory cells in the tumor microenvironment and local inflammation and

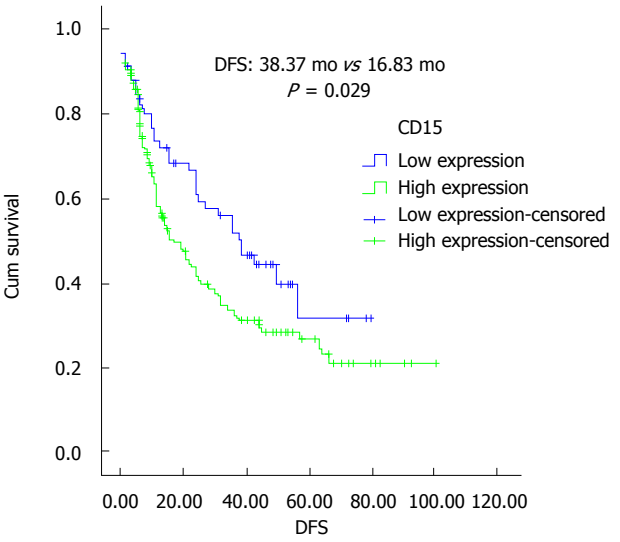

Figure 3 CD15 expression in tumor tissues and disease free survival time. DFS: Disease free survival time.

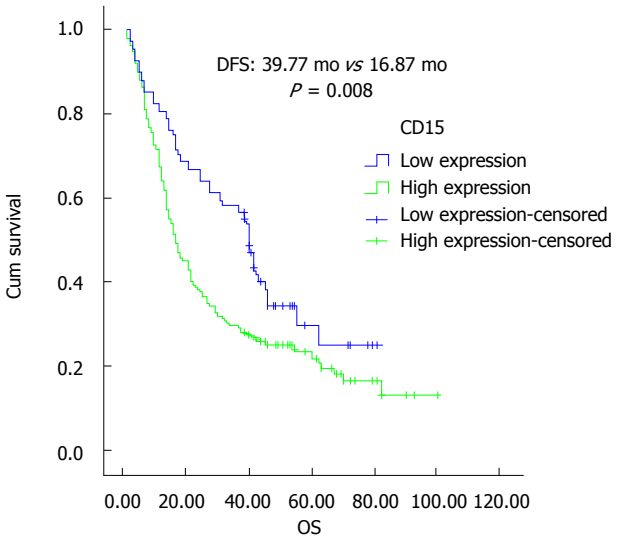

Figure 4 CD15 expression in tumor tissues and overall survival. OS: overall survival.

prognosis, to reflect the relationship between inflammation and cholangiocarcinoma.

There are currently no approved targeted agents for cholangiocarcinoma[44]. However, drug-related genes have been detected, including KRAS, PIK3CA, MET, EGFR, BRAF and NRAS[45]. In some cholangiocarcinomas, gene mutations occur, but targeted therapy is still not effective, possibly because cholangiocarcinoma gene mutation is relatively complex, involving mutations of two or more genes[46]. Yoshikawa *et al*[47] used Vandetanib to treat cholangiocarcinoma and inhibit both VEGFR and EGFR signalling appears a promising therapeutic approach for cholangiocarcinoma. The absence of KRAS mutation and the presence of EGFR amplification may be potential predictive molecular markers of sensitivity to EGFR-targeted therapy in cholangiocarcinoma.

El-Khoueiry *et al*[48] used Sorafenib in two clinical studies for unresectable or metastatic gallbladder carcinoma and cholangiocarcinoma treatment, but did not achieve objective responses. Patients with biliary cancers receiving this drug showed some therapeutic benefit. Additional studies with Sorafenib in combination with chemotherapy or other targeted agents may be warranted. Takahashi *et al*[49] suggested that Axitinib is a promising therapy for vascular endothelial growth factor-expressing cholangiocarcinoma, irrespective of tumor origin and Gemcitabine sensitivity. El-Khoueiry *et al*[50] reported that Sorafenib and Erlotinib did not have promising clinical activities in an unselected population of patients with biliary cancers. Improved patient selection, based on tumor biology and molecular markers, is critical for future evaluation of targeted therapies in this disease. Based on several promising phase Ⅱ clinical trials currently underway, the most promising targeted agents for cholangiocarcinoma thus far include anti-EGFR agents, MEK inhibitors and anti-angiogenic agents[44].

In summary, distribution of local inflammatory cells in patients with cholangiocarcinoma in the tumor microenvironment is valuable to assess prognosis. The local inflammatory state, in varying degrees, reflects the *in vivo* state of chronic inflammation in patients with malignant tumors. The chronic inflammatory state and the resulting immune status deserve further study and are meaningful for subsequent treatment of cancers. These results indicate that neutrophil distribution in various cancers might indicate poor prognosis and may serve as a target for clinical treatment.

COMMENTS

Background

Cholangiocarcinoma accounts for 3% of all gastrointestinal tumors and is the second most common tumor in hepatobiliary cancers. A full understanding of the biological characteristics of cholangiocarcinoma and the adoption standardized, individualized treatment are the key to cholangiocarcinoma treatment. Clinicians and scientists around the world are committed to determining the etiology of cholangiocarcinoma to permit early diagnosis and treatment of cholangiocarcinoma. This study investigated the inflammatory cell distribution in the tumor microenvironment and further elaborated the mechanisms of inflammation in cholangiocarcinoma occurrence, development and metastasis, to provide new insights into the treatment of cholangiocarcinoma.

Research frontiers

In this study, the authors investigated the expression of CD15 by immunohistochemical (IHC) staining to examine inflammatory state of cholangiocarcinoma. The results are helpful to understand the occurrence, development and metastasis of cholangiocarcinoma.

Innovations and breakthroughs

In this study, the authors explored the expression of CD15 to reflect inflammatory state of cholangiocarcinoma: the expression of CD15 had been seldom detected in cholangiocarcinoma. The results could help explain the occurrence, development and metastasis of cholangiocarcinoma.

Applications

IHC staining of CD15 to mark neutrophils and to reflect the inflammation caused by cholangiocarcinoma is a fast, convenient and economical method in clinical applications.

Terminology

Cholangiocarcinoma originates from epithelial tumors. Diagnosis is often

delayed and prognosis is poor. Cholangiocarcinoma accounts for 3% of all gastrointestinal cancers, and is the second most common tumor in hepatobiliary cancers.

Peer-review

The authors conducted an IHC analysis of CD15 to assess the neutrophil distribution and to further reflect the inflammatory state of cholangiocarcinoma. The study is very interesting.

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ORIGINAL ARTICLE

Retrospective Study

Laparoscopic resection of lower rectal cancer with telescopic anastomosis without abdominal incisions

Shi-Yong Li, Gang Chen, Jun-Feng Du, Guang Chen, Xiao-Jun Wei, Wei Cui, Fu-Yi Zuo, Bo Yu, Xing Dong, Xi-Qing Ji, Qiang Yuan

Shi-Yong Li, Gang Chen, Jun-Feng Du, Guang Chen, Xiao-Jun Wei, Wei Cui, Fu-Yi Zuo, Bo Yu, Xing Dong, Xi-Qing Ji, Qiang Yuan, Department of General Surgery, General Hospital of PLA Beijing Military Command, Beijing 100700, China

Author contributions: Li SY, Chen Gang, Yu B and Ji XQ designed the surgical strategy; Li SY, Chen Gang, Cui W, Yuan Q and Du JF performed the operation; Wei XJ, Cui W, Dong X, Zuo FY and Chen G analyzed data; Li SY and Du JF wrote the paper.

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Ethics approval: This is a retrospective clinical study, therefore, no approval from the Ethics Committee is required due to the nature of the study design. Authors apply an exemption of clinical trial registration.

Informed consent: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest: The authors confirm that this article content has no conflict of interest.

Data sharing: No additional data are available.

Correspondence to: Shi-Yong Li, MD, Director, Department of General Surgery, General Hospital of PLA Beijing Military Command, 5 Nanmencang, Dongsi Shitiao, Beijing 100700, China. lisybz@163.com
Telephone: +86-10-66721188
Fax: +86-10-66721188

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Abstract

AIM: To assess laparoscopic radical resection of lower rectal cancer with telescopic anastomosis through transanal resection without abdominal incisions.

METHODS: From March 2010 to June 2014, 30 patients (14 men and 16 women, aged 36-78 years, mean age 59.8 years) underwent laparoscopic radical resection of lower rectal cancer with telescopic anastomosis through anus-preserving transanal resection. The tumors were 5-7 cm away from the anal margin in 24 cases, and 4 cm in six cases. In preoperative assessment, there were 21 cases of $T_1N_0M_0$ and nine of $T_2N_0M_0$. Through the middle approach, the sigmoid mesentery was freed at the root with an ultrasonic scalpel and the roots of the inferior mesenteric artery and vein were dissected, clamped and cut. Following the total mesorectal excision principle, the rectum was separated until the anorectal ring reached 3-5 cm from the distal end of the tumor. For perineal surgery, a ring incision was made 2 cm above the dentate line, and sharp dissection was performed submucosally towards the superior direction, until the plane of the levator ani muscle, to transect the rectum. The rectum and distal sigmoid colon were removed together from the anus, followed by a telescopic anastomosis between the full thickness of the proximal colon and the mucosa and submucosal tissue of the rectum.

RESULTS: For the present cohort of 30 cases, the mean operative time was 178 min, with an average of 13 positive lymph nodes detected. One case of postoperative anastomotic leak was observed, requiring temporary colostomy, which was closed and recovered 3 mo later. The postoperative pathology showed T_1-$T_2N_0M_0$ in 19 cases and $T_2N_1M_0$ in 11 cases. Twelve months after surgery, 94.4% patients achieved anal function Kirwan grade 1, indicating that their anal

function returned to normal. The patients were followed up for 1-36 mo, with an average of 23 mo. There was no local recurrence, and 17 patients survived for > 3 years (with a survival rate of 100%).

CONCLUSION: Laparoscopic radical resection of lower rectal cancer with telescopic anastomosis through transanal resection without abdominal incisions is safe and feasible.

Key words: Laparoscopic resection; Rectal neoplasms; Anus-preserving rectectomy; Telescopic anastomosis

Core tip: This study assesses the laparoscopic radical resection of lower rectal cancer with telescopic anastomosis through transanal resection without abdominal incisions. Thirty cases of lower rectal cancer have been treated using this procedure with satisfactory outcomes in follow-up observation. We concluded that laparoscopic radical resection of lower rectal cancer with telescopic anastomosis through transanal resection without abdominal incisions is safe and feasible.

Li SY, Chen G, Du JF, Chen G, Wei XJ, Cui W, Zuo FY, Yu B, Dong X, Ji XQ, Yuan Q. Laparoscopic resection of lower rectal cancer with telescopic anastomosis without abdominal incisions. *World J Gastroenterol* 2015; 21(16): 4969-4974 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4969.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4969

INTRODUCTION

Procedures for anus-preserving radical resection for lower rectal cancer are of current research interest[1-5]. In recent years, laparoscopic radical surgery of rectal cancer has been widely carried out[6-8], but it generally requires an assisting abdominal incision for removal of tumor specimens, which prevents it from being completely minimally invasive[9-11]. We have completed 420 cases of telescopic anastomosis for anal preservation in radical resection of lower rectal cancer[3]. We first reported laparoscopic radical resection of lower rectal cancer with telescopic anastomosis through transanal resection without abdominal incisions in 2011[12,13], which is an abdominal incision-free and scar-free completely minimally invasive technique. So far, 30 cases of lower rectal cancer have been treated using this procedure with satisfactory outcomes in follow-up observation.

MATERIALS AND METHODS

Patient data

Thirty patients, including 14 men and 16 women, were recruited. The ages ranged from 36 to 78 years (mean: 59.8 years). The tumors were 5-7 cm away from the anal margin in 24 cases, and 4 cm in six cases. They were all confirmed as high-grade rectal adenocarcinoma by preoperative pathology. The diagnoses were confirmed by colonoscopy biopsies in all cases. Barium enema, digital rectal examination, and rectal endoscopy were used to determine the distance between the lower tumor edge and the anal margin. Pelvic and liver magnetic resonance imaging or computed tomography (CT) examination, as well as transrectal ultrasonography, was carried out to assess the depth of tumor invasion, presence of liver metastasis and lymph node enlargement. Preoperative assessment was $T_1N_0M_0$ in 21 cases and $T_2N_0M_0$ in nine.

Study inclusion and exclusion criteria

Inclusion criteria were: (1) lower rectal cancer with the lower edge 5-7 cm from the anal margin; (2) T1/T2N0M0 highly differentiated small adenocarcinomas; (3) early rectal cancer confined to the bowel wall, tumor diameter $\leqslant$ 3 cm, invasion less than or equal to half the circle of the intestinal wall; and (4) tubular adenomas, which are villous tubular and glandular cancerous tumors for which the resection margin is > 1 cm; highly differentiated adenocarcinomas with resection of the lower edge > 2 cm; and poorly differentiated adenocarcinomas with resection of the lower edge > 3 cm.

Exclusion criteria were: (1) the lower edge of tumor < 5 cm from the anal margin; (2) the preoperative stage of tumor > T1/T2N0M0; (3) tumor diameter > 3 cm or invasion more than half the circle of the intestinal wall; and (4) overweight patient with BMI > 30 or with narrow pelvis.

Surgical techniques

Abdominal surgical procedures: After successful anesthesia, the patient was placed in a bladder lithotomy position. Following the four-port trocar operation, the umbilical camera system was placed in the observation port, and the working port was created at the right lower quadrant. A trocar was placed at each incision of the right middle and left lower quadrants of the abdomen. With the patient's head facing down, the surgical field was revealed. Through the middle approach, the sigmoid mesentery was freed at the root with an ultrasonic scalpel, the adipose tissue and lymph nodes around the roots of the inferior mesenteric artery and vein were dissected, and the vascular root was clamped and cut. Bilateral ureteral tracts were protected. Following the total mesorectal excision (TME) principle, the rectum was dissected along the connective tissue between the visceral and parietal layers of the presacral space posterior to the rectum, to the anorectal ring and 3-5 cm from the distal end of the tumor. Caution

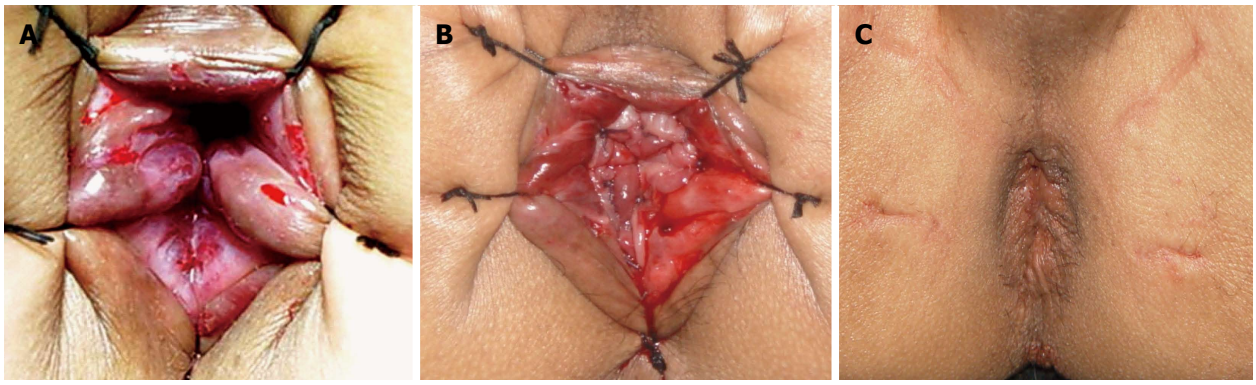

Figure 1 Anal operation. A: Surgical field exposed above the dentate line using the five-stitch suspension method; B: Telescopic anastomosis is completed; C: Anus after removal of the suspension sutures.

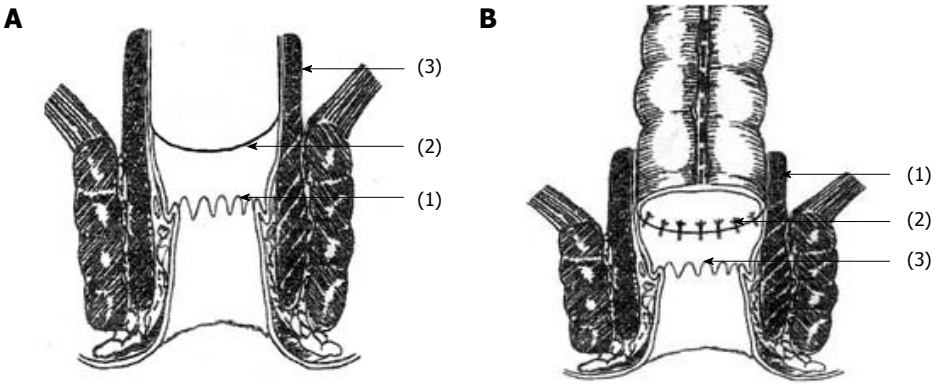

Figure 2 Rectal mucosa and telescopic anastomosis. A: Rectal mucosa is peeled off while retaining the rectal sheath: (1) Dentate line; (2) Rectal mucosa is peeled 2-4 cm from 2 cm above the dentate line; (3) Intact rectal muscle sheath 2-4 cm; B: Telescopic anastomosis: (1) Four stitches for telescopic colon seromuscular layer and rectal sheath; (2) Telescopic full-thickness colon and residual rectal submucosa; (3) Anatomosis is 1-2 cm above the dentate line.

was exercised to protect the pelvic autonomic nerve plexus. The cutting margin was 10 cm superior to the tumor. The corresponding mesentery was dissected using an ultrasonic scalpel. After completion of the perineal surgery, a large amount of warm distilled water was used to rinse the surgical field and a port was made to the right lower abdomen for placement of pelvic tube drainage. The trocars were removed and all ports closed. As a result, no auxiliary abdominal incision was made, leaving only four 0.5-1.0-cm small holes.

Anal operation: After extraepidural administration to fully relax the anal sphincter, the anus was dilated up to 4-6 fingers. Using the five-stitch suspension method[3,14] to reveal the field of the anal canal above the dentate line (Figure 1A), the anorectal tract was exposed using a double-leaf anal retractor. After submucosal circular injection of 1:10000 adrenaline saline 1.0 cm above the dentate line, to significantly elevate the submucosal tissue, an electric knife was used to cut open the mucosa 1.5-2.0 cm above the dentate line. Sharp dissection was performed along the rectal mucosa, upwards, to peel off the rectal mucosa for 2-4 cm, to reach the levator ani muscles, and the rectum was cut circularly[3]. The rectal tumor and distal sigmoid colon were removed from the anus together, and the sigmoid colon was cut proximally about 10 cm from the tumor, and the proximal colon was dragged out of the anus through the rectum. The distal rectal margin was sent for frozen section examination. The intact rectal muscular sheath of 2-4 cm was preserved about 2 cm above the dentate line, as well as the entire internal and external anal sphincter (Figure 2A). The distal colon was pulled out of the anus through the rectal muscular sheath, and at 2.0 cm above the dentate line set between the distal colon and rectal muscle sheath and fixed using the four-pin fixation method, and sutured intermittently at 12, 3, 6 and 9 points, respectively[5], to reduce tension and fix the connection. The full thickness of the proximal colon and the rectal and intestinal mucosa above the dentate line were connected using absorbable sutures with 8-12 stitches (Figure 1B and Figure 2B). After confirmation that the anastomosis had a satisfactory blood supply in a tension-free state, a piece of Vaseline gauze was inserted into the anal canal for support. The suspension sutures were removed, and the anastomotic portion was gently

Table 1 Patient data

	No. of patients (n = 30)
Age (yr)	36-78 (mean 59.8)
Sex	
Male	14
Female	16
Distance from lower tumor edge to anal margin (cm)	
5-7	24
4	6
Preoperative tumor stage (n)	
Ⅰ	28
Ⅱ	2
Ⅲ	0
Ⅳ	0
Postoperative tumor stage (n)	
Ⅰ	19
Ⅱ	0
Ⅲ	11
Ⅳ	0
Postoperative diagnosis of rectal cancer (n)	
Intermediately to highly differentiated	18
Intermediately differentiated	8
Poorly differentiated	2
Adenoma	2
Preventive colostomy (n)	
No preventive colostomy	29
Preventive colostomy	1
Operative time (min)	178 (range: 150-210)
Blood loss (mL)	60 (range: 30-300)
Hospital stay (d)	12 (range: 7-21)

returned into the anus to complete the anastomosis between the full-thickness colon and rectal mucosa and submucosa (Figure 1C). The perineum was then covered with sterile dressing. Double-lumen presacral drainage was placed as routine, penetrating the anal skin, fixed on the skin, and connected with suction or a sterile bag, which was usually removed after 2-5 d.

Follow-up

According to the 2011 National Comprehensive Cancer Network guidelines for postoperative monitoring and follow-up, a medical history was taken and a physical examination was conducted once every 3 mo for 2 years, then once every 6 mo for 3 years, and once every 12 mo afterwards. Carcinoembryonic antigen assessment was carried out for tumors at T2 or above once every 3-6 mo after surgery for 2 years, and once every 6 mo until 5 years after surgery. Colonoscopy or proctoscopy of the anastomoses was performed once every 6 mo for 5 years for early detection of local recurrence. At the first 2-5 years after surgery, patients at Stages Ⅱ or Ⅲ received thoracic, abdominal, and pelvic CT scans each year for early detection of lung and liver metastases. The endpoint of follow-up was death or the end of the study.

Anal functional assessment

A postoperative questionnaire survey was conducted for the Kirwan anal function assessment.

RESULTS

Surgery was completed successfully in all 30 patients, with an average length of operation of 178 min, including about 127 min for abdominal surgery and about 51 min for anal anastomosis. Mean intraoperative blood loss was 76 mL, and 13 positive lymph nodes were detected on average. Postoperative bowel movement occurred 3 d after surgery. There were no postoperative complications related to the surgery. The patients were discharged 12 d after surgery on average. The postoperative pathology showed T_1-$T_2N_0M_0$ in 19 cases and $T_2N_1M_0$ in 11. In the present study, no patient received preoperative radiotherapy, and patients with greater than T2 stage received 7-12 cycles of postoperative systemic chemotherapy with the mFOLFOX6 protocol (oxaliplatin, 5-fluorouracil and calcium folinate). Eleven patients with $T_2N_1M_0$ stage were given postoperative pelvic radiotherapy at a total dosage of 10-20 Gy before adjuvant chemotherapy. No abdominal incision was made, thereby achieving completely minimally invasive scar-free cosmetic outcomes. All of the 30 patients underwent a radical resection without positive circumferential resection of the margin and without positive distal resection margin (resection of the lower edge > 2 cm). The patients were followed up for 1-36 mo, with a mean of 23 mo. Early after surgery, defecation was 2-6 times/d, and the patients received two compound diphenoxylate tablets orally daily. Twelve months after surgery, 91.3% (21/23) of patients achieved anal function Kirwan grade 1, indicating that their anal function returned to normal. No patient received intraoperative diverting stoma, and one case of postoperative anastomotic leak was observed, requiring temporary colostomy, which was closed and recovered 3 mo later. No anastomotic stenosis or local tumor recurrence occurred. The quality of life was good for all patients, and 17 of them survived for > 3 years (with a survival rate of 100%). The patient data are shown in Table 1.

Anal function

Kirwan classification[15] includes the following five grades: 1: normal; 2: occasionally needs an enema, with gas incontinence; 3: needs drug control, daily enema, needs to wear panty liner with a small amount of leakage or occasionally mild leakage of excrement; 4: underwear is often contaminated; and 5: requires colostomy. In this cohort, 6-9 bowel movements per day were observed after early intake after surgery. After administration of two compound diphenoxylate tablets orally 3 times daily for 2-3 d, the defecation was controlled at 3-6 times daily. Self-control bowel movements were significantly improved 2-3 mo after surgery. Twelve months after surgery, 91.3% (21/23) patients achieved anal function Kirwan grade 1, indicating that their anal function returned to normal (Table 2).

Table 2 Kirwan anal function results 6 and 12 mo after surgery

Time	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	Total
6 mo after surgery	17 (65.4)	8 (30.8)	1 (3.8)	0	0	26
12 mo after surgery	21 (91.3)	2 (8.7)	0	0	0	23

Results are expressed as percentages.

DISCUSSION

Laparoscopic anus-preserving radical resection of rectal cancer has been widely carried out in recent years[16,17], but an abdominal incision is always required to remove the specimens, regardless of transumbilical single-port or assisted laparoscopic surgery[18,19], undoubtedly making it difficult to achieve the optimal goal of completely minimally invasive, incision-free, scar-free surgery. Based on the experience of open telescopic anastomosis for anal preservation in radical resection of lower rectal cancer[3,14], we have established laparoscopic radical resection of lower rectal cancer with telescopic anastomosis, through transanal resection without abdominal incisions, which enables incision-free, scar-free, cosmetic and minimally invasive treatment[12,13]. This innovative surgery is a major innovation for rectal cancer surgery, which has not been reported previously.

Telescopic anastomosis for anal preservation in radical resection of lower rectal cancer[12] preserves the anus and bowel movement control for most patients with lower or ultralow rectal cancer. The innovations of this surgery include the following: (1) the critical technique in the telescopic anastomosis is the four-stitch suture between the full-thickness colon and the residual rectal muscle sheath, which plays a role in reducing tension and reinforcing the fixation. This is the key to preventing the occurrence of anastomotic leakage, effectively reducing the incidence of anastomotic complications; (2) the anorectal ring and the entire internal and external sphincters are retained intact, so bowel control is preserved at 3-6 mo after surgery, improving postoperative physiological and quality of life; (3) under laparoscopy, sigmoid and rectal dissection is completed with an ultrasonic scalpel to 3-5 cm from the distal end of the tumor. The specimen is removed through and from the anus, which enables incision-free, scar-free, cosmetic and minimally invasive treatment; and (4) due to the minimal invasiveness, patients experience less postoperative pain, and can achieve earlier ambulation and faster recovery. Laparoscopic radical resection of lower rectal cancer with telescopic anastomosis, through transanal resection without abdominal incisions, is safe and feasible, which achieves the goal of minimally invasive and function-preserving surgery. This technique may be used for anus-preserving laparoscopic surgical treatment of lower rectal cancer.

Laparoscopic radical resection of lower rectal cancer with telescopic anastomosis, through transanal resection without abdominal incisions, includes the following features: (1) when using the scalpel to dissect the lymph nodes and fat tissue at the root of the mesenteric vessels, caution should be taken to avoid damage to the autonomic nervous bundle; (2) when dividing the inferior mesenteric artery and vein, attention should be paid to retain the mesenteric vascular bow, and ensure an adequate blood supply and length of the distal bowel segments, so that the anastomosis can be connected after the tumor is removed from the anus; (3) according to TME principles, the rectum should be dissected along the connective tissue between the visceral and parietal layers of the presacral space, posterior to the rectum, to the anorectal ring and 3-5 cm from the distal end of the tumor, to ensure complete tumor resection and lymph node dissection; (4) perineal surgery should be performed under adequate anesthesia[3,12], so that the anal sphincter achieves complete relaxation. For this, we use the five-stitch suspension method, which fully exposes the field above the dentate line, conducive to the surgical procedure. However, the suspension suture must only be closed when the sphincter is completely relaxed, to avoid damage to the anal skin and structure, thus affecting postoperative recovery of bowel function[12]; (5) to remove the mucosa completely while avoiding damage to internal and external sphincters, scissors can be used for sharp dissection along the superficial surface of the internal sphincter, otherwise it will damage the sphincter; (6) to ensure no fat residue along the colonic anastomosis, but a good blood supply, the colon ends should be trimmed to 0.5 cm, so that there is no residual fat tissue, which is conducive to anastomotic healing; (7) the telescopic full-thickness colon should be connected and fixed with the residual rectal sheath using interrupted four-stitch sutures[5], for reinforcement and reduced tension, which is the key to prevention of anastomotic fistula; and (8) the telescopic anastomosis should be closed with 8-12 stitches without too dense sutures in case of anastomotic stenosis.

Laparoscopic radical resection of lower rectal cancer with telescopic anastomosis, through transanal resection without abdominal incisions, is indicated for the same conditions as open telescopic anastomosis[3,13]. The TME principles should be strictly followed in the selection of personalized anus-preserving surgery according to the specific circumstances of the patient.

The lack of a control group is the main limitation of this study. More cases and longer follow-up will be included for the observation of oncological outcome, and we will carry out randomized controlled trials in future studies.

In conclusion, laparoscopic radical resection of lower rectal cancer with telescopic anastomosis, through transanal resection without abdominal incisions, is safe and feasible, which enables incision-free, scar-

free, cosmetic and minimally invasive treatment. This technique may become an option for anus-preserving laparoscopic surgical treatment of low colorectal cancer.

COMMENTS

Background

Procedures for anus-preserving radical resection for lower rectal cancer are of current research interest. Although laparoscopic radical surgery of rectal cancer has been widely carried out recently, it generally requires an assisting abdominal incision for removal of tumor specimens, which prevents it from being completely minimally invasive.

Research frontiers

The authors have completed 420 cases of telescopic anastomosis for anal preservation in radical resection of lower rectal cancer. They first reported laparoscopic radical resection of lower rectal cancer with telescopic anastomosis through transanal resection without abdominal incisions, which is an abdominal incision-free and scar-free completely minimally invasive technique, in 2011. So far, they have treated 30 cases of lower rectal cancer using this procedure with satisfactory outcomes in follow-up observation.

Innovations and breakthroughs

This study assesses the laparoscopic radical resection of lower rectal cancer with telescopic anastomosis through transanal resection without abdominal incisions. Thirty cases of lower rectal cancer have been treated using this procedure with satisfactory outcomes in follow-up observation. The authors concluded that laparoscopic radical resection of lower rectal cancer with telescopic anastomosis through transanal resection without abdominal incisions is safe and feasible.

Applications

Laparoscopic radical resection of lower rectal cancer with telescopic anastomosis, through transanal resection without abdominal incisions, is safe and feasible, which enables incision-free, scar-free, cosmetic and minimally invasive treatment. This technique may become an option for anus-preserving laparoscopic surgical treatment of low colorectal cancer.

Terminology

Telescopic anastomosis for anal preservation in radical resection of lower rectal cancer preserves the anus and bowel movement control for most patients with lower or ultralow rectal cancer.

Peer-review

This is an interesting study regarding incision free laparoscopic rectal resection for rectal cancer.

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ORIGINAL ARTICLE

Clinical Trials Study

Metadoxine improves the three- and six-month survival rates in patients with severe alcoholic hepatitis

Fátima Higuera-de la Tijera, Alfredo I Servín-Caamaño, Aurora E Serralde-Zúñiga, Javier Cruz-Herrera, Eduardo Pérez-Torres, Juan M Abdo-Francis, Francisco Salas-Gordillo, José L Pérez-Hernández

Fátima Higuera-de la Tijera, Eduardo Pérez-Torres, Juan M Abdo-Francis, Francisco Salas-Gordillo, José L Pérez-Hernández, Liver Clinic, Gastroenterology Department, Hospital General de México, Dr. Eduardo Liceaga, Mexico City 06720, Mexico
Alfredo I Servín-Caamaño, Javier Cruz-Herrera, Internal Medicine Department, Hospital General de México, Dr. Eduardo Liceaga, Mexico City 06720, Mexico
Aurora E Serralde-Zúñiga, Fundación Mexicana para la Salud A.C, Mexico City 06720, Mexico
Author contributions: Higuera-de la Tijera F designed the study, provided financial support though the "Angeles Espinosa Yglesias 2010" stimulus, supervised the study, reviewed the statistical methods and wrote the final manuscript; Servín-Caamaño AI and Serralde-Zúñiga AE contributed to the acquisition, analysis and interpretation of the data and wrote the manuscript; Cruz-Herrera J, Pérez-Torres E, Abdo-Francis JM and Salas-Gordillo F contributed to the acquisition of data and cared for the patients; Pérez-Hernández JL reviewed the statistical methods and wrote the final manuscript; all of the authors read and approved the final manuscript.
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Informed consent: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.
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Data sharing: Technical appendix, statistical code, and dataset available from the corresponding author at fatimahiguera@yahoo.com.mx. Consent for data sharing was not obtained but the presented data are anonymized and risk of identification is low. No additional data are available.

Correspondence to: Fátima Higuera-de la Tijera, MD, MSc, Liver Clinic, Gastroenterology Department, Hospital General de México, Dr. Eduardo Liceaga, Dr. Balmis 148, Mexico City 06720, Mexico. fatimahiguera@yahoo.com.mx
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Abstract

AIM: To evaluate the impact of metadoxine (MTD) on the 3- and 6-mo survival of patients with severe alcoholic hepatitis (AH).

METHODS: This study was an open-label clinical trial, performed at the "Hospital General de México, Dr. Eduardo Liceaga". We randomized 135 patients who met the criteria for severe AH into the following groups: 35 patients received prednisone (PDN) 40 mg/d, 35 patients received PDN+MTD 500 mg three times daily, 33 patients received pentoxifylline (PTX) 400 mg three times daily, and 32 patients received PTX+MTD 500 mg three times daily. The duration of the treatment for all of the groups was 30 d.

RESULTS: In the groups treated with the MTD, the

survival rate was higher at 3 mo (PTX+MTD 59.4% *vs* PTX 33.3%, *P* = 0.04; PDN+MTD 68.6% *vs* PDN 20%, *P* = 0.0001) and at 6 mo (PTX+MTD 50% *vs* PTX 18.2%, *P* = 0.01; PDN+MTD 48.6% *vs* PDN 20%, *P* = 0.003) than in the groups not treated with MTD. A relapse in alcohol intake was the primary independent factor predicting mortality at 6 mo. The patients receiving MTD maintained greater abstinence than those who did not receive it (74.5% *vs* 59.4%, *P* = 0.02).

CONCLUSION: MTD improves the 3- and 6-mo survival rates in patients with severe AH. Alcohol abstinence is a key factor for survival in these patients. The patients who received the combination therapy with MTD were more likely to maintain abstinence than those who received monotherapy with either PDN or PTX.

Key words: Alcoholic hepatitis; Metadoxine; Survival; Alcohol abstinence

Core tip: Severe alcoholic hepatitis (AH) has a high mortality rate. Oxidative stress and the depletion of mitochondrial glutathione are factors implicated in injury to the liver. Metadoxine (MTD), an antioxidant that participates in the synthesis of glutathione and inhibits hepatic lipid accumulation, appears to be a novel therapeutic agent in patients with severe AH because it improves their 3- and 6-mo survival. The patients who received MTD were also better able to abstain from alcohol use, which is a key factor contributing to the improved survival in patients with severe AH.

Higuera-de la Tijera F, Servín-Caamaño AI, Serralde-Zúñiga AE, Cruz-Herrera J, Pérez-Torres E, Abdo-Francis JM, Salas-Gordillo F, Pérez-Hernández JL. Metadoxine improves the three- and six-month survival rates in patients with severe alcoholic hepatitis. *World J Gastroenterol* 2015; 21(16): 4975-4985 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4975.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4975

INTRODUCTION

Severe alcoholic hepatitis (AH) has a high mortality rate despite its standard therapy[1,2]. Some populations, such as Hispanics, have responded poorly to standard therapy and show a mortality rate similar to those treated with placebos, particularly in patients classified as Age-Bilirubin-International normalized ratio-Creatinine (ABIC) classes B and C[3-5]. Therefore, it is important to search for new therapeutic options that are effective and safe.

Acute and chronic alcohol exposure is associated with high oxidative stress[6] and liver injury mediated by acetaldehyde[7,8]. Reactive oxygen species (ROS) are responsible for activating redox-sensitive transcription factors, such as nuclear factor-kappa B (NF-κB), thereby maintaining a pro-inflammatory profile[7].

Metadoxine (MTD), an antioxidant, participates in the synthesis of glutathione (GSH) and inhibits hepatic steatosis[9]. The preliminary findings in patients with severe AH have demonstrated that MTD in combination with glucocorticoids improves the survival rates at 30 and 90 d as well as the response to steroid therapy, according to the Lille score[10].

The aim of this study was to evaluate the impact of MTD added to standard therapy using prednisone (PDN) or pentoxifylline (PTX) compared with monotherapy on the 3- and 6-mo survival rates of patients with severe AH.

MATERIALS AND METHODS

Study design

This study was a randomized, open-label clinical trial, performed at the "Hospital General de Mexico, Dr. Eduardo Liceaga", Mexico City, from April 2010 to December 2013.

Sample size calculation

We used the Epidat 3.1 (Galicia, Spain 2006) statistical program to calculate the sample size, considering a difference in the survival rate at 3 mo of 30% between the groups receiving MTD and those receiving the standard treatment to be significant. We also considered a two-tailed confidence level of 95%, a potency of 80%, and an additional 20% of subjects as potential losses. There were a total of 35 patients per group.

Inclusion criteria

Patients between 18 and 65 years old who met the clinical and biochemical criteria of severe AH[11,12], as characterized by a history of chronic and heavy alcohol intake (> 80 g/d for the previous 5 years), the rapid onset of jaundice in the absence of a biliary tract obstruction, painful hepatomegaly and ascites, transaminases ⩾ two times above the normal value, an aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio ⩾ 2, neutrophilia, a total bilirubin > 5 mg/dL, and a Maddrey's discriminant function > 32 (calculated with the formula [4.6 × (patient prothrombin time (PT)-control PT, in seconds) + total bilirubin in mg/dL]), were included in the study.

Exclusion criteria

Patients with acquired immunodeficiency syndrome; neoplasms; autoimmune diseases; psychiatric disorders other than alcoholism, such as depression and anxiety according to the Diagnostic and Statistical

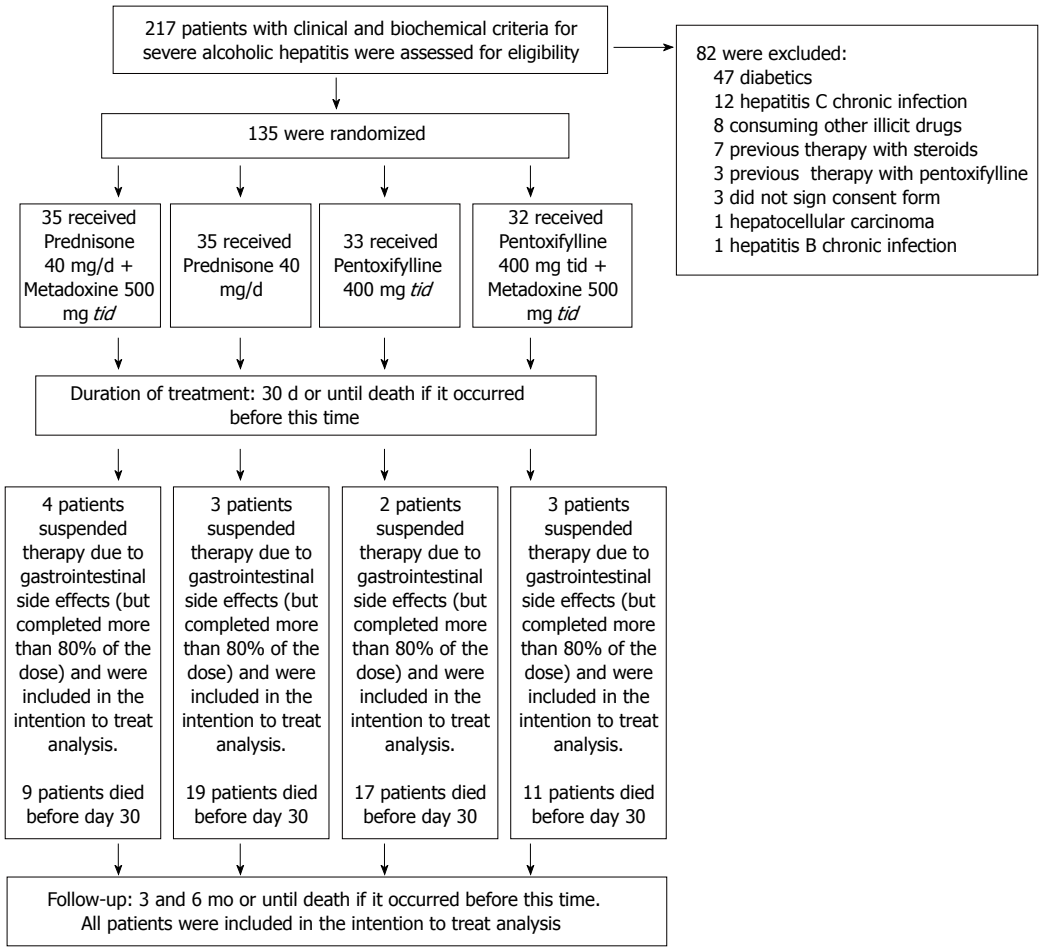

Figure 1 Enrollment and outcomes.

Manual of Mental Disorders, Fourth Edition (DSM-Ⅳ); a history of atopy or asthma; diabetes; obesity; pregnancy; hepatitis B or C virus infection; or tuberculosis were excluded from the study.

Patients with an intake of illicit drugs, herbal products, antioxidant supplements (multivitamins, S-adenosyl-L-methionine, MTD, silymarin), or previous treatment with steroids or PTX within the previous two years were excluded.

Patients without family support or without access to telephone communication were also excluded.

Pre-inclusion screening

We obtained written informed consent from all of the patients before they were enrolled in the study. On the day of admission, we performed a clinical history and collected peripheral blood samples to determine each patient's glucose, urea, creatinine, total bilirubin, alkaline phosphatase, gamma glutamyl transpeptidase, ALT, AST, sodium, potassium, albumin, leucocytes, neutrophils, hemoglobin, platelets, PT and international normalized ratio levels. The patients were also tested for HBsAg, anti-HBs, anti-HBc, IgM anti-HBc, with serological screening for the hepatitis C virus and h*uman immunodeficiency virus*. The screening for bacterial infections included urine, blood and ascites cultures, as well as chest radiography and a neutrophil count in ascites. A liver ultrasound and endoscopy were performed to determine the presence of varices in all of the patients.

Randomization and treatment groups

The patients were randomized into four treatment groups. For the randomization, we used the Epidat 3.1 statistical program (Galicia, Spain 2006) to construct a table of random numbers to compose four groups of equal size (Figure 1).

We evaluated 217 patients who met clinical and biochemical criteria for severe AH. However, 82 subjects met one or more exclusion criteria: 47 were diabetics, 12 had hepatitis C chronic infection, 8 consuming illicit drugs, 7 were previously treated with steroids, 3 were previously treated with PTX, 3 did not sign consent form, 1 had also hepatocellular carcinoma, and 1 had also hepatitis B chronic infection. Therefore, 135 subjects who met all eligibility criteria and provided written informed consent were randomly assigned to one of four groups of treatment: A group receiving PDN (at a dose of 40 mg once daily); a group receiving PDN (at a dose of 40 mg once daily) and MTD (at a dose of 500 mg three times daily); a group receiving PTX (at a dose of 400 mg three times

daily); a group receiving PTX (at a dose of 400mg three times daily) and MTD (at a dose of 500 mg three times daily). In all cases, the duration of treatment was 30 d, or until death if it occurs earlier. The 500-mg metadoxine tablets (Abrixone®) were provided by Eurodrug Laboratories as a donation for our institution. The 40-mg prednisone tablets and the 400-mg pentoxifylline tablets provided by our institution. Eurdorug Laboratories provided comments regarding the study design but were not involved in the writing of the protocol, the conduct of the trial, the decision-making with respect to the trial, the analysis of the data, or the preparation of the manuscript. The doses were selected on the basis of previous studies[1,2,13]. The dose for metadoxine was selected on the basis of the study conducted by Caballería *et al*[13].

Study phase and endpoints

The primary endpoints were the 3- and 6-mo survival rates. The secondary endpoints were the development or progression of acute renal failure (ARF), variceal bleeding (VB), hepatic encephalopathy (HE), bacterial or fungal infections, adverse effects and a relapse to alcohol consumption between 30 d and 6 mo of the follow-up. We also performed a sub-analysis stratifying the patients according to their ABIC class.

The patients were monitored weekly during the first month, two times per month during the second and third months, and monthly thereafter until 6 mo. Each visit included a clinical examination and the collection of peripheral blood samples. During the first two weeks, all of the patients were hospitalized; subsequently, each investigator determined the duration of the hospitalization. For the hospitalized patients, the medications were administered under the supervision of physicians and nurses. For outpatients, adherence to treatment was monitored by a family member and reported in a control diary. To increase compliance with the medication regimen, the patients were required to return the empty blisters at each visit.

In patients who were suspected of developing an infection during the study, cultures and chest radiography were performed if necessary. In patients who developed odynophagia or dysphagia, an endoscopic study was performed to identify esophageal candidiasis; when suspicious lesions were observed, brushing and mycological examinations were performed.

Management of complications

ARF: This condition was defined according to the criteria of the *Acute Kidney Injury Network* as an abrupt reduction (48 h) in renal function characterized by an increase of 0.3 mg/dL in the serum creatinine compared with the baseline value. The patients who had a baseline value of serum creatinine $\geqslant$ 1.5 mg/dL at the time of admission were considered to have ARF[14,15]. These patients were treated with intravascular volume expansion using an albumin infusion at 1 g/kg for 48 h. The patients who did not respond to this treatment were evaluated for hepatorenal syndrome (HRS) according to the *Ascites International Club* criteria and were treated with a vasopressor (terlipressin or norepinephrine) and intravascular volume expansion with albumin[16,17].

HE: This condition was defined clinically by both neuropsychiatric alterations and neuromuscular signs according to the West-Haven criteria[18]. Patients with an HE grade of Ⅰ or Ⅱ were treated orally with L-ornithine-L-aspartate. Patients with an HE grade of Ⅲ or Ⅳ were treated intravenously with L-ornithine-L-aspartate. In patients for whom L-ornithine-L-aspartate was contraindicated, oral lactulose for HE grades Ⅰ or Ⅱ and lactulose enemas for HE grades Ⅲ or Ⅳ were prescribed.

VB: This condition was defined by the presence of melena or hematemesis associated with gastroesophageal varices as determined by an endoscopy. These patients were treated with terlipressin or octreotide, and fresh frozen plasma and blood were transfused as necessary. An endoscopic band ligation for esophageal varices or cyanoacrylate injection for gastric varices was performed, and antibiotic prophylaxis was prescribed. Subsequently, the patients received a secondary prophylaxis[19].

Spontaneous bacterial peritonitis: This condition was defined and treated according to the most recent guidelines of the European Association for Study of Liver Diseases[17].

Other infections: Urinary tract infections were diagnosed in the patients having urinary symptoms that were associated with abnormal urinary examinations and urinary cultures, including a bacterial count greater than 100000 CFU. An antibiotic therapy was prescribed based on the results of the urinary cultures.

Pneumonia was diagnosed in the patients who developed a cough with expectoration and confirmation on the chest X-ray. Treatment was initiated with ceftriaxone and clarithromycin, after which the antibiotic therapy was adjusted depending on the sputum culture results.

When patients developed odynophagia or dysphagia, esophageal candidiasis was suspected. The diagnosis was confirmed through oral cavity examinations and an endoscopy indicating the presence of compatible lesions; brushing was performed for a mycological examination in all of the cases, and treatment with fluconazole 100 mg twice daily was prescribed.

Patients with diarrhea were evaluated by microscopic examinations of fresh stool and stool cultures. These patients were treated empirically with ciprofloxacin

Table 1 Baseline characteristics of the patients

Characteristic	PDN (*n* = 35)	PDN+MTD (*n* = 35)	PTX (*n* = 33)	PTX+MTD (*n* = 32)	*P* value
Male, *n* (%)	33 (94.3)	32 (91.4)	31 (93.9)	28 (87.5)	0.40
Age, yr	43.1 ± 9.5	43.4 ± 9.0	43.4 ± 9.9	44.4 ± 9.1	0.94
Alcohol intake, g/d	313.7 ± 157.1	328.9 ± 142.5	365.9 ± 196.2	346.5 ± 173.6	0.61
Child-Pugh	12.4 ± 1.0	12.4 ± 0.9	12.6 ± 1.0	12.5 ± 1.0	0.82
Maddrey´s modified discriminant function	70.9 ± 25.6	67.3 ± 19.3	93.4 ± 84.7	78.3 ± 40.2	0.14
MELD	28 ± 4	29 ± 6	31 ± 9	31 ± 7	0.19
ABIC	8.199 ± 1.3	8.604 ± 2.9	8.675 ± 1.8	8,677 ± 1.6	0.73
Urea, mg/dL Log_{10}	1.60 ± 0.34	1.64 ± 0.31	1.66 ± 0.32	1.64 ± 0.42	0.94
Creatinine, mg/dL	1.5 ± 0.7	1.5 ± 0.8	1.7 ± 1.1	1.9 ± 1.5	0.21
Sodium, mEq/L	131.3 ± 5.8	132.7 ± 5.8	130.9 ± 5.1	131.7 ± 4.7	0.57
Albumin, mg/dL	1.9 ± 0.4	1.9 ± 0.5	1.8 ± 0.4	1.8 ± 0.5	0.89
Total bilirubin, mg/dL	24.4 ± 10.5	24.5 ± 10.1	23.0 ± 9.5	25.9 ± 11.6	0.73
Alkaline phosphatase, UI/L Log_{10}	2.3 ± 0.2	2.3 ± 0.2	2.2 ± 0.2	2.4 ± 0.2	0.29
Gamma-glutamyltransferase, UI/L Log_{10}	2.5 ± 0.3	2.4 ± 0.3	2.4 ± 0.4	2.4 ± 0.3	0.73
Aspartate aminotransferase, UI/L Log_{10}	2.3 ± 0.2	2.3 ± 0.2	2.2 ± 0.2	2.2 ± 0.3	0.39
Alanine aminotransferase, UI/L Log_{10}	1.7 ± 0.2	1.7 ± 0.2	1.7 ± 0.2	1.7 ± 0.4	0.28
Leucocytes, cell/mm^3	20.9 ± 8.0	18.5 ± 8.0	19.5 ± 9.0	19.2 ± 10.3	0.71
Neutrophils, cell/mm^3	16.2 ± 7.0	15.7 ± 7.5	16.6 ± 8.5	17.0 ± 9.9	0.92
Hemoglobin, g/dL	11.6 ± 2.7	11.8 ± 2.5	10.9 ± 3.0	11.4 ± 2.4	0.52
Platelets, cell/mm^3	178.1 ± 119.5	186.0 ± 113.6	182.8 ± 117.7	153.0 ± 84.0	0.61
Prothrombin time in seconds	22.0 ± 5.8	21.1 ± 3.6	27.6 ± 18.5	23.2 ± 8.8	0.06
INR	1.9 ± 0.5	1.8 ± 0.3	2.4 ± 1.9	2.0 ± 0.7	0.06
Cirrhosis on liver ultrasound, *n* (%)	25 (71.4)	20 (57.1)	26 (78.8)	20 (62.5)	0.24

ABIC: Age-bilirubin-international normalized ratio-creatinine; INR: International normalized ratio; MELD: Model for end-stage liver disease; PDN: Prednisone; PDN+MTD: Prednisone + metadoxine; PTX: Pentoxifylline; PTX+MTD: Pentoxifylline + metadoxine.

and/or metronidazole.

Statistical analysis

The statistical methods of this study were reviewed by Fátima Higuera-de la Tijera, MD, MSc. and José L. Pérez Hernández, MD, MSc. From the "Hospital General de México, Dr. Eduardo Liceaga". The distribution of variables was analyzed; in cases of quantitative variables with a non-normal distribution base, a 10-logarithmic transformation was performed to normalize their distribution for the analysis using parametric tests. Descriptive statistics were used. The quantitative variables were expressed as the mean ± SD, and the qualitative variables were expressed as proportions and percentages. To compare the basal characteristics between the groups, a one-way ANOVA was performed for the quantitative variables. Tukey's or Tamhane's T2 tests were used according to the homogeneity of the variance for the post hoc tests, and a χ^2 test with Yates correction or Fisher's exact test were used for the qualitative variables. To compare the primary and secondary endpoints between the groups, an analysis with an intention to treat (ITT) was conducted. The χ^2 test with a Yates correction, Fisher's exact test or Student's *t*-test was used when needed, based on the variable type. A survival analysis was performed using Kaplan-Meier curves to evaluate the 3- and 6-mo survival and to evaluate the alcohol intake relapse between 30 d and 6 mo of follow-up and compare it with the log-rank test. To identify the main risk factors associated with 6-mo mortality, a multivariate analysis using a Cox regression was conducted. SPSS version 18.0 (Chicago, IL, United States 2009) and Epidat 3.1 (Galicia, Spain 2006) were used to perform the statistical analyses. A two-sided *P* value of 0.05 was considered to be statistically significant.

RESULTS

The baseline characteristics of the patients are listed in Table 1. In the groups receiving MTD, the survival rate was significantly higher at 3 mo than in the groups not receiving MTD: PTX+MTD 19/32 (59.4%) *vs* PTX 11/33 (33.3%), *P* = 0.04; PDN+MTD 24/35 (68.6%) *vs* PDN 7/35 (20%), *P* = 0.0001 (Figure 2).

In the groups receiving the MTD, the survival rate was significantly higher at 6 mo than in the groups not receiving MTD: PTX+MTD 16/32 (50%) *vs* PTX 6/33 (18.2%), *P* = 0.01; PDN+MTD 17/35 (48.6%) *vs* PDN 7/35 (20%), *P* = 0.003; (Figure 3).

There was no difference in the survival rates between the PDN and PTX monotherapy groups at 3 and 6 mo. There was no difference in the survival rate between the PDN+MTD and PTX+MTD groups at 3 and 6 mo.

According to their ABIC class, 13 patients (9.6%) were classified as class A, 82 patients (60.7%) as class B, and 40 patients (29.6%) as class C. The global survival according to the ABIC class was 10 patients (76.9%) for class A, 42 patients (51.2%) for class B, and 9 patients (22.5%) for class C. We performed a sub-analysis stratifying patients according to their ABIC class according to the two different groups of

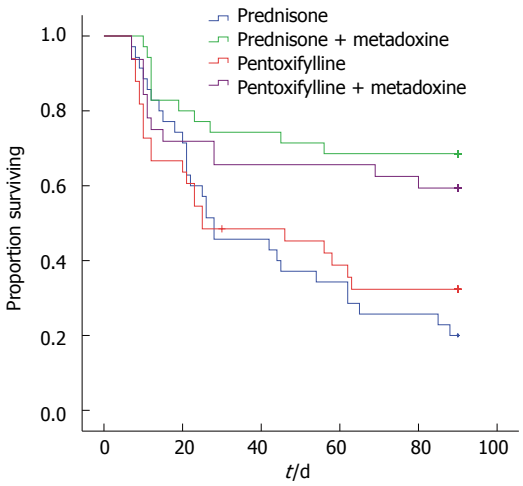

Figure 2 Kaplan Meier curves showing the 3-mo survival according to the treatment groups. Pentoxifylline + Metadoxine 59.4% *vs* Pentoxifylline 33.3%, Log rank test $P = 0.04$; Prednisone + Metadoxine 68.6% *vs* Prednisone 20%, Log rank test $P = 0.0001$.

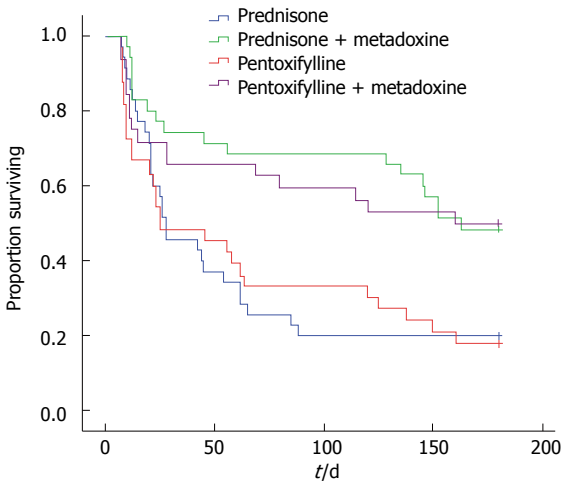

Figure 3 Kaplan Meier curves showing the 6-mo survival according to the treatment groups. Pentoxifyne + Metadoxine 50% *vs* Pentoxifylline 18.2%, Log rank test $P = 0.01$; Prednisone + Metadoxine 48.6% *vs* Prednisone 20%, Log rank test $P = 0.003$.

treatment into the group of 67 patients who received concomitant therapy with MTD (MTD Group) and the group of 68 patients who did not receive MTD (the standard therapy or ST Group). The improvement in the survival in the MTD Group was observed primarily in the ABIC class B: the MTD Group 30/38 (78.9%) *vs* the ST Group 12/44 (27.3%), $P = 0.0001$. There were no significant differences between the treatment groups in either the ABIC class A or ABIC class C patients: the MTD Group 6/6 (100%) *vs* ST Group 4/7 (57.1%) $P = 0.19$, and the MTD Group 7/23 (30.4%) *vs* ST Group 2/17 (11.8%) $P = 0.25$, respectively.

Regarding the development of complications at 3 mo of follow-up, there was significantly less development of HE and HRS in the patients who received the concomitant therapy *vs* the patients who received the PDN alone. There was no difference between the PTX+MTD *vs* the PTX group. Neither were there any differences among the groups regarding the development of VB or infections (Table 2)

The occurrence of adverse effects was similar in all of the groups, principally consisting of epigastric burning, nausea and vomiting, due to which 12 patients dropped out of the study. The patients who dropped out included 4 patients in the PDN and MTD group, 3 patients in the PDN group, 2 patients in the PTX group, and 3 patients in the PTX and MTD group. These patients were included in the ITT analysis because we verified that they had received at least 80% of the treatment. Serious adverse effects were not reported in any of the groups.

Maintenance of abstinence: Seventy-nine of the patients survived after 30 d (the end of therapy), 54 (68.4%) of whom had maintained alcohol abstinence and 25 (31.6%) of whom had relapsed into alcohol intake at 6 mo of follow-up. When we compared the groups, the patients receiving MTD were better able to maintain abstinence than the patients who did not receive MTD; MTD Group 35/47 (74.5%) *vs* ST Group 19/32 (59.4%), $P = 0.02$.

In the multivariate analysis, a relapse in alcohol intake was the primary independent factor predicting mortality at 6 mo. Additionally, the coexistence of cirrhosis on the ultrasound was identified as a predictor factor that was associated with mortality at 6 mo. In this study, the quantity of the alcohol intake was not associated with the 6-mo mortality. On the other hand, the treatment with MTD was identified as a protective factor (Table 3).

DISCUSSION

The mortality rate in our patients was high despite the treatment with PDN or PTX. However, other studies in Mexican population have also shown a high mortality rate and a poor response to steroid therapy. In a cross-sectional study, Ruíz-Zavála A reported a failure to respond to corticosteroids, evidenced by a Lille score greater than 0.45 in 90% of the patients diagnosed with alcoholic hepatitis (a mean Lille score of 0.80 ± 0.18)[20]. Additionally, in a clinical trial in Mexican patients with severe AH that compared treatment with PTX *vs* treatment with PDN, the mortality rate at 30 d was high, at 46.6% *vs* 59.9%, respectively, and there was no difference between the groups ($P = 0.30$)[3]. If we compare the mortality rates according to the ABIC class, Mexican patients have a higher mortality rate than other populations despite the treatment with steroids or PTX. The survival rate in the Mexicans *vs* the Europeans according to their ABIC class was as follows: an ABIC class of A, 81% *vs* 100%; an ABIC class of B, 50% *vs* 70%; and an ABIC class of C, 13% *vs* 25%, respectively[4,5]. The quantity of the alcohol intake may be an explanation for the higher mortality observed in the Mexican population, as Altamirano *et al*[5] demonstrated that the consumption of more than 120 g per day of alcohol is associated with greater

Table 2 Development of complications at the 3-mo follow-up *n* (%)

Complication	PDN+MTD (*n* = 35)	PDN (*n* = 35)	PTX+MTD (*n* = 32)	PTX (*n* = 33)	*P* value[2]	HR (95%CI)[3]	*P* value[4]	HR (95%CI)[5]
HE	10 (28.6)	21 (60.0)	13 (40.6)	17 (51.5)	0.008[1]	0.2 (0.1-0.7)	0.38	0.6 (0.2-1.7)
HRS	11 (31.4)	19 (54.3)	11 (34.4)	16 (48.5)	0.05[1]	0.3 (0.1-1.0)	0.25	0.5 (0.2-1.5)
VB	10 (28.6)	13 (37.1)	11 (34.4)	14 (42.4)	0.44	0.6 (0.2-1.8)	0.51	0.7 (0.2-1.9)
Infections	11 (31.4)	14 (40.0)	11 (34.4)	12 (36.4)	0.45	0.6 (.02-1.8)	0.87	0.9 (0.3-2.5)
None	24 (68.6)	21 (60)	21 (65.6)	21 (63.6)				
UTI	0 (0)	3 (8.6)	3 (9.4)	6 (18.25)				
SBP	2 (5.7)	2 (5.7)	3 (9.4)	3 (9.15)				
Pneumonia	9 (25.7)	7 (20)	1 (3.1)	1 (3.0)				
EC	0 (0)	1 (2.85)	3 (9.4)	1 (3.0)				
Diarrhea	0 (0)	1 (2.85)	1 (3.1)	1 (3.0)				

[1]Significant difference ($P < 0.05$); [2]*P* value comparing the PDN *vs* the PDN+MTD groups; [3]HR and 95%CI comparing the PDN *vs* the PDN+MTD groups; [4]*P* value comparing the PTX *vs* the PTX+MTD groups; [5]HR and 95%CI comparing the PTX *vs* the PTX+MTD groups. EC: Esophageal candidiasis; HE: Hepatic encephalopathy; HR: Hazard ratio; HRS: Hepatorenal syndrome; PDN: Prednisone; PDN+MTD: Prednisone + metadoxine; PTX: Pentoxifylline; PTX+MTD: Pentoxifylline + metadoxine; SBP: Spontaneous bacterial peritonitis; UTI: Urinary tract infection; VB: Variceal bleeding.

Table 3 Multivariate analysis: predictors of mortality at 6 mo in patients with severe alcoholic hepatitis

Variable	HR (95%CI)	*P* value
Relapse in alcohol intake[1]	8.9 (3.9-20.2)	0.0001
Cirrhosis[1]	2.3 (1.1-4.7)	0.02
Treatment with metadoxine[2]	0.3 (0.2-0.7)	0.005
Quantity of alcohol intake (> 150 g/d)	1.8 (0.4-7.7)	0.45

[1]Risk factor; [2]Protective factor. HR: Hazard ratio; 95%CI: 95% confidence interval.

mortality[5]. In our study, the mean alcohol intake was greater than 300 g per day. Moreover, Mexican-American males have a higher prevalence of alcoholic cirrhosis and a higher mortality rate compared with Caucasians[21].

Controversial results exist concerning whether steroids or PTX is superior for improving the survival of patients with severe AH. In our study, there was no difference in the survival between the PDN and the PTX groups. Neither was there a difference in the survival between the PDN+MTD and PTX+MTD groups. However, in 2009, De *et al*[22] performed a randomized, double-blind, controlled clinical trial to compare the efficacy of PTX and prednisolone in the treatment of severe AH. In this study, the probability of dying at 3 mo was higher in the prednisolone group compared with the PTX group (35.29% *vs* 14.71%, $P = 0.04$; log rank test). In 2013, a systematic review by Parker *et al*[2] that included ten trials and a total of 884 patients found that PTX appears to be superior to a placebo in the prevention of fatal HRS and thus may be an effective treatment for severe AH when corticosteroids are contraindicated. However, multiple trials have failed to show the superiority of either PTX or steroids. More recently, Park *et al*[23] found that PTX was not superior to prednisolone for improving the 6-mo survival rate (64.5% *vs* 72.9%, respectively; $P = 0.23$). The investigators in the "Steroids or Pentoxifyllline for Alcoholic Hepatitis" (STOPAH) trial[24], recently presented their findings at the Liver Meeting 2014 in Boston, Massachusetts. The STOPAH trial was a multicenter, double-blind, factorial (2 × 2) trial that included 1103 patients who were randomized to one of four groups: prednisolone + placebo, PTX + placebo, prednisolone + PTX, or a double placebo group. The investigators found that prednisolone, but not PTX, was associated with a lower risk of 28-d mortality. In contrast, the mortality rate in the group that received PTX was similar to the mortality rate of those who received the double placebo. Beyond 28 d, neither of the drugs was associated with a survival benefit, and infections were approximately twice as frequent in the prednisolone group.

Our study shows that treatment with MTD may have a protective role, as it improves 3- and 6-mo survival rates. MTD is the ion pair between pyridoxine and pyrrolidone carboxylate, the cyclic amide of glutamic acid that is responsible for the synthesis and catalysis of GSH[25]. Alcohol exposure is associated with high oxidative stress[6]. The oxidative pathway for metabolizing alcohol involves alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase, and both of these enzymatic reactions reduce nicotinamide dinucleotide (NAD) to its reduced form of NADH. An excess of NADH causes several metabolic disorders, including the inhibition of the Krebs cycle and fatty acid oxidation, which favors steatosis and hyperlipidemia[8]. Acetaldehyde participates in alcohol-mediated liver injury by causing cellular damage, inflammation, and fibrogenesis[7]; it promotes cell death by depleting the concentration of reduced GSH, inducing lipoperoxidation, and increasing the toxic effect of the free radicals. The ROS can oxidize and damage the DNA, proteins and unsaturated fatty acids, thereby altering cell function[6].

The oxidation of alcohol also occurs through cytochrome P450's generation of ROS, such as hydrogen peroxide and superoxide ions. In particular, cytochrome P450 2E1 (CYP2E1) is increased several-fold and contributes to lipoperoxidation and liver injury. CYP2E1 also converts alcohol to acetaldehyde. The ROS are responsible for activating the redox-sensitive

transcription factors, such as NF-κB, and maintaining a pro-inflammatory profile[7]. Other cytochromes, such as CYP1A2 and CYP3A4, may also contribute to the metabolism of ethanol[8].

Several studies have demonstrated that MTD increases the metabolism and depuration of ethanol[26,27] and acetaldehyde in the liver and plasma and prevents the damage caused by ethanol and acetaldehyde in both hepatocytes and hepatic stellate cells[28]. MTD also restores the concentrations of NAD, GSH[29] and adenosine triphosphate in the brain and liver[30,31] and acts as an antioxidant because the ion-pair molecule is capable of dissociating into N-oxide, which acts as scavenger to trap the ROS and free radicals[32,33]. MTD inhibits the synthesis of the fatty acid esters in the liver, reduces the hepatic content of the triglycerides and prevents the injuries associated with lipoperoxidation[13,34-36].

The global survival according to the ABIC class in our patients was similar to that reported by Altamirano *et al*[5] in a previous cohort of Mexican patients. The majority of our patients (60.7%) were categorized as ABIC class B. We believe that the greatest benefit that we observed using MTD therapy in the ABIC class B may have occurred because this group had the largest proportion of the patients compared with the ABIC classes A and C. However, further studies that include more patients who are categorized as ABIC classes A and C are needed to validate this assumption.

In regard to the development of complications, there was significantly less development of HRS in patients who had received PDN+MTD compared with those who received PDN alone at the 3-mo follow-up. Although there was no significant difference between PTX+MTD and PTX alone, 14.1% fewer patients in the group treated with PTX+MTD developed HRS compared with the patients who received PTX alone (34.4% *vs* 48.5%, respectively). In our study, MTD had a protective effect on renal function. Previous studies have shown that MTD decreases the formation of acetaldehyde macromolecular adducts in all targets of ethanol toxicity, including the brain, liver and kidneys. The effect in the kidneys is due to two mechanisms of action: the inhibition of adduct formation and the increased excretion rate of acetaldehyde[37].

The impaired renal function is closely associated with the elevation of inflammatory markers (tumor necrosis factor-α, interleukin-1β, and interleukin-6), leading to both an increase in markers of oxidative stress and a decreased in antioxidants[38-40]. All of these mechanisms are involved in the pathophysiology of SAH and could be modulated by MTD therapy.

There was less development of HE in the patients receiving therapy with PDN+MTD compared with those who received PDN alone. Moreover, 10.9% fewer patients developed HE in the group treated with PTX+MTD compared with the patients who received PTX alone (51.5% *vs* 40.6%, respectively). In our study, MTD demonstrated a protective effect over the patients' mental status. Pyrrolidone carboxylate is an intermediate in the γ-glutamyl cycle, which is an amino acid transport system into the cell through the cell membrane. Unlike glutamic acid, the uptake of pyrrolidone carboxylate by the central nervous system (CNS) is possible because it crosses the hemato-encephalic barrier. In the CNS, it exerts a number of actions on the cognitive and memory functions that are affected by alcohol, and it is important and clinically relevant to restore those superior functions. Once hydrolyzed by oxoprolinase, the open glutamic acid becomes available for important metabolic processes. Its derivative, N-acetyl glutamate, which is released in the subsequent metabolic steps, plays an essential role in maintaining the nitrogen balance because it activates the carbamoyl-synthetase I, a key enzyme in the urea cycle. Furthermore, by reacting with oxaloacetate, an intermediate of the Kreb's cycle, it participates in the biosynthesis of aspartate, an essential element in the urea cycle, which is therefore activated from two different entry points. In addition, glutamate may react with ammonia to form glutamine, thereby contributing to the elimination of toxic ammonia and to the nitrogen fixation by the organism[25,41-43].

In our study, a relapse in alcohol intake was the primary independent factor predicting mortality at 6 mo. Alcohol abstinence is considered to be the cornerstone of the management of AH. In the results from the STOPAH trial[24], a relapse in alcohol consumption had a deleterious effect; at 1 year, the patients who either did not reduce or who increased their alcohol consumption had a 3-fold risk for death compared with the patients who abstained (OR = 2.99; $P < 0.001$). The patients who reduced their alcohol consumption but not below a safe level still had a more than a 2-fold risk for death at 1 year compared with the patients who abstained (OR = 2.28; $P = 0.032$), as did the patients who reduced their alcohol consumption to below a safe level (OR = 2.17; $P = 0.031$). Wang *et al*[44] demonstrated that alcohol abstinence ameliorates AH by decreasing the liver enzyme and fibrotic markers and improving hepatic steatosis. In our study, the therapy with MTD helped patients to maintain alcohol abstinence. This finding is similar to those reported by several studies that have demonstrated that MTD is an effective therapy for abstinence[45-49]. Currently, disulfiram, naltrexone and acamprosate are approved for the treatment of alcoholic dependency; however, all of these medications are contraindicated in patients with severe liver disease[44], such as our patients. Patients who have recovered from an episode of severe alcoholic hepatitis must be supported in maintaining alcohol abstinence without risk or compromise to their liver function. Bono *et al*[49] found that alcoholic patients who received treatment with MTD achieved alcohol abstinence in a greater

proportion compared with those who did not receive it. More recently, an interesting retrospective analysis by Leggio *et al*[45] found that patients with ALD who were treated with MTD had a significant decrease in drinks per week and demonstrated an improvement in the AST/ALT ratio compared with those who did not receive it. The beneficial neurological effects of MTD therapy in patients with attention-deficit/hyperactivity disorder have been demonstrated in several studies conducted by Manor *et al*[50-52]. In animal models, the effects of MTD on CNS have been studied. Ethanol and acetaldehyde increase the activity of dopamine neurons in the reward areas of the CNS, and these actions are associated with the rewarding and reinforcing properties of the ethanol. MTD may favor abstinence through its ability to metabolize and to clear ethanol and its metabolites from the organism, as well as through its direct effect on neurotransmitters such as gamma-aminobutyric acid, acetylcholine and dopamine, all of which are involved in the neurobiology of alcohol craving[45-52].

In this study, the presence of cirrhosis on the ultrasound was identified as a predictive factor associated with 6-mo mortality. In a study by Altamirano *et al*[53], the degree of fibrosis, degree of neutrophil infiltration, type of bilirubinostasis, and presence of megamitochondria were independently associated with 90-d mortality.

In conclusion, MTD improves the 3- and 6-mo survival in patients with severe AH, and it has a tendency to improve serious complications, such as HRS and HE, particularly when it is added to PDN. The greatest benefit of MTD therapy was observed in the ABIC class B patients. However, further studies including a greater sample size with a larger number of severe AH patients categorized as ABIC classes A and C are needed to demonstrate whether MTD also improves survival in these groups. This study reaffirms the knowledge that alcohol abstinence is a key factor for survival in severe AH patients and that MTD is a safe therapy that helps to achieve this objective.

COMMENTS

Background

Severe alcoholic hepatitis is a disease with a high mortality rate despite the use of standard therapy with steroids or pentoxifylline. Oxidative stress plays a key role in the physiopathology of alcoholic hepatitis and therefore represents a novel therapeutic target that must be investigated.

Research frontiers

Previous studies have demonstrated that metadoxine increases the metabolism and depuration of ethanol and acetaldehyde in the liver and the plasma and prevents the damage caused by ethanol and acetaldehyde in the hepatocytes and hepatic stellate cells. Metadoxine also acts as an antioxidant because its ion-pair molecule is capable of dissociating into N-oxide, which acts as scavenger to trap the reactive oxygen species and free radicals. Furthermore, metadoxine can prevent the steatosis and injury associated with lipoperoxidation. Metadoxine is a drug currently indicated for treating acute alcohol intoxication; several studies have also validated its use for treating alcohol dependence. However, until the current study, metadoxine had not been evaluated as a therapy for patients with severe alcoholic hepatitis.

Innovations and breakthroughs

In the current study, the authors found that metadoxine is an effective therapy for severe alcoholic hepatitis; the patients treated with metadoxine had better survival at 3 and at 6 mo compared with those treated with standard therapy with steroids or pentoxifylline. Furthermore, it is well known that alcohol abstinence is an important factor associated with long-term survival in these patients. In this study, the authors found that the patients who received metadoxine were more likely to maintain alcohol abstinence, and their greater abstinence may be related to the improvements in the 6-mo survival in the patients treated with this drug.

Applications

The results of this study suggest that metadoxine could be used as an effective therapy for patients with severe alcoholic hepatitis, and the validated results of other previous studies have found that metadoxine is an effective therapy to achieve alcohol abstinence.

Terminology

Severe alcoholic hepatitis is a condition characterized by a rapid onset of jaundice in the absence of biliary tract obstruction, painful hepatomegaly and ascites, transaminases $\geqslant$ two times above the normal values, an aspartate aminotransferase/alanine aminotransferase ratio $\geqslant$ 2, neutrophilia, a total bilirubin > 5 mg/dL, and a Maddrey's discriminant function > 32 (calculated with the formula [4.6 × (patient prothrombin time (PT)-control PT, in seconds) + total bilirubin in mg/dL]), which occurs in patients with a history of chronic and heavy alcohol intake. Metadoxine is the ion pair between pyridoxine and pyrrolidone carboxylate, the cyclic amid of glutamic acid, which is responsible for the synthesis and catalyzation of glutathione.

Peer-review

This is an interesting study, focusing on a therapeutic area with largely unmet needs. This is a single-center open-label clinical trial comparing pentoxifylline, prednisone or metadoxine alone or in combination to assess their efficacy in severe alcoholic hepatitis. The results showed that the groups that received metadoxine achieved better survival. As in other studies, the maintenance of alcohol abstinence was the best predictor of survival. This study found that alcohol abstinence is an independent prognostic factor of the six-month mortality and that patients treated with metadoxine were more likely not to relapse into alcohol consumption. However, intervention did not prevent the complications associated with cirrhosis, which may be because the study was underpowered or that the effect of abstinence itself rather than the intervention was the main predictor for survival.

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ORIGINAL ARTICLE

Clinical Trials Study

Moxibustion combined with acupuncture increases tight junction protein expression in Crohn's disease patients

Hai-Xia Shang, An-Qi Wang, Chun-Hui Bao, Huan-Gan Wu, Wei-Feng Chen, Lu-Yi Wu, Rong Ji, Ji-Meng Zhao, Yin Shi

Hai-Xia Shang, An-Qi Wang, Lu-Yi Wu, Rong Ji, Ji-Meng Zhao, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China
Chun-Hui Bao, Huan-Gan Wu, Yin Shi, Shanghai Institute of Acupuncture-Moxibustion and Meridian, Shanghai 200030, China
Wei-Feng Chen, Zhongshan Hospital affiliated to Fudan University, Shanghai 200032, China
Author contributions: Shang HX, Wang AQ and Bao CH contributed equally to this work; Shi Y designed the study; Wang AQ, Bao CH and Wu LY contributed to the patient enrollment; Bao CH and Wu LY performed the moxibustion and acupuncture procedure; Chen WX performed enteroscopy and tissue sampling; Ji R and Shang HX performed the assays; Zhao JM analyzed the data; Wang AQ and Shang HX drafted the figures and wrote the manuscript; Wu HG and Shi Y conducted the study and revised the manuscript.
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Informed consent: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.
Conflict-of-interest: All authors stated that there is no conflict of interest related to the manuscript.
Data sharing: Technical appendix, statistical code, and dataset available from the corresponding author at flysy0636@163.com. Participants gave informed consent for data sharing.

Correspondence to: Yin Shi, MD, PhD, Shanghai Institute of Acupuncture-Moxibustion and Meridian, No. 650 South Wanping Road, Xuhui District, Shanghai 200030, China. flysy0636@163.com
Telephone: +86-21-64383910
Fax: +86-21-64644238
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Abstract

AIM: To investigate the effect of herb-partitioned moxibustion combined with acupuncture on the expression of intestinal epithelial tight junction (TJ) proteins.

METHODS: Sixty patients diagnosed with mild to moderate Crohn's disease (CD) were allocated into the herb-partitioned moxibustion combined with acupuncture (HMA) group (n = 30) or the mesalazine (MESA) group (n = 30) using a parallel control method. There were 2 sets of acupoints used alternately for HMA treatment. The following points were included in Set A: ST25 (*Tianshu*), RN6 (*Qihai*), and RN9 (*Shuifen*) for herb-partitioned moxibustion and ST36 (*Zusanli*), ST37 (*Shangjuxu*), LI11 (*Quchi*), and LI4 (*Hegu*) for acupuncture. The points for Set B included BL23 (*Shenshu*) and BL25 (*Dachangshu*) for herb-partitioned moxibustion and EX-B2 of T6-T1 (*Jiajixue*) for acupuncture. The patients received the same treatment 6 times a week for 12 consecutive weeks. The MESA group received 1 g of mesalazine enteric coated tablets 4 times daily for 12 consecutive weeks. Intestinal

tissues were stained and examined to compare the morphological and ultrastructural changes before and after the treatment session. Immunohistochemistry and *in situ* hybridization assays were used to detect the expression of intestinal epithelial TJ proteins zonula occludens-1 (ZO-1), occludin, and claudin-1. The mRNA levels were also evaluated.

RESULTS: After the treatment, both herb-partitioned moxibustion combined with acupuncture and mesalazine improved intestinal morphology and ultrastructure of CD patients; the patients treated with HMA showed better improvement. HMA significantly increased the expression of ZO-1 (P = 0.000), occludin (P = 0.021), and claudin-1 (P = 0.016). MESA significantly increased the expression of ZO-1 (P = 0.016) and occludin (P = 0.026). However, there was no significant increase in the expression of claudin-1 (P = 0.935). There was no statistically significant difference between the two groups for the expression of occludin and claudin-1 (P > 0.05). The HMA group showed a significant improvement in ZO-1 expression compared to the MESA group (2333.34 ± 352.51 *vs* 2160.38 ± 307.08, P = 0.047). HMA significantly increased the expression of *ZO-1* mRNA (P = 0.000), occludin mRNA (P = 0.017), and claudin-1 mRNA (P = 0.017). MESA significantly increased the expression of ZO-1 mRNA (P = 0.000), occludin mRNA (P = 0.042), and claudin-1 mRNA (P = 0.041). There was no statistically significant difference between the two groups in the expression of occludin and claudin-1 mRNA (P > 0.05). However, the HMA group showed a significant improvement in *ZO-1* mRNA expression compared with the MESA group (2378.17 ± 308.77 *vs* 2200.56 ± 281.88, P = 0.023).

CONCLUSION: HMA can repair intestinal epithelial barrier lesions and relieve inflammation by upregulating the expression of TJ proteins and their mRNAs.

Key words: Crohn's disease; Herb-partitioned moxibustion; Acupuncture; Intestinal epithelial cells; Tight junction proteins

Core tip: Crohn's disease (CD) is a chronic relapsing inflammatory condition involving all layers of the gastrointestinal tract. Although its etiopathogenesis remains unclear, increased permeability of the intestinal epithelial barrier is one of the crucial factors in CD onset. Tight junctions (TJs) within intestinal epithelial cells form the structural basis of the intestinal epithelial barrier, and reduced expression of TJ proteins is positively correlated with CD severity. This study investigated the therapeutic effect of herb-partitioned moxibustion combined with acupuncture on CD. We found that this treatment upregulated the expression of intestinal epithelial TJ proteins and their mRNAs.

Shang HX, Wang AQ, Bao CH, Wu HG, Chen WF, Wu LY, Ji R, Zhao JM, Shi Y. Moxibustion combined with acupuncture increases tight junction protein expression in Crohn's disease patients. *World J Gastroenterol* 2015; 21(16): 4986-4996 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4986.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4986

INTRODUCTION

Crohn's disease (CD) is a chronic relapsing inflammatory condition involving all layers of the gastrointestinal (GI) tract and can affect any part of the GI tract from the mouth to anus[1]. Clinical epidemiological studies have shown the prevalence of CD in mainland China is 1.4 cases per 100000 person-years, and the incidence has increased steadily and rapidly in recent years[2].

The clinical management of CD involves conventional medical treatments including non-steroidal anti-inflammatory drugs, such as 5-aminosalicylate (5-ASA), corticosteroids and immunosuppressive drugs, such as thiopurines[3]. However, the European Crohn's and Colitis Organization has recognized the efficiency of 5-ASA as "limited"[4] in improving patients' CD Activity Index (CDAI). The inappropriate use of large amounts of corticosteroids and insufficient amounts of thiopurines is common among Asian physicians[5] and results in drug resistance and adverse effects. One new treatment approach in Western countries is biologic therapy, such as anti-tumor necrosis factor (anti-TNF) agents or infliximab[6]. However, in Asia, the use of biologics is limited by economic burdens due to the strict coverage range of medical insurance.

Complementary and alternative medicines (CAMs), such as Chinese medicine, flourish in Asia and especially in East Asia. In China, more than half of CD patients receive concomitant traditional Chinese therapies[7] including Chinese materia medica decoction, acupuncture and moxibustion. The efficacy and safety of these therapies have been frequently questioned because of the indiscriminate use of CAMs in patients with CD. As a result, many attempts have been made to obtain concrete evidence of their therapeutic effect and to investigate their possible mechanisms. Recent studies[8] have shown that apart from the undeniable placebo effect, acupuncture and moxibustion offer additional therapeutic benefits in patients with mild to moderately active CD. Compared with the conventional 5-ASA treatment, moxibustion and acupuncture have a significant advantage in improving quality-of-life ratings and CDAI scores of patients with mild to moderate CD[9]. In further clinical studies, we found that herb-partitioned moxibustion combined with acupuncture can inhibit intestinal epithelial cell apoptosis by decreasing the overexpression of intestinal mucosa tumor

necrosis factor alpha (TNF-α), tumor necrosis factor 1 (TNFR1), and tumor necrosis factor 2 (TNFR2)[10]. The treatments also reduce intestinal inflammation by increasing hemoglobin (HGB) counts and by decreasing C-reactive protein levels and erythrocyte sedimentation rates in CD patients[11].

Previous studies have suggested that increased intestinal permeability may appear precede clinical manifestations[12] and could be used to predict clinical relapses[13] of CD. Animal experiments have been performed to understand the pathological basis underlying the occurrence of CD and to investigate the cause of increased intestinal permeability from two aspects. One aspect is excessive epithelial cell apoptosis[14,15]. The other aspect is intestinal mucosal epithelial barrier dysfunction[16]. The main function of the intestinal epithelial barrier is to maintain intestinal permeability, and the barrier depends on the dynamically changing formation of tight junctions (TJs) reacting to varied extracellular stimuli[17]. Through protein-protein interactions, the cytoplasmic protein zonula occludens-1 (ZO-1) and the transmembrane proteins claudin-1 and occludin are able to modulate and associate with different forms of multimolecular complexes to regulate the formation of TJs[18]. In previous study, we observed decreased intestinal permeability and reduced expression of TJ proteins ZO-1, claudin-1 and occludin in a TNBS-induced CD rat model. However, treatment with herb-partitioned moxibustion and acupuncture increased the expression of TJ proteins ZO-1, claudin-1 and occludin. Additionally, the inflammatory reaction in the intestinal mucosa was improved based on histological observation[19].

Therefore, we hypothesized that herb-partitioned moxibustion combined with acupuncture could increase intestinal permeability by upregulating the expression of TJ proteins ZO-1, claudin-1 and occludin in CD patients. In this study, we examine the mechanism of herb-partitioned moxibustion combined with acupuncture in CD patients and try to verify the feasibility of acupuncture and moxibustion methods for treating mild to moderate CD.

MATERIALS AND METHODS

Patients and group allocation

The study was performed from July 2009 through March 2010 in the CD clinic of the Shanghai Research Institute of Acupuncture within the Moxibustion and Meridian Endoscopy Center of Zhongshan Hospital affiliated to Fudan University. Ethics approval was obtained from the Chinese Clinical Trial Register Center (registration number: ChiCTR-TRC-10000950). All patients provided written informed consent prior to the beginning of the trial. Patients with CD were recruited in accordance with Jinan diagnostic criteria (revised by the National Conference on Inflammatory Bowel Disease in 2007[20]). Patients with mild to moderate disease and CDAI scores between 150 and 450 were included. The patients were not treated with other relevant pharmacological therapies and signed an informed consent. The exclusion criteria included the following: pregnant or lactating patients, psychotic patients, and patients with severe heart, brain, liver, kidney, or hematopoietic system diseases and other severe diseases. This study was conducted as a controlled trial with 2 parallel treatment groups. Patients from the Shanghai Research Institute of Acupuncture-Moxibustion and Meridian were enrolled in the herb-partitioned moxibustion combined with acupuncture group (HMA group, n = 30). The patients from the endoscopy center of Zhongshan Hospital affiliated with Fudan University were included in the mesalazine group (MESA group, n = 30).

Treatments

The patients in the HMA group received herb-partitioned moxibustion combined with acupuncture. There were 2 sets of acupoints used for treatment. The points in Set A included the following: ST25 (*Tianshu*), RN6 (*Qihai*), and RN9 (*Shuifen*) for herb-partitioned moxibustion and ST36 (*Zusanli*), ST37 (*Shangjuxu*), LI11 *(Quchi*), and LI4 (*Hegu*) for acupuncture. The points in Set B were BL23 (*Shenshu*) and BL25 (*Dachangshu*) for herb-partitioned moxibustion and EX-B2 of T6 - T1 (*Jiajixue*) for acupuncture. These acupoints were located based on the national GB-12346-90 acupoint standard. The procedure used involved placing moxa cones (1.7 cm in height and 1.8 g in weight; Hanyi, Henan, China) on a herbal cake (2.3 cm in diameter and 0.5 cm in length). The herbal cake consisted of 3 g of Shaoxing wine and 2.5 g of herbal powder [medicinal formula: Aconite preparata (radix), Cinnamomi (cortex), *etc.*]. Four sets of moxa cones and herbal cakes were used for each treatment. The skin was cleaned with a tincture of iodine and alcohol, and then sterile single-use acupuncture needles (Φ 0.30 mm × 40 mm specification, Huatuo, Suzhou, China) were inserted between 20 mm and 40 mm into the acupoints. Acupuncture was performed by the same qualified and skilled physician. When two moxa cones burned out, the moxa cones, herbal cake and needles were removed. The treatment was applied once per day, 6 times per week for 12 consecutive weeks. The MESA group received mesalazine enteric coated tablets four times a day for 12 consecutive weeks.

Tissue sampling

On a voluntary basis and after signing an informed consent form, intestinal mucosa tissue samples from 10 patients of each group were removed *via* painless enteroscopy before and after the treatment session.

Morphological observation

Pieces of intestinal mucosa tissues (0.5 cm^3 for each)

were collected from CD patients. The intestinal mucosa tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin and then sectioned into 4-μm-thick tissue sections. The tissue sections were then stained with hematoxylin and eosin, dehydrated in 95%, 90% and 70% ethanol and cleared in xylene. The stained sections were mounted in Permount or Histoclad and observed using an Olympus DP73 microscope (Olympus, Tokyo, Japan).

Ultrastructural observation

The intestinal mucosa tissues were cut into 1-mm^3 strips, fixed for 4 h at 4 ℃ in 5% glutaraldehyde and then washed 3 times in 0.1 mol/L phosphate buffered saline (PBS). The tissues were then postfixed for 2 h at 4 ℃ in 2% osmium tetroxide and dehydrated in a graded ethanol series. The tissues were embedded in Epon 812 and then cut into ultrathin sections (75 nm) and stained with uranyl acetate and lead citrate. The sections were viewed in a HITACHI H-600 electron microscope at 80 kV (HITACHI, Tokyo, Japan).

Immunohistochemical assay

The expression levels of occludin, claudin-1, and ZO-1 were detected by immunohistochemical assays. The sections were dewaxed, hydrated, and then pretreated in a microwave (antigen retrieval). The endogenous peroxidase activity was inhibited with 0.3% H_2O_2. The nonspecific binding was blocked with 20% normal goat serum. All sections were incubated with ZO-1 (Rabbit anti-ZO-1 polyclonal antibody 1:50, Invitrogen, New York, United States), occludin (Rabbit anti-Occludin polyclonal antibody 1:100, Invitrogen, New York, United States) and claudin-1 antibodies (Mouse anti-Claudin-1 monoclonal antibody 1:100, Invitrogen, New York, United States) for 2 h at 37 ℃. The samples were washed and then incubated for 30 min at room temperature with appropriate preabsorbed biotinylated secondary antibody. The antigen was visualized using the streptavidin-peroxidase method (JRDUN Biotechnology Co., Ltd., Shanghai, China), and 3,3-diaminobenzidine (DAB) (Liquid DAB-Plus Substrate Kit, JRDUN Biotechnology Co., Ltd., Shanghai, China) was used as a chromogen. The slides were washed in distilled water and counterstained with Mayer's hematoxylin before they were dehydrated and mounted. The primary antibody was replaced with PBS for a negative control. A semi-quantitative analysis of the staining results was conducted using the IMS medical image quantitative analysis system (JRDUN Biotechnology Co., Ltd., Shanghai, China). Positive results for ZO-1, occludin and claudin-1 were brown or yellow particles stained among intestinal epithelium. The positive area and the optical density (OD) values in 3 high-power optical fields (× 200) of every slice were measured. The immune positive area index (positive area/total area × OD) values of ZO-1, occludin and claudin-1 were calculated in every high-power optical field.

In situ hybridization

The expression levels of ZO-1, occludin and claudin-1 mRNAs were detected by *in situ* hybridization. Digoxigenin-labelled RNA probes were generated with a DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany) and a relevant plasmid (provided by JRDUN Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's protocol. The tissues were dewaxed and hydrated. The sections were heated at 98 ℃ for 20 min in EDTA solution (0.01 mol/L, pH 8.0) and pre-treated with the following solutions: 2 μg/mL proteinase K in TE at 37 ℃ for 8 min; 0.1 mol/L glycine in PBS for 10 min; a graded ethanol series (each concentration for 1 min); and 0.2 × SSC and 50% formamide to pre-hybridize at 37 ℃ for 30 min. Pre-treated sections were covered with 20-30 mL of hybridization buffer (1 μg/mL RNA probe) and incubated at 48 ℃ for 8-12 h under a coverslip. The slides were washed twice at 45 ℃ for 5 min in 2 × SSC and twice at 37 ℃ for 5 min in 1 ×, 0.5 ×, 0.1 × SSC. The samples were then blocked in 10% serum. The sections were then incubated at 37 ℃ for 30 min in mouse McAb anti-Dig IgG (cat#84-0146, lot#305359, Invitrogen). The slides were washed three times in PBS and further incubated at 37 ℃ for 40 min with goat anti-Mouse IgG-HRP antibody (cat#84-01450, lot#651053A, Invitrogen). The sections were washed three times in PBS for 3 min each. The staining detection was performed using 0.04% DAB and 0.03% H_2O_2 according to the manufacturer's recommendations. The tissues were dehydrated and mounted after color development. We performed semi-quantitative analyses of the results, and the positive expression area index (positive area/total area × OD) values of ZO-1, occludin and claudin-1 mRNA were calculated in 3 high-power optical fields.

Statistical analysis

All measurement data are presented as the mean ± SD. All statistical analyses were performed using SPSS 16.0 (SPSS Inc. Illinois, United States). The comparison of sex and severity from the baseline data were analyzed using the χ^2 test. The changes in age, duration of disease from the baseline data and between group differences after treatment were compared using two independent sample *t*-tests. A paired-samples *t*-test was used for within group comparisons. All two-sided *P* values < 0.05 were considered statistically significant.

RESULTS

Baseline subject characteristics

Table 1 presents the baseline characteristics of the two groups by age, sex, duration of disease, and severity of disease. There were no differences between the HMA group and the MESA group before the procedure.

Table 1 Baseline characteristics

	HMA n = 30	MESA n = 30	P value
Age (yr)			
Min	22	18	
Max	56	65	
mean ± SD	31.77 ± 8.77	36.93 ± 13.25	0.080
Sex (M/F)	23/7	19/11	0.260
Duration of disease (yr)			
Min	1	1	
Max	12	18	
mean ± SD	6.33 ± 3.63	5.67 ± 4.08	0.507
Severity of disease (mild/moderate)	22/8	18/12	0.273

HMA: Herb-partitioned moxibustion combined with acupuncture group; MESA: Mesalazine group.

Intestinal morphological observations

Figure 1 shows the morphological observations of intestinal mucosa tissues from both groups before and after treatment. Figure 1A shows that in the HMA group before treatment, the mucosal epithelium was seriously damaged. The intestinal glands were rare, and there were ulcerations and obvious submucosal hyperemia and edema. Tissue damage was observed in the mucosa, submucosa and muscular layer, and there was substantial eosinophil and inflammatory cell infiltration in the intestinal mucosa and submucosa. Figure 1B shows that in the HMA group after treatment, there was only a small amount of hyperemia and inflammatory cell infiltration in the intestinal mucosa and submucosa. The intestinal glands were arranged in an orderly manner. Figure 1C shows that in the MESA group before treatment, the mucosal epithelium was also seriously damaged. There was obvious hyperemia and edema, and eosinophils and inflammatory cells had infiltrated into the intestinal mucosa and submucosa. There was also ulceration and tissue damage in the mucosa, submucosa and muscular layer. Figure 1D shows that in the MESA group after treatment, there was less severe hyperemia and edema in intestinal mucosa and submucosa. Additionally, some of the intestinal glands were restored but some were poorly organized.

Intestinal ultrastructural observations

Figure 2 presents ultrastructural images of the intestinal mucosa tissues from both groups before and after treatment. Figure 2A shows that in the HMA group before treatment, the connections between the epithelial cells were loose and that there was a significant broadening of intercellular spaces. Furthermore, the cell membranes were partly injured, and intestinal epithelial cells contained a small number of bubbles inside of the cytoplasm. Figure 2B shows that after HMA treatment the connections between the epithelial cells were relatively tight and that the intercellular spaces between cells were not broadened. A small amount of particle secretion was observed, and a few villi could be observed on the cell surfaces. Figure 2C shows that in the MESA group before treatment, the connections between the epithelial cells were quite loose and that there was significant broadening of intercellular spaces. Particle secretion was detected. Figure 2D shows that after MESA treatment the connections between the epithelial cells were tight. However, there was broadening of some intercellular spaces, and particle secretion was detected.

Expression of TJ proteins occludin, claudin-1 and ZO-1

After treatment, the HMA group showed significantly increased expression of occludin (P = 0.021), claudin-1 (P = 0.016), and ZO-1 (P = 0.000) (Figure 3A, B and E). The MESA group showed a significant increase in the expression of occludin (P = 0.026) and ZO-1 (P = 0.016). However, there was no significant increase in the expression of claudin-1 (P = 0.935) (Figure 3C-E). There was no statistical difference for the expression of occludin (P = 0.512) and claudin-1 (P = 0.055) between groups. The HMA group showed a significant improvement in ZO-1 expression compared to the MESA group (2333.34 ± 352.51 *vs* 2160.38 ± 307.08, P = 0.047) (Figure 3B, D and E).

Expression of TJ proteins occludin, claudin-1 and ZO-1 mRNAs

The HMA group showed significant increases in the expression of *ZO-1* mRNA (P = 0.000), occludin mRNA (P = 0.017), and claudin-1 mRNA (P = 0.017) (Figure 4A, B and E). The MESA group showed significant increases in the expression of *ZO-1* mRNA (P = 0.000), occludin mRNA (P = 0.042), and claudin-1 mRNA (P = 0.041) (Figure 4C-E). There was no difference between groups in the expression of occludin mRNA and claudin-1 mRNA (P = 0.748, P = 0.388). The HMA group showed a significant increase in *ZO-1* mRNA expression compared to the MESA group (2378.17 ± 308.77 *vs* 2200.56 ± 281.88, P = 0.023) (Figure 4B, D and E).

DISCUSSION

The dominant symptom of CD is "leak-flux diarrhea" due to epithelial barrier dysfunction, which results in increased epithelial permeability and a continuous loss of solutes[21]. Previous studies have shown that increases in intestinal permeability not only act as an etiological factor in CD[22] but also precede clinical relapses in CD and are an indicator of subclinical disease[13,23]. However, increased intestinal permeability has also presented in first-degree relatives of CD patients in the absence of clinical symptoms[24,25]. This result suggests that increased intestinal permeability might be one of several pathogenic factors in CD. In recent decades, new findings have revealed the

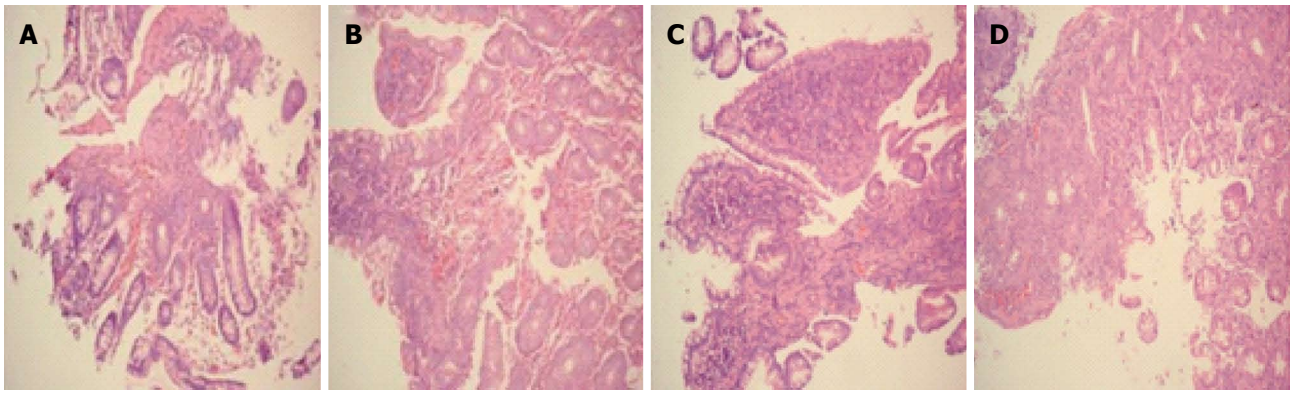

Figure 1 Morphological observation of patient intestinal mucosa tissue from two groups before and after treatment. A: HMA group before treatment; B: HMA group after treatment; C: MESA group before treatment; D: MESA group after treatment. Magnification × 200 (A-D). HMA: Herb-partitioned moxibustion combined with acupuncture group; MESA: Mesalazine group.

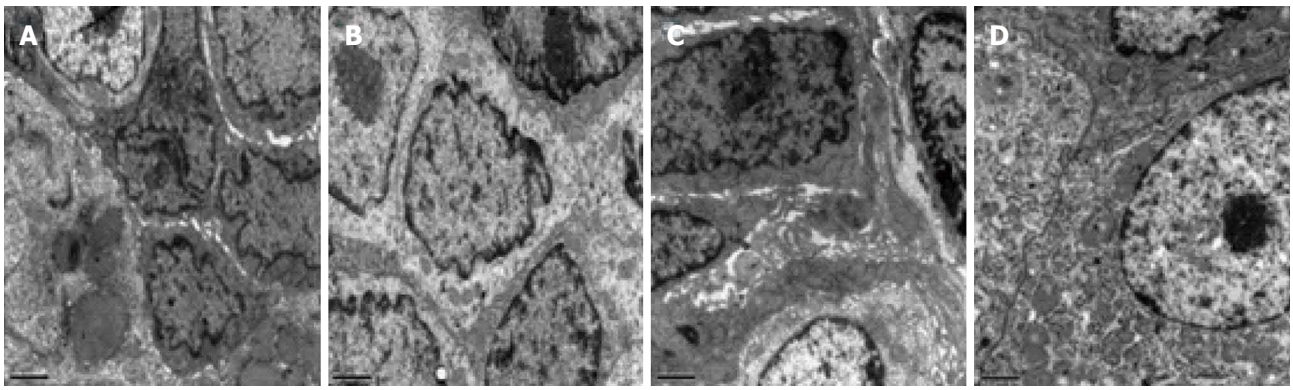

Figure 2 Ultrastructural observation of patient intestinal mucosa tissue from two groups before and after treatment. A: HMA group before treatment; B: HMA group after treatment; C: MESA group before treatment; D: MESA group after treatment. Magnification × 10000 (A-D). HMA: Herb-partitioned moxibustion combined with acupuncture group; MESA: Mesalazine group.

major factors underlying CD etiopathogenesis. These factors include the following: excessive bacterial translocation caused by intestinal epithelial barrier dysfunction[26], infection resulting in dysfunction of immunotolerance and aggressive immune response to bacteria[27,28], significant loss of complexity in species of the Firmicutes and the Bacteroidetes phyla and increased Enterobacteriaceae, particularly *Escherichia coli* species[29,30]. The epithelial barrier is the primary defense against exogenous pathogens. Thus, maintaining and repairing the epithelial barrier is crucial for the treatment and prevention of CD.

The epithelial barrier is a single layer of epithelial cells that lines the entire digestive tract. A TJ seals the intercellular space between adjacent epithelial cells[31], thus, serve as the major determinant of epithelial permeability[32]. The crucial feature of TJs is a fibril-like protein structure called TJ strands. The strands are connected with each other to create a continuous network. The TJ strands act as a diffusion barrier to regulate the transport of ions, macromolecules and immune cells in the paracellular pathway[33]. A TJ is a membrane-associated multimolecular complex composed of three transmembrane protein families[34]. The protein families consist of the claudin family[35], the junctional adhesion molecule (JAM) protein family[36] and the TJ-associated Marvel domain proteins (TAMPs) family, which includes occludin, tricellulin and Marvel D3[37]. Claudins are responsible for the charge[38] and size-selectivity[39,40] of the TJ barrier. The JAM proteins and TAMPs are mainly responsible for the stabilization of TJs and the regulation of epithelial permeability[41,42]. ZO-1 is a member of the membrane-associated guanylate kinases family. ZO-1 is a multi-domain scaffolding protein with an important role in the assembly of the TJ barrier and in the maintenance of the cytoskeleton[32] because it establishes a connection between the TJ barrier and perijunctional actomyosin[43].

Previous studies have shown that the expression of TJ proteins occludin, claudin-1, and ZO-1 were significantly decreased in the lamina propria in both active and chronic CD patients. The decrease in ZO-1 leads to increased intestinal epithelial permeability and TJ barrier dysfunction[44,45]. In this study, we evaluated occludin, claudin-1, and ZO-1 as indicators of intestinal epithelial barrier dysfunction during CD pathologic processes. We also examined the impact of

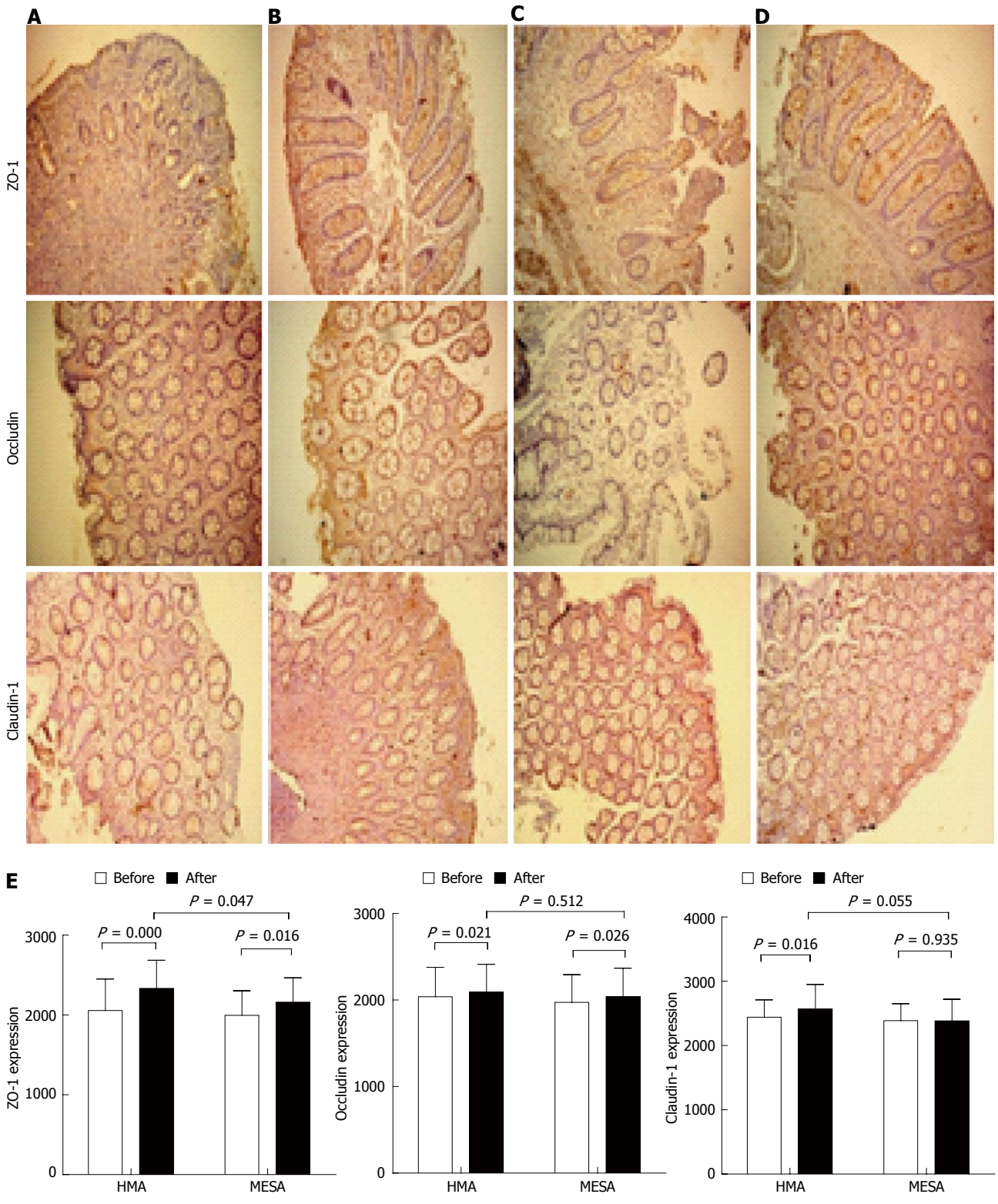

Figure 3 Expression of protein zonula occludens-1, occludin and claudin-1 in two groups before and after the treatment session. A: HMA group before treatment; B: HMA group after treatment; C: MESA group before treatment; D: MESA group after treatment; E: Bar graphs of the expression of protein zonula occludens-1, occludin and claudin-1 in HMA group and MESA group before and after the treatment session. HMA: Herb-partitioned moxibustion combined with acupuncture group; MESA: Mesalazine group; ZO-1: Zonula occludens-1.

HMA therapy on the intestinal epithelium. We chose mesalazine treatment for the control group patients because it is one of the most commonly used 5-ASA therapies used for treating mild to moderate CD. We compared HMA with mesalazine treatment to evaluate the possible application of HMA in CD management.

In this study, we evaluated the morphology and ultrastructure of intestinal mucosa tissue before and

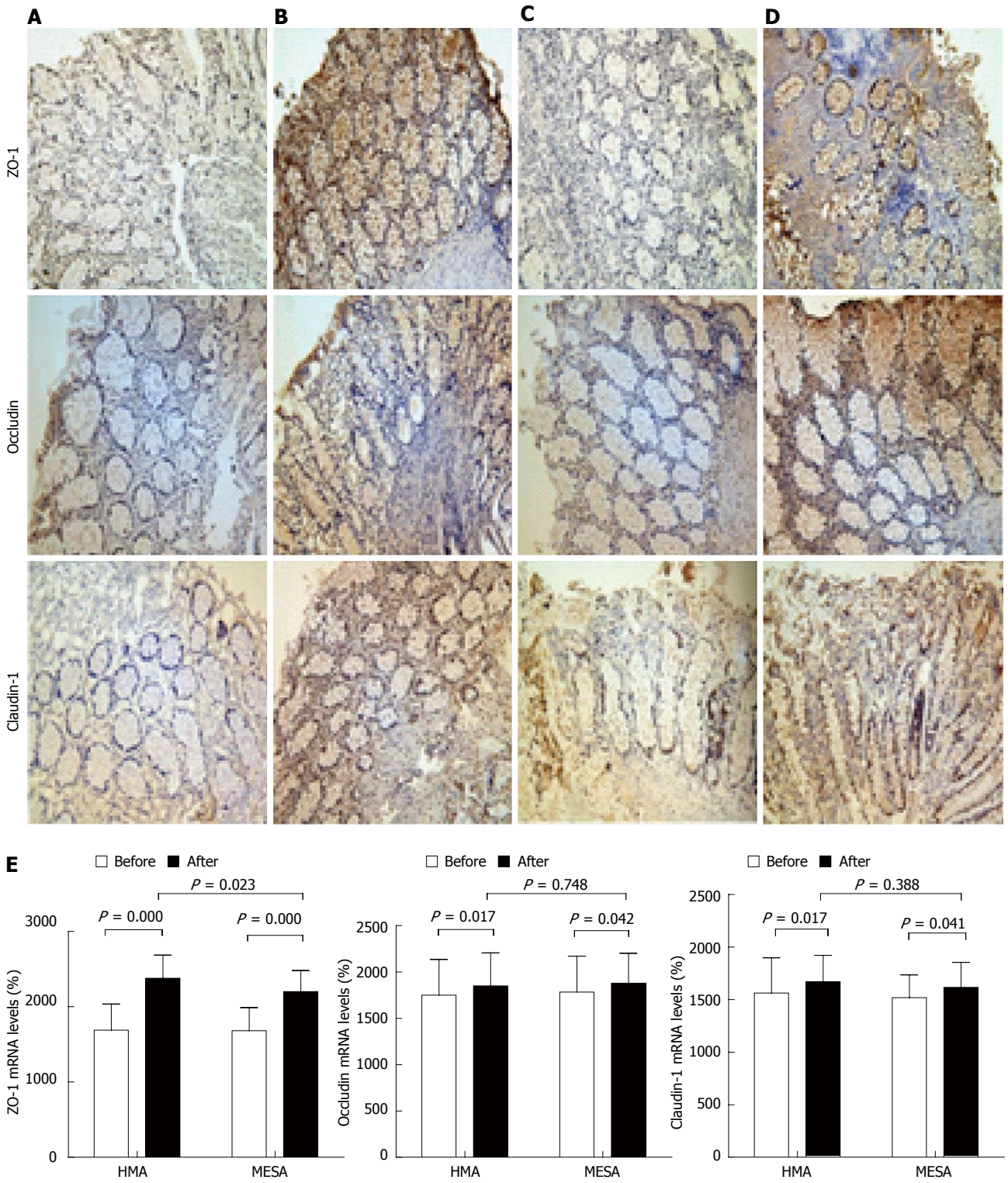

Figure 4 Expression of zonula occludens-1 mRNA, occludin mRNA and claudin-1 mRNA in two groups before and after the treatment session. A: HMA group before treatment; B: HMA group after treatment; C: MESA group before treatment; D: MESA group after treatment; E: Bar graphs of the expression of zonula occludens-1 mRNA, occludin mRNA and claudin-1 mRNA in HMA group and MESA group before and after the treatment session. HMA: Herb-partitioned moxibustion combined with acupuncture group; MESA: Mesalazine group; ZO-1: Zonula occludens-1.

after treatment. Before treatment, the morphological observations using light microscopy showed epithelia impairment, intestinal gland loss and inflammatory cellular infiltration. These findings suggested that the basic structure of the epithelial mucosa was damaged and that mucosal inflammation occurred. Furthermore, analysis of the tissue ultrastructure by electron microscopy showed intercellular connection loss and broadened intercellular spaces. These results suggested that TJs were damaged and failed

to strengthen the intercellular connections and seal the intercellular spaces. These defects may lead to a continuous loss of solute and compromise resistance against pathogens in the gut lumen. After the treatments, the morphological and ultrastructural observations of the MESA group showed partially recovered but still disorganized intestinal glands. There was also limited inflammatory cellular infiltration and comparatively strengthened intercellular connections with partially narrowed intercellular spaces. These results suggest that mesalazine ameliorate inflammation in the intestinal mucosa and can restore the TJ barrier function. In the HMA group, the morphological and ultrastructural observations revealed regularly arranged intestinal glands, mild and localized inflammatory cellular infiltration, tight intercellular connections and few broadened intercellular spaces. These findings suggest that HMA induces mucosa inflammation remission and repairs the TJ barrier structure. Our comparisons between the MESA group and the HMA group indicate that HMA achieves inflammatory remission similar to mesalazine and surpasses mesalazine in repairing the TJ barrier structure in intestinal epithelial mucosa.

We compared the expression of the TJ proteins and their mRNAs before and after treatment. The data show that the expression of occludin, claudin-1, and ZO-1 and their mRNAs are significantly increased in both the MESA and HMA group after treatment. This result in combination with morphological evidence suggests the mechanism of mesalazine and HMA is to repair the TJ barrier by upregulating the expression of TJ proteins such as occludin, claudin-1, and ZO-1 and their mRNAs. The expression of occludin and claudin-1 and their mRNAs showed no significant differences between the MESA group and the HMA group. However, the HMA group showed a significant increase in ZO-1 (P = 0.047) and ZO-1 mRNA (P = 0.023) expression compared to the MESA group.

ZO-1 mediates the assembly of TJs by organizing components of TJs and linking them to the cortical actin cytoskeleton[46]. We found that there was a significant difference between groups in the expression *ZO-1* and its mRNA, which may suggest a different mechanism of HMA and mesalazine in CD patients. However, the specific functional mechanism of HMA still requires further investigation.

This study is only an initial attempt to investigate the effectiveness of HMA treatment and its underlying mechanism. We should examine several questions in future studies. What is the indication for HMA in CD treatment - mild or moderate CD? Is HMA alone effective in controlling CD symptoms, or does HMA only function as a supplement to conventional management? Is there any short-term or long-term adverse reaction to HMA, and what is the corresponding measurement?

In conclusion, HMA improves intestinal epithelial barrier repair and reduces inflammation in CD patients by upregulating the expression of the TJ proteins occludin, claudin-1, and ZO-1 and their mRNAs.

COMMENTS

Background

Crohn's disease (CD) is a chronic, recurrent inflammatory bowel disease. Although its etiopathogenesis remains obscure, compromised permeability of the intestinal epithelial barrier is recognized to play a pivotal role in CD pathology.

Research frontiers

Tight junctions (TJs) within intestinal epithelial cells form the structural basis of the intestinal epithelial barrier and are the major determinant of epithelial permeability. Recent studies have confirmed that the decreased expression of TJ proteins is positively correlated with CD severity.

Innovations and breakthroughs

Previous studies by Shang *et al* have demonstrated the efficiency of acupuncture and herb-partitioned moxibustion in improving CD patients' clinical symptoms and pathological changes. In the current study, they found that herb-partitioned moxibustion combined with acupuncture (HMA) can improve epithelial barrier repair and reduce inflammation by upregulating the expression of TJ proteins in CD patients.

Applications

This study provides initial evidence of the therapeutic effect of HMA on CD by upregulating the expression of intestinal epithelial TJ proteins and their mRNAs. Therefore, HMA might be considered as an alternative option to treat mild to moderate CD.

Terminology

The intestinal epithelial barrier is a monolayer lining the entire digestive tract. Tight junctions are fibril-like structures connecting adjacent cells, sealing the intercellular spaces to control paracellular transportation of ions, macromolecules and immune cells.

Peer-review

This study evaluated whether herb-partitioned moxibustion combined with acupuncture changes the permeability of the intestinal epithelial barrier by affecting the expression of colonic epithelial TJ-related proteins in mild to moderate CD patients. The question posed by the authors is well defined and the methods are described appropriately. The data and figures in the manuscript appear to be genuine.

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ORIGINAL ARTICLE

Observational Study

Role of colonoscopy in the diagnostic work-up of bowel endometriosis

Marco Milone, Antonio Mollo, Mario Musella, Paola Maietta, Loredana Maria Sosa Fernandez, Olena Shatalova, Alessandro Conforti, Gianni Barone, Giuseppe De Placido, Francesco Milone

Marco Milone, Mario Musella, Paola Maietta, Olena Shatalova, Gianni Barone, Francesco Milone, Department of Advanced Biomedical Science, University of Naples "Federico II", 80131 Naples, Italy
Antonio Mollo, Loredana Maria Sosa Fernandez, Giuseppe De Placido, Department of Neuroscience, Reproductive Science and Odonstostomatology, University of Naples "Federico II", 80131 Naples, Italy
Gianni Barone, Department of Surgery, Fatebenefratelli Hospital, 80123 Naples, Italy
Author contributions: Milone M and Musella M contributed equally to this manuscript; Milone M was the main investigator and wrote the first manuscript draft; Milone M, Mollo A, Musella M, Maietta P, Sosa Fernandez LM, Shatalova O, Conforti A and Barone G designed the study; Maietta P, Sosa Fernandez LM, Shatalova O, Conforti A and Barone G were involved in patient recruitment and collection of the data; Milone M, Mollo A, Musella M and Barone G significantly contributed to the interpretation of findings; Milone F and De Placido G share the co-seniorship, approved the research and significantly contributed to the final version of the article; all the authors read and approved the final manuscript.
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Correspondence to: Marco Milone, MD, Department of Advanced Biomedical Science, University of Naples "Federico II", Via Pansini 5, 80131 Naples, Italy. milone.marco@alice.it
Telephone: +82-39-817463064
Fax: +82-39-817462896
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Abstract

AIM: To evaluate the accuracy of colonoscopy for the prediction of intestinal involvement in deep pelvic endometriosis.

METHODS: This prospective observational study was performed between September 2011 and July 2014. Only women with both a clinical and imaging diagnosis of deep pelvic endometriosis were included. The study was approved by the local ethics committee and written informed consent was obtained in all cases. Both colonoscopy and laparoscopy were performed by expert surgeons with a high level of expertise with these techniques. Laparoscopy was performed within 4 wk of colonoscopic examination. All hypothetical colonoscopy findings (eccentric wall thickening with or without surface nodularities and polypoid lesions with or without surface nodularities of endometriosis) were compared with laparoscopic and histological findings. We calculated the sensitivity, specificity, positive predictive value and negative predictive value for the presence of colonoscopic findings of intestinal endometriosis.

RESULTS: A total of 174 consecutive women aged between 21-42 years with a diagnosis of deep pelvic endometriosis who underwent colonoscopy and

surgical intervention were included in our analysis. In 76 of the women (43.6%), intestinal endometrial implants were found at surgery and histopathological examination. Specifically, 38 of the 76 lesions (50%) were characterized by the presence of serosal bowel nodules; 28 of the 76 lesions (36.8%) reached the muscularis layer; 8 of the 76 lesions (10.5%) reached the submucosa; and 2 of the 76 lesions (2.6%) reached the mucosa. Colonoscopic findings suggestive of intestinal endometriosis were detected in 7 of the 174 (4%) examinations. Colonoscopy failed to diagnose intestinal endometriosis in 70 of the 76 women (92.1%). A colonoscopic diagnosis of endometriosis was obtained in all cases of mucosal involvement, in 3 of 8 cases (37.5%) of submucosal involvement, in no cases of muscularis layer involvement and in 1 of 38 cases (2.6%) of serosa involvement. The sensitivity, specificity, positive predictive and negative predictive values of colonoscopy for the diagnosis of intestinal endometriosis were 7%, 98%, 85% and 58%, respectively.

CONCLUSION: Being an invasive procedure, colonoscopy should not be routinely performed in the diagnostic work-up of bowel endometriosis.

Key words: Endometriosis; Colonoscopy; Intestinal; Bowel; Laparoscopy

Core tip: Endometriosis is common gynecological condition that in a substantial number of cases injures intestinal tissue and causes remarkable morbidity among affected individuals. A surgical approach is still the most effective, but preoperative assessment is often challenging even for expert physicians and requires several diagnostic techniques for a clear definition of the location and extent of endometrial implants. The aim of the present study was to evaluate the role of colonoscopy in the diagnostic work-up of bowel endometriosis.

Milone M, Mollo A, Musella M, Maietta P, Sosa Fernandez LM, Shatalova O, Conforti A, Barone G, De Placido G, Milone F. Role of colonoscopy in the diagnostic work-up of bowel endometriosis. *World J Gastroenterol* 2015; 21(16): 4997-5001 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/4997.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.4997

INTRODUCTION

Intestinal endometriosis is a condition that causes significant morbidity in affected individuals and, despite our current knowledge of this disease, it continues to be a challenging diagnosis to make preoperatively[1]. Although a precise diagnosis regarding the presence, location and extent of endometrial implants should be required during the preoperative evaluation in order to ensure the best therapeutic approach and treatment planning[2], there is a notable absence of agreed upon disease-specific endoscopic and radiological features[3].

The reference standard for the diagnosis of endometriosis is the laparoscopic visualization of suspicious lesions, which also provides correct staging of the disease, as established by the American Fertility Society[4-6].

Conversely, the role of colonoscopy in the assessment of bowel involvement is still controversial.

Despite some authors believing that the paucity of mucosal involvement makes colonoscopy more useful in excluding other diagnoses rather than confirming the diagnosis[7,8], other authors identify the colonoscopic findings of intestinal endometriosis[9].

The aim of the present study was to evaluate the accuracy of colonoscopy for the prediction of intestinal involvement in deep pelvic endometriosis using laparoscopic and histological data as the reference standard.

MATERIALS AND METHODS

This prospective observational study was carried out between September 2011 and July 2014 in women with a clinical and radiological diagnosis of deep pelvic endometriosis. Written informed consent was obtained in all cases and was approved by the local ethics committee.

The inclusion criteria were as follows: clinical symptoms, such as chronic pelvic pain, dysmenorrhea, dyspareunia and infertility; gastrointestinal disorders suggestive of bowel involvement, such as rectal pain coincident with menses and cramping abdominal pain before or during the passage of stools; defecation disorders without signs of bowel obstructions; and video laparoscopy within 4 wk of the colonoscopic examination. The patients who did not undergo video laparoscopy within 4 wk of the imaging were excluded.

Colonoscopy was performed in all cases by an expert operator with over 10 years of experience in intestinal endoscopy, focusing on all hypothetical colonoscopic findings of endometriosis, according to previous literature (eccentric wall thickening with or without surface nodularities and polypoid lesions with or without surface nodularities). The exam was performed again until accurate bowel cleaning was obtained. No biopsies were taken and the diagnosis was made at bowel resection. Of interest, the endoscopist was blinded about the previous radiological diagnosis.

In all surgeries, after adequate adhesiolysis, the presence, location, number of nodules and extent of endometriosis were noted during laparoscopic surgery

Table 1 Colonoscopy and deep pelvic endometriosis

	Colonoscopy and deep pelvic endometriosis (n = 174)	
	Presence of intestinal endometriosis	Absence of intestinal endometriosis
Presence of colonoscopic findings	6	1
Absence of colonoscopic findings	70	97
Sensitivity = 7% Specificity = 98%		
Positive predictive value = 85%		
Negative predictive value = 58%		

performed by expert laparoscopic surgeons (more than 200 laparoscopic procedures were performed). All specimens obtained were evaluated histologically for the presence of endometrial tissue, particularly focusing on intestinal wall involvement. Diagnosis of rectosigmoid endometriosis was based on the presence of ectopic endometrial and stromal tissue penetrating at least into the serosal layer of the bowel wall. Colonoscopic findings were compared with laparoscopic and histological findings. Of interest, bowel resection was not influenced by colonoscopic findings; bowel involvement was assessed by laparoscopic evaluation.

We calculated the sensitivity (those with both presence of colonoscopic findings and diagnosis of intestinal endometriosis/those with diagnosis of intestinal endometriosis), specificity (those without the presence of either colonoscopic findings or diagnosis of intestinal endometriosis/those without diagnosis of intestinal endometriosis), positive predictive value (those with the presence of both colonoscopic findings and diagnosis of intestinal endometriosis/ those with the presence of colonoscopic findings) and negative predictive value (those without the presence of colonoscopic findings or diagnosis of intestinal endometriosis/those without diagnosis of intestinal endometriosis) for the presence of colonoscopic findings of intestinal endometriosis.

Statistical analysis

Statistical methods should be described when they are used to verify the results. Suitable techniques are chosen for the statistical treatments; for example, *t* test (group or paired comparisons), χ^2 test, Ridit, probit, logit, regression (linear, curvilinear or stepwise), correlation, analysis of variance (ANOVA), analysis of covariance, *etc*.

RESULTS

One hundred and seventy-four consecutive women in the age range 21-42 years (mean age 29.7 ± 5.2 years) with diagnosis of deep pelvic endometriosis (by echography and magnetic resonance) who underwent colonoscopy and surgical intervention were included in our analysis. In 76 women (43.6%), intestinal endometrial implants were found at surgery and histopathological examination. Colonoscopy and video laparoscopy were concordant in 103 out of 174 cases (59.1%). Colonoscopic findings suggestive of intestinal endometriosis were detected in 7 out of 174 (4%) examinations. Colonoscopy failed to diagnose intestinal endometriosis in 70 out of 76 women (92.1%).

In detail, 38 out of 76 lesions (50%) were characterized by the presence of serosal bowel nodules; 28 out of 76 lesions (36.8%) reached the muscularis layer; 8 out of 76 lesions (10.5%) reached the submucosa and 2 out of 76 lesions (2.6%) reached the mucosa.

Of interest, diagnosis of intestinal endometriosis by colonoscopy was obtained in all 2 cases of mucosa involvement, in 3 out of 8 cases (37.5%) of submucosa involvement, in no cases of muscularis layer involvement and in 1 out of 38 cases (2.6%) of serosa involvement.

We found 2 cases of polypoid lesions without surface nodularities which were confirmed to be intestinal endometriosis and 5 cases of wall thickening without surface nodularities, of which one was not confirmed to be an intestinal endometriosis.

Six out of 174 cases (3.4%) were true positive, 97 out of 174 cases (55.7%) were true negative, 70 out of 174 cases (40.2%) were false negative and 1 out of 174 (0.5%) were false positive. The sensitivity, specificity, positive predictive and negative predictive values of colonoscopy for the diagnosis of intestinal endometriosis were 7%, 98%, 85% and 58%, respectively (Table 1).

DISCUSSION

Endometriosis is a common gynecological disease defined as the presence of endometrial glands and stroma outside the uterus which induces a chronic inflammatory reaction. The most common locations of endometriosis are the ovaries and the pelvic peritoneum. Peritoneal lesions can be superficial or deep[10].

Deep pelvic endometriosis is defined as the presence of endometrial implants, fibrosis and muscular hyperplasia more than 5 mm below the peritoneum[11]. Rectovaginal endometriosis is deep infiltrating endometriosis that infiltrates the vagina, rectum and the rectovaginal septum and obliterates the posterior cul-de-sac or the pouch of Douglas[12].

It is much less common than ovarian or peritoneal endometriosis and affects between 3.8% and 37% of all patients with endometriosis. Anywhere from 5.3%-12% of patients are estimated to have bowel endometriosis. The rectosigmoid is the most common site of gastrointestinal involvement, affecting 74% of patients[12,13].

Preoperative diagnosis can be challenging. There is a notable absence of agreed upon disease-specific

endoscopic and radiological features. However, several diagnostic methods have been proposed and studied in the literature, including digital rectovaginal examination, transvaginal/transrectal ultrasounds, magnetic resonance imaging (MRI) colonoscopy, computed tomography (CT) colonography and, ultimately, laparoscopic excision with histological confirmation[14-16].

Laparoscopy is the gold standard for the diagnosis of endometriosis and histological confirmation can be beneficial due to high false positive rates of visual diagnosis. Due to the invasiveness of the procedure, other methods are often employed to detect the lesion and to aid with preoperative planning and patient counseling. Transvaginal ultrasound, transrectal ultrasound, CT colonography and MRI are examples of the preoperative methods available to detect deep infiltrating RVE[14].

There is varying data on which offers the highest sensitivity, specificity, PPV and NPV, and accuracy in cases of deep rectovaginal endometriosis. On the other hand, this is the first study, to our best knowledge, evaluating the usefulness of colonoscopy.

Although colonoscopy is often performed in many patients with IE to evaluate presenting complaints, most authors believe that the paucity of mucosal involvement makes colonoscopy more useful in excluding other diagnoses rather than confirming the diagnosis. Bowel endometriosis refers to a condition in which endometrial glands and stroma infiltrate the bowel wall inward from the serosa, reaching at least the subserosal fat tissue. It is particularly common in the subserosa and muscularis propria of the colon. The submucosa may be involved but the infiltration of the lesion into the mucosa is thought to be rare[7].

However, several case reports described the diagnosis of colorectal endometriosis by colonoscopy. Furthermore, Kim *et al*[9] described the colonoscopic finding of colorectal endometriosis, concluding that eccentric wall thickening is the most common colonoscopic finding of colorectal endometriosis and the histological diagnostic yield of endoscopic biopsy is high when lesions are accompanied by surface nodularities.

Regarding the study by Kim *et al*[9], several limitations have to be addressed. It is a retrospective observational study on a small representative study population that includes only intestinal endometriosis, not using laparoscopic and/or histological data as the reference standard. Different from this previous experience, we designed a prospective observational study including all women with deep pelvic endometriosis, confirming the colonoscopic findings by certain laparoscopic and histological diagnosis.

At present, recognized endoscopic findings of colorectal endometriosis include distortion, narrowing or inward bulging of the bowel lumen, polyps or masses, and mucosal changes such as erythema and granularity[9,17,18].

We can confirm that the colonoscopic findings of intestinal endometriosis are wall thickening and polypoid lesions. However, the incidence of the presence of colonoscopic findings of intestinal endometriosis in deep pelvic endometriosis is quite low (4%); therefore, we cannot justify routine colonoscopy in all women with deep pelvic endometriosis. With the sensitivity being very low (7%), we cannot identify intestinal endometriosis by colonoscopy. Furthermore, the negative predictive value is quite low (58%) and we cannot exclude the need for a bowel resection based on a negative colonoscopy examination alone.

Thus, colonoscopy could be considered useless in the identification of bowel involvement in deep pelvic endometriosis. Although colonoscopy should be performed in patients with intestinal symptoms such as rectal bleeding as the differential diagnoses, we can hypothesize that, being an invasive procedure, it should not be routinely performed. However, further studies are needed to validate its effectiveness. Furthermore, further studies could be useful to evaluate the potential role of virtual colonoscopy and compare the accuracy of these procedures, with virtual colonoscopy a non-invasive diagnostic tool[19,20].

COMMENTS

Background

Preoperative assessment of deep pelvic endometriosis is often challenging even for expert physicians, requiring several diagnostic techniques for a clear definition of location and extension of endometrial implants.

Research frontiers

The aim of the present study is to evaluate the role of colonoscopy in the diagnostic work-up of bowel endometriosis.

Innovations and breakthroughs

This is the first study evaluating the usefulness of colonoscopy for the prediction of intestinal involvement in deep pelvic endometriosis.

Applications

Being an invasive procedure, colonoscopy should not be routinely performed in the diagnostic work-up of bowel endometriosis.

Peer-review

This is an interesting paper that adds to the literature. The authors need to clarify how the bowel endometriosis diagnosis was made. The topic of this paper, with regards to the diagnosis of endometriosis, especially when compromising the bowel or the rectum, is a very challenging field. Some minor revisions and language polishing are needed.

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ORIGINAL ARTICLE

Prospective Study

In vivo gastric mucosal histopathology using endocytoscopy

Hiroki Sato, Haruhiro Inoue, Haruo Ikeda, Chiaki Sato, Chainarong Phlanusittepha, Bu'Hussain Hayee, Esperanza Grace R Santi, Yasutoshi Kobayashi, Shin-ei Kudo

Hiroki Sato, Haruhiro Inoue, Haruo Ikeda, Chiaki Sato, Chainarong Phlanusittepha, Shin-ei Kudo, Digestive Disease Center, Showa University, Northern Yokohama Hospital, Yokohama 224-8503, Japan
Bu'Hussain Hayee, Department of Gastroenterology, King's College Hospital NHS Foundation Trust, London SE5 9RS, United Kingdom
Esperanza Grace R Santi, Department of Gastroenterology, De La Salle University Medical Center, Dasmariñas City 4114, The Philippines
Yasutoshi Kobayashi, Kobayashi Internal Medicine Clinic, Kobe 652-0812, Japan
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Clinical trial registration: This study was registered at https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=history&action=list&type=summary&recptno=R000009120&language=J.
Informed consent: All study participants provided informed written consent prior to study enrollment.
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Correspondence to: Hiroki Sato, MD, PhD, Digestive Disease Center, Showa University, Northern Yokohama Hospital, 35-1 Chigasakichuo, Tsuzuki-ku, Yokohama 224-8503, Japan. pyloki@yahoo.co.jp
Telephone: +81-45-9497000
Fax: +81-45-9497000
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Abstract

AIM: To study the ability of endocytoscopy to identify normal gastric mucosa and to exclude *Helicobacter pylori* (*H. pylori*) infection.

METHODS: Endocytoscopic examination of the gastric corpus and antrum was performed in 70 consecutive patients. Target biopsy specimens were also obtained from the assessed region and multiple *H. pylori* tests were performed. The normal endocytoscopy patterns of the corpus and antrum were divided into the normal pit-dominant type (n-Pit) or the normal papilla-dominant type (n-Pap), respectively characterized as either regular pits with capillary networks or round, smooth papillary structures with spiral capillaries. On the other hand, normal mucosa was defined as mucosa not demonstrating histological abnormalities, including inflammation and atrophy.

RESULTS: The sensitivity and specificity of n-Pit for normal mucosa in the gastric corpus were 94.4% and 97.1%, respectively, whereas those of n-Pap for normal mucosa in the antrum were 92.0% and 86.7%, respectively. The positive predictive values of n-Pit and n-Pap for *H. pylori*-negative tissue were 88.6% and 93.1%, respectively, and their negative predictive values for *H. pylori*-negative tissues were 42.9% and 41.5%, respectively. The inter-observer agreement for determining n-Pit and n-Pap for normal mucosa were 0.857 and 0.769, respectively, which is considered reliable.

CONCLUSION: N-Pit and n-Pap, seen using EC, are considered useful predictors of normal mucosa and the

absence of *H. pylori* infection.

Key words: Atrophy; Endocytoscopy; Gastric mucosa; *Helicobacter pylori*; *In vivo* histopathology

Core tip: The identification of minute inflammatory or atrophic changes is very difficult using conventional endoscopy. This is because these changes are usually predicted using non-specific endoscopic findings, such as superficial color, edema and erosions. However, endocytoscopy enables real-time histology, which corresponds well with conventional histopathology. The procedure is also simple (the endocytoscope only has to make contact with the gastric wall) and is therefore expected to be used regularly globally in the future.

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INTRODUCTION

Endocytoscopy (EC) was developed as an ultra-magnifying technology for *in vivo* pathological diagnoses[1,2]. As a result of *in vivo* staining, EC enables detailed examinations, with images comparable to those obtained using microscopy. In our practice, EC is primarily used for the differential diagnosis of neoplastic and non-neoplastic lesions because the atypical cellular and nuclear structures can be clearly identified[3-10].

Additionally, EC can be expected to definitively distinguish between normal mucosa and pathological gastritis [most commonly induced by *Helicobacter pylori* (*H. pylori*) infection]. Conventional endoscopy allows the recognition of gastritis dependent upon non-specific and indirect findings, such as changing patterns of superficial colors, edema and erosions. However, EC is expected to be able to more accurately identify minute changes than any other endoscopic modality. EC should also be able to differentiate clearly between the fundic and antral glands, similar to the capability of narrow band imaging (NBI).

Therefore, the purpose of the present study was to assess the ability of EC to identify normal gastric patterns, corresponding to histopathologically normal mucosa, as well as to exclude cells with possible *H. pylori* infections.

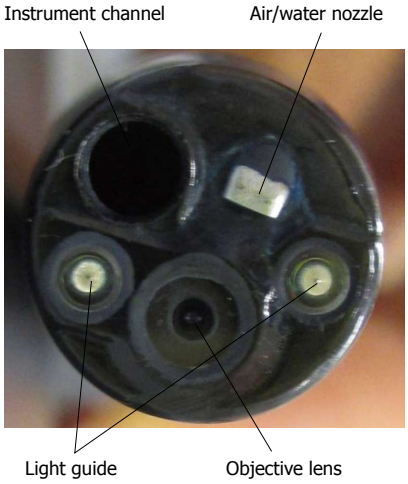

Figure 1 Single charged-couple device integrated type endocytoscope (GIF-Y0002, a prototype from Olympus, Tokyo, Japan).

MATERIALS AND METHODS

Patients

This study was performed at Showa University Northern Yokohama Hospital, a tertiary referral center in Japan, between December 2013 and February 2014. Study participants were prospectively and consecutively recruited from the cohort of patients undergoing endoscopic examination for any indication. Patients who had undergone gastric surgery, were receiving anticoagulant therapy, or had other significant co-morbidities that might affect endoscopic examination were excluded from the study.

The study was approved by our Institutional Review Board and conducted as part of a large study registered in the University Hospital Medical Information Network in Japan (UMIN000007745). Written informed consent was obtained from all patients.

EC materials

All EC examinations were performed using an integrated-type endocytoscope (GIF-Y0002, a prototype from Olympus, Tokyo, Japan). The endocytoscope used has one lens that can increase the image magnification from that of a conventional endoscope to 380 × magnification (tissue field of view, 700 μm × 600 μm). A hand-operated lever is used to allow gradual magnification at the center of the monitor, ensuring that the area of interest is accurately located (Figure 1). The clinical use of the prototype endoscope was also approved by the hospital's ethics committee.

For EC examination, a previously reported mixture of 0.1% methylene blue and 0.05% crystal violet was used to stain *in vivo* tissues in a manner approximating conventional hematoxylin/eosin-stained histopathological specimens. Crystal violet effectively

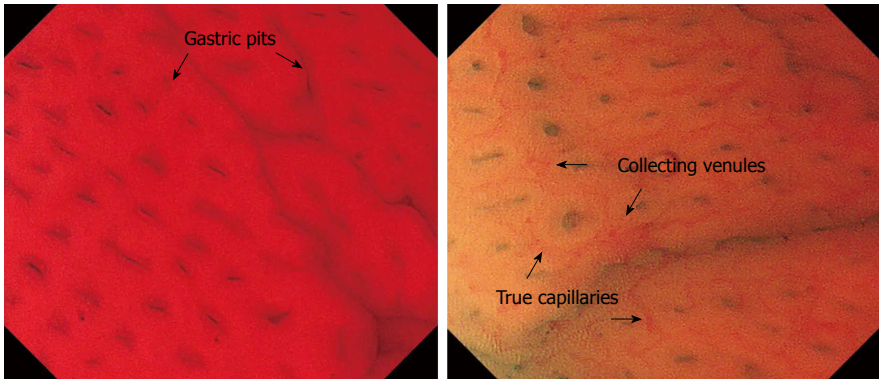

Figure 2 Normal pit-dominant type in the gastric corpus. A regular arrangement of gastric pits is observed, with true capillary network.

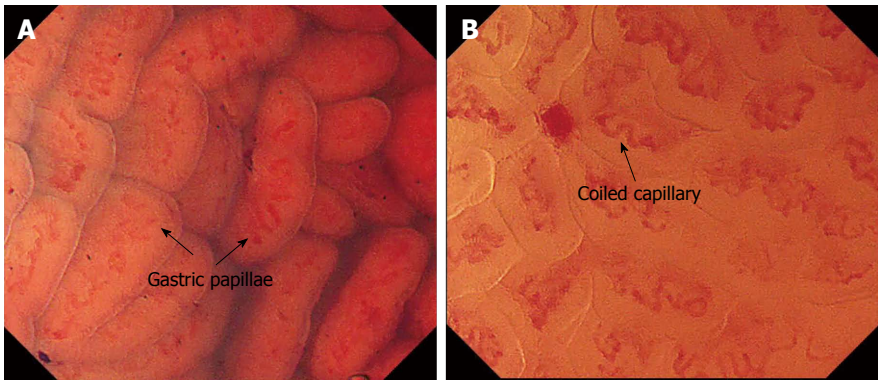

Figure 3 Normal papilla-dominant type in the gastric antrum (A) and the rounded papillae are tightly-packed and regularly arranged, surrounding coiled capillaries (B).

dyes the cytoplasm, while methylene blue staining reveals cell structure details, including the nucleus and cytoplasm[11].

EC criteria for normal gastric mucosa

The normal EC pattern was divided into two types: the normal pit-dominant type (n-Pit) and the normal papilla-dominant type (n-Pap). In the n-Pit type, regular pits are observed as "pin-holes" or short linear structures with capillary networks present around the pit, corresponding to a normal fundus gland (Figure 2). In the n-Pap type, each papillary structure has a round, smooth surface with a spiral capillary contained within the pit; these structures are regularly and closely arranged. The finding is considered to be indicative of a normal antral gland (Figure 3).

There are also some key points common to the both types. Normal gastric epithelium does not consist of intestinal epithelium and does not have an absorptive function; hence, dye uptake is poor. During EC of normal mucosa, the staining solution is observed to pool or accumulate within the gastric pits. Moreover, active mucus production also prevents staining. These characteristics are affected by pathological changes. Atrophic epithelium is superficially stained owing to the opening of the pit and to the decreased mucus production. In the presence of atrophic changes, the gastric pits widen due to shortening of the secretory duct (the duct forming the pit) and shrinkage of the stroma (the stroma forms the papilla), allowing dye uptake. In the presence of intestinal metaplasia, the superficial epithelium is completely stained, allowing goblet cell identification. During active gastritis, necrotic tissue and bacterial infiltration can be appreciated following adequate removal of the surface mucus.

Abnormal EC images were classified as having irregular EC patterns: i-Pit or i-Pap, respectively (Figure 4).

Endoscopic procedure and assessment

All endoscopic procedures were carried out by one endoscopist with 2 years of experience involving more than 100 EC procedures. Conventional upper gastrointestinal tract endoscopy, using an H260Z (Olympus, Tokyo, Japan), was performed prior to EC under intravenous anesthesia. The surface mucus was cleared using dimethicone/water irrigation (Gascon, Kissei Medical, Tokyo, Japan).

Staining was then achieved using 2 mL of the methylene blue and crystal violet mixture, described above. The time interval between staining and EC examination was 5 s. EC of the antrum and corpus (greater curvature side) was performed at maximum magnification (380 ×) and recorded as high-definition video and image files. EC assessment was performed

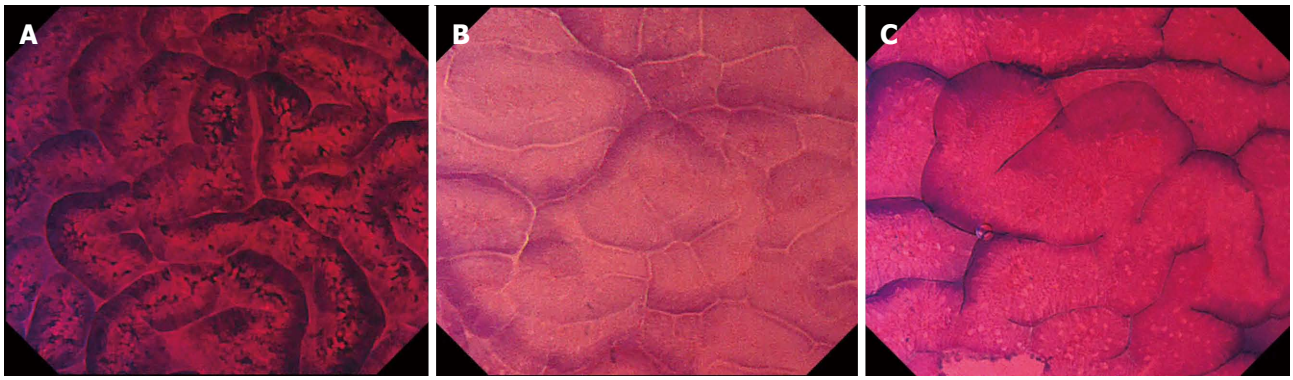

Figure 4 Irregular endocytoscopic patterns. A: Unclear surface epithelium with necrotic tissue; B: Gastric pits (forming sulcus) are visualized when open and more easily stained; C: Completely stained epithelium, with goblet cells.

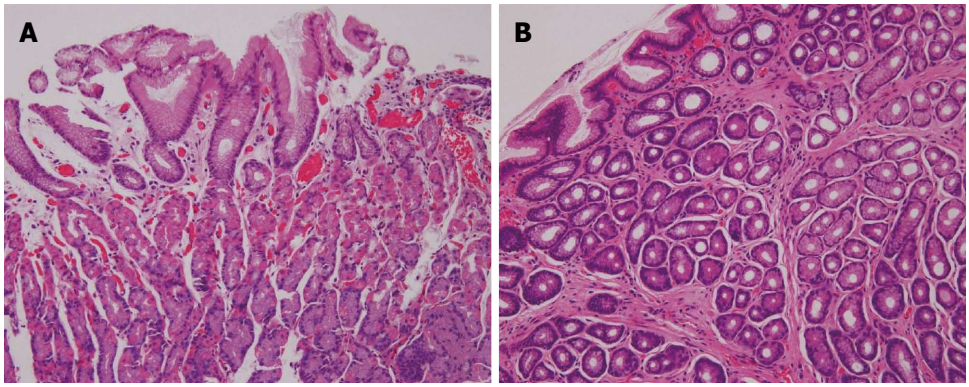

Figure 5 Histopathology of normal fundus gland and normal antral gland. A: Histopathology showing the arrangement of normal fundus gland in the corpus. The arrangement of the surface mucosal epithelium is regular, with little inflammation; B: Histopathology showing the arrangement of normal antral gland. There is no inflammation and the arrangement of the surface mucosal epithelium is regular.

in real time, with the corpus images being classified as demonstrating either n-Pit or i-Pit; images from the antral region were similarly classified as n-Pap or i-Pap.

EC assessment was performed in real-time by one reviewer. To calculate inter-observer agreement in image classification, the EC image files, excluding conventional endoscopy, were divided according to the area of observation (gastric corpus or antral region), with 2 EC images in each case. An independent external reviewer, blinded to the patient information, conventional endoscopy results and histopathological diagnosis, reviewed and reclassified the EC images (n-Pit/i-Pit or n-Pap/i-Pap in the corpus or antral regions, respectively).

After EC examination, two forceps biopsy specimens were obtained from each region, corresponding to the area of EC examination, for pathological assessment and for microscopic *H. pylori* examination. Two random biopsies of the antrum and corpus were also taken for use in the rapid urease test (Pyloritek, Eidia, Tokyo Japan). A total of four biopsies were performed in each case. A serum pepsinogen test[12,13] was concomitantly performed. A positive test result in any *H. pylori* test was interpreted as indicating the presence of an *H. pylori* infection.

To calculate inter-observer agreement, the EC image files not including conventional endoscopy were divided into two parts (gastric corpus and antral region), with two EC images in each. A second, independent external reviewer, blinded to the patient information, results of conventional endoscopy and histopathological diagnosis, reviewed the EC images, classifying them as n-Pit or i-Pit in the gastric corpus or as n-Pap or i-Pap in the antral region.

Histopathological assessment

Fixed biopsy specimens were assessed for the presence or absence of active inflammation and atrophic changes. *H. pylori* infection was evaluated according to the updated Sydney system[14]. Regular arrangement of the surface mucosal epithelium, with absent or few inflammatory cells, were the criteria for normal mucosal assessment of the histopathology specimens (Figure 5).

Statistical analysis

All analyses were performed using STATA, version 11.2 (Stata, College Station, TX, United States). Continuous variables are expressed as mean ± SD and confidence intervals (CI) are given as 95%CI in parentheses.

Table 1 Association between endocytoscopy findings and histological assessment and *Helicobacter pylori* infection - examination in gastric corpus

EC findings (*n*)	Active inflammation		Atrophic change		*Helicobacter pylori* infection	
	(+)	(-)	(+)	(-)	(+)	(-)
n-Pit (35)	0	35	0	35	4	31
i-Pit (35)	19	16	16	19	15	20

The category in non-active inflammation and no atrophy is defined as normal mucosa. Sensitivity and specificity of n-Pit to detect normal mucosa was 94.4% (95%CI: 81.3-99.3), 97.1% (84.7-99.9), respectively. Sensitivity, specificity, positive predictive value and negative predictive value of n-Pit for *Helicobacter pylori*-negative was 60.8% (46.1-74.2), 78.9% (54.4-94.0), 88.6 (73.3-96.8) and 42.9% (26.3-60.6), respectively. EC: Endocytoscopy; n-Pit: Normal pit-dominant type.

Table 2 Association between endocytoscopy findings and histological assessment and *Helicobacter pylori* infection - examination in antral region

EC findings (*n*)	Active inflammation		Atrophic change		*Helicobacter pylori* infection	
	(+)	(-)	(+)	(-)	(+)	(-)
n-Pap (29)	2	27	4	25	2	27
i-Pap (41)	19	22	35	6	17	24

The category in non-active inflammation and no atrophy is defined as normal mucosa. Sensitivity and specificity of n-Pap to detect normal mucosa was 92.0% (95%CI: 74.0-99.0), 86.7% (73.2-95.0), respectively. Sensitivity, specificity, positive predictive value and negative predictive value of n-Pap for *Helicobacter pylori*-negative was 52.9% (38.5-67.1), 89.5% (66.9-98.7), 93.1% (77.2-99.2), 41.5 (26.3-57.9), respectively. EC: Endocytoscopy; n-Pit: Normal pit-dominant type.

Inter-observer agreement was assessed using kappa statistics and interpreted as proposed by Landis and Koch[15]. A kappa value = 0 demonstrated the absence of agreement; < 0.20, slight agreement; 0.21-0.40, fair; 0.41-0.60, moderate; 0.61-0.80, substantial; and > 0.81, almost perfect agreement.

RESULTS

A total of 70 consecutive patients were enrolled in this study (mean age 66.5 ± 12.8 years, 45 males). Six participants had esophageal cancer and 15 had gastric cancer not involving the examined areas of the corpus and antrum; these individuals were referred to our hospital for treatment. The other indications for EC were achalasia (n = 6); screening for other diseases, *e.g.*, liver cirrhosis and preoperative testing due to colon cancer (n = 13), endoscopic surveillance following endoscopic treatment (n = 13), medical examinations (n = 8) and upper endoscopy due to non-specific abdominal symptoms (n = 9). Each EC procedure, including biopsy, was performed within 7 min; adverse events were not observed.

EC correlation with histological assessment and H. pylori infection

The gastric corpus examination results are shown in Table 1. The sensitivity of the n-Pit classifications for determining a histopathologically normal mucosa (fundus gland) was 94.4% (range: 81.3%-99.3%), with a specificity of 97.1% (range: 84.7%-99.9%). The mean inter-observer kappa score between the two reviewers was 0.857 (range: 0.737- 0.978). Although 4 *H. pylori*-positive patients were included among the patients with n-Pit, they exhibited closed-type atrophic gastritis that was confined to the gastric antrum[16]. The mean sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for n-Pit classifications corresponding to normal (*H. pylori*-negative) tissue were 60.8% (range: 46.1%-74.2%), 78.9% (range: 54.4%-94.0%), 88.6% (range: 73.3%-96.8%) and 42.9% (range: 26.3%-60.6%), respectively.

The results of the antral region examination are described in Table 2. The sensitivity and specificity of the n-Pap determinations corresponding to histopathologically normal mucosa were 92.0% (range: 73.4%-99.0%) and 86.7% (range: 73.2%-95.0%), respectively.

The mean kappa score for inter-observer agreement between the two independent reviewers was 0.769 (range: 0.620-0.919). The mean sensitivity, specificity, PPV and NPV for the n-Pap determinations corresponding to normal (*H. pylori*-negative) histopathology were 52.9% (range: 38.5%-67.1%), 89.5% (range: 66.9%-98.7%), 93.1% (range: 77.2%-99.2%) and 41.5% (range: 26.3%-57.9%), respectively.

DISCUSSION

Endoscopic observation of microorganisms, including *H. pylori*, is technically difficult. In an *in vivo* setting, an EC magnification of 1100 × is reportedly needed to detect *H. pylori*[3]. Therefore, using microscopy or EC to look for *H. pylori* is impractical for the diagnosis of an *H. pylori* infection. Moreover, tissue stains, *e.g.*, Giemsa stain, used for the diagnosis of *H. pylori* infections have not yet been proven safe for *in vivo* use and further evaluation is required to address their toxicity and long-term effects prior to their clinical implementation. At present, other diagnostic tests for *H. pylori* infection are widely available commercially[17] (*i.e.*, urease test, serum pepsinogen, stool antigen test). These tests require that patients not be currently taking any proton pump inhibitors prior to testing. To date, the most widely used test, currently considered the gold standard, is the urea breath test. Although considered to be a non-invasive procedure, the breath test requires additional scheduling. Amongst its other attributes, EC has the potential to be a "one-step" diagnostic tool for diagnosing *H. pylori* infection.

The identification of minute changes in the microstructure of the gastric mucosa during endoscopy is a key for determining the status of an *H. pylori* infection[18-20]. Yagi *et al*[21,22] reported that the regular arrangement of collecting venules (RAC) is a practical marker for differentiating normal mucosa from *H. pylori*-associated gastritis. RAC consists of collecting venules and true capillaries (which form networks) that are visible using conventional endoscopy. The authors also reported that the identification of a "well-defined ridge pattern" (wDRP) in the antral mucosa has 100% specificity for normal (*H. pylori*-negative) mucosa but a sensitivity of only 54.5%. Recently, a combination of magnifying endoscopy (ME) and NBI has been shown to allow the visualization of small, round pits with a regular arrangement of capillary networks, indicative of normal mucosa[23-26]. The presence of RAC and wDRP, observed using conventional endoscopy, and a normal pit pattern using ME-NBI are considered to be useful markers for identifying normal mucosa in the absence of an *H. pylori* infection.

EC of the gastric mucosa revealed two different patterns, n-Pit and n-Pap, in the gastric corpus and antral regions, respectively. These differences are considered to arise due to differences in the glandular structures and arrangements between the two regions[27]. In the gastric corpus, the collecting venules and the true capillaries form networks that surround the gastric glands, feeding into the collecting venules which descend vertically. In the antrum, the collecting venules tend to run obliquely and the capillaries are expanded. Furthermore, surface mucous cells form papillary structures and expand deeply into the lamina propria. We can also identify cellular level information about the goblet cells using endocytoscopy. Additionally, due to the higher magnification that is possible, more structural change can be seen than when using ME-NBI, although the microvessels are less clear than in ME-NBI.

Our results demonstrate that the presence of the n-Pit morphology in the gastric corpus, observed using EC, corresponds well with histopathologically proven normal mucosa, with a high sensitivity and specificity. Moreover, the excellent inter-observer agreement shows that the observation of the n-Pit mucosal pattern in the corpus is a clear-cut, practical indicator of normal mucosa, even for non-specialists. The sensitivity and specificity of n-Pap for identifying normal antral region mucosa were 92.0% and 86.7%, respectively. Although the sensitivity of n-Pap was much higher than that of wDRP, as reported by Yagi *et al*, n-Pap was not as reliable for identifying normal mucosa as n-Pit in our series. However, for identifying closed-type gastritis[16,28,29], n-Pap in the antral region appears to predict the presence of histological gastritis.

The specificities and PPVs of n-Pit and n-Pap for identifying *H. pylori*-negative mucosa were considered to be sufficient to be useful for excluding the presence of *H. pylori* infection. The sensitivities and NPVs of n-Pit and n-Pap associated with normal mucosa were not as reliable, probably due to cases of natural *H. pylori* eradication or false negative *H. pylori* test results.

In conclusion, EC determinations of n-Pit and n-Pap are useful predictors of normal mucosa and the absence of *H. pylori* infection. The results also correspond well with the presence of normal fundic and antral glands. This was a pilot study and further study is needed.

COMMENTS

Background

Endocytoscopy (EC) was developed as an ultra-magnifying technology for *in vivo* pathological diagnoses.

Research frontiers

EC enables detailed examinations, with images comparable to those obtained using microscopy.

Applications

EC can differentiate gastric mucosal patterns of minimal change and appears to reliably exclude *Helicobacter pylori* (*H. pylori*) infection.

Terminology

EC of the normal gastric mucosa revealed two different patterns, normal pit-dominant type (n-Pit) and normal papilla-dominant type (n-Pap), in the gastric corpus and antral regions, respectively.

Peer-review

This is a study about endocytoscopic examination of gastric mucosa according to the presence of *H. pylori* infection. The results showed that EC determinations of n-Pit and n-Pap are useful predictors of normal mucosa and the absence of *H. pylori* infection.

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ORIGINAL ARTICLE

Prospective Study

Pathophysiology of functional heartburn based on Rome Ⅲ criteria in Japanese patients

Yasuhiro Tamura, Yasushi Funaki, Shinya Izawa, Akihito Iida, Yoshiharu Yamaguchi, Kazunori Adachi, Naotaka Ogasawara, Makoto Sasaki, Hiroshi Kaneko, Kunio Kasugai

Yasuhiro Tamura, Yasushi Funaki, Shinya Izawa, Akihito Iida, Yoshiharu Yamaguchi, Kazunori Adachi, Naotaka Ogasawara, Makoto Sasaki, Kunio Kasugai, Department of Gastroenterology, Division of Internal Medicine, Aichi Medical University School of Medicine, Aichi 480-1195, Japan
Yasushi Funaki, Department of Clinical Laboratory, Aichi Medical University School of Medicine, Aichi 480-1195, Japan
Hiroshi Kaneko, Department of Internal Medicine, Hoshigaoka Maternity Hospital, Aichi 480-1195, Japan
Author contributions: Tamura Y, Izawa S, Funaki Y and Kasugai K designed the research; Tamura Y, Yamaguchi Y, Adachi K and Iida A performed the experiments; Kaneko H and Sasaki M analyzed the data; and Ogasawara N, Tamura Y and Kasugai K wrote the paper.
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Correspondence to: Yasushi Funaki, MD, Department of Gastroenterology, Division of Internal Medicine, Aichi Medical University School of Medicine, 1-1 Yazakokarimata, Nagakute, Aichi 480-1195, Japan. momomaru@aichi-med-u.ac.jp
Telephone: +81-561-623311
Fax: +81-561-621508
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Abstract

AIM: To investigate the pathophysiology of functional heartburn (FH) in Japanese patients.

METHODS: A total of 111 patients with proton pump inhibitor (PPI)-refractory non-erosive gastroesophageal reflux disease underwent intraesophageal pressure testing and 24-h multichannel intraluminal impedance-pH (24MII-pH) testing. The patients also completed several questionnaires while they were receiving the PPI treatment, including the questionnaire for the diagnosis of reflux disease (QUEST), the frequency scale for the symptoms of gastroesophageal reflux disease (FSSG), the gastrointestinal symptoms rating scale (GSRS), SF-36, and the Cornell Medical Index (CMI). The subjects were classified into FH and endoscopy-negative reflux disease (ENRD) groups based on the Rome Ⅲ criteria.

RESULTS: Thirty-three patients with esophageal motility disorder were excluded from this study, while 22 patients with abnormal esophageal acid exposure time (pH-POS) and 34 with hypersensitive esophagus (HE) were included in the ENRD group. The FH group included 22 patients with no reflux involvement. Sex, age, and body mass index did not differ significantly between the groups. The mean SF-36 values were < 50 (normal) for all scales in these groups, with no significant differences. The GSRS scores in these groups were not different and showed overlap with other gastrointestinal symptoms. The QUEST and the FSSG scores did not differ significantly between the groups.

Neuroticism was diagnosed using the CMI questionnaire in 17 of the 78 included subjects within the pH-POS (n = 4), HE (n = 8), and FH (n = 5) groups, with no significant differences.

CONCLUSION: Clinical characteristics of the FH and PPI-refractory ENRD groups were similar. Therefore, esophageal function should be examined via manometry and 24MII-pH testing to differentiate between them.

Key words: Functional heartburn; Endoscopy-negative reflux disease; Proton pump inhibitor-resistant; Rome Ⅲ criteria; 24-h multichannel intraluminal impedance-pH testing

Core tip: The Rome Ⅲ criteria define functional heartburn (FH) by normal esophageal acid exposure time, with no relationship between symptoms and reflux, and no response to proton pump inhibitor (PPI) treatment. However, in Japanese clinical practice, PPI-refractory non-erosive reflux disease is often treated as FH, thought the pathophysiology of these diseases is not clear. In this study, we found no differences in the clinical characteristics of FH and PPI-refractory endoscopy-negative reflux disease, and recommend using manometry and 24-h multichannel intraluminal impedance-pH testing to differentiate between these two conditions.

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INTRODUCTION

Non-erosive reflux disease (NERD) is characterized by the absence of esophageal mucosal damage during upper gastrointestinal endoscopy, despite the presence of classic symptoms of gastroesophageal reflux, such as heartburn and acid reflux[1]. In addition, NERD accounts for more than half of all cases of gastroesophageal reflux disease (GERD)[2]. Furthermore, acid reflux is known to have only a minor effect on the pathophysiologic mechanism of NERD[3]. For this reason, NERD patients who receive proton pump inhibitors (PPIs), which are the first-line therapy for GERD, show a low symptom-improvement rate, and almost 50% of NERD patients fail to respond to standard acid-suppression therapy that uses PPIs[2,4-6].

Heartburn symptoms that are defined as functional heartburn (FH) have been described as belonging to one of the functional gastrointestinal disorders, which present with chronic gastrointestinal symptoms despite the absence of organic gastrointestinal disease. According to the latest Rome Ⅲ criteria[7], a diagnosis of FH must fulfill all of the following criteria: (1) burning retrosternal discomfort or pain; (2) no evidence that gastroesophageal acid reflux is the cause of the symptoms; (3) no histopathologic evidence of esophageal motility disorders; and (4) these criteria have been fulfilled for the past 3 mo, with symptom onset at least 6 mo before the diagnosis. It has also been shown that a presumptive diagnosis of heartburn can be made in cases of endoscopy-negative reflux disease (ENRD) if 24-h intraesophageal pH monitoring reveals abnormal acid reflux, or acid reflux within the normal range that is associated with symptoms that abate after a course of PPI treatment[8]. In all other cases, the diagnosis should be FH. Regarding the putative mechanism of FH, various mechanisms that are directly related, indirectly related, or unrelated to reflux can be cited. Possible reflux-related mechanisms include acidic factors, such as weak acid reflux, and other factors, such as weak alkaline (bile) reflux. The latter may result from hypersensitivity to physiologic stimuli, abnormal central processing of esophageal signals, hypervigilance, or emotional factors[7].

According to the Rome Ⅲ criteria, FH might be involved in esophageal motility disorders that exhibit no histopathologic abnormalities. However, esophageal motility disorders are present in 4% of patients who have FH and NERD, and their prevalence increases according to the GERD severity[9]. We have reported that patients with PPI-refractory NERD have esophageal motility disorders, and that gastroesophageal reflux plays a role in the symptoms' onset[10]. Therefore, it is important to differentiate esophageal motility disorders from diagnoses of FH during pathophysiologic investigation. We planned a prospective study of patients with PPI-refractory NERD, which is routinely diagnosed as FH, to investigate the pathophysiology of FH and to define rigorous diagnostic criteria for FH in Japanese patients.

MATERIALS AND METHODS

Subjects

This prospective study was conducted at Aichi Medical University Hospital between January 2007 and March 2014. All patients who complained of heartburn at least twice per week for more than 6 mo, and who did not receive antisecretory therapy, underwent upper gastrointestinal endoscopy. If no organic abnormalities (excluding esophageal hiatus hernia) were detected during the endoscopy, the patients were diagnosed as having NERD and were prescribed the standard dose of PPI. PPI-refractory NERD was defined as NERD with no improvements in heartburn symptoms after ⩾ 8 wk of PPI treatment at the standard dose, and

all patients with PPI-refractory NERD were enrolled in this study. All medical interviews and examinations were conducted while the patients were receiving a continuous course of PPI at the standard doses (30 mg of lansoprazole, 20 mg of rabeprazole, or 20 mg of omeprazole per day) for ⩾ 8 wk.

This study was approved by the Ethics Committee of Aichi Medical University School of Medicine, and was performed with the written, informed consent of the patients.

Intraesophageal pressure test

Intraesophageal pressure was measured using a Polygraf ID multiparameter gastrointestinal motility function measurement system (Sierra Scientific Instruments, Los Angeles, CA, United States) and an 8-channel, water-perfused, Dent's sleeve catheter that was inserted nasally into the esophagus. With the patient recumbent, the lower esophageal sphincter (LES) position from the nasal cavity, LES pressure, and the primary peristaltic wave was observed over ten water swallows. Esophageal motility disorders were classified as achalasia, diffuse esophageal spasm, nutcracker esophagus, hypertensive LES, ineffective esophageal motility, and nonspecific esophageal motility disorders, according to the classifications of Castell *et al*[11].

Twenty-four-hour multichannel intraluminal impedance-pH testing

Patients in whom esophageal motility disorders were not detected after the intraesophageal pressure testing subsequently underwent twenty-four-hour multichannel intraluminal impedance-pH (24MII-pH) testing (Sleuth multi impedance pH monitoring system; Sandhill Scientific, Highlands Ranch, CO, United States)[12]. This system includes a portable data logger with impedance-pH amplifiers and a catheter with one antimony pH electrode and eight impedance electrodes at 2, 4, 6, 8, 10, 14, 16, and 18 cm from the tip of the catheter. Each pair of adjacent electrodes represents an impedance-measurement segment (length, 2 cm) that corresponds to one recording channel. The one pH signal and six impedance signals were recorded at a frequency of 50 Hz on a 128-MB CompactFlash memory card (SanDisk, Milpitas, CA, United States). The single-use MII-pH catheter was transnasally inserted into the esophagus and positioned with the pH electrode 5 cm above the upper margin of the LES. The MII-pH data were continuously recorded for 24 h while the patient was in the hospital. All patients ate three meals and were asked to record their posture (*e.g.*, recumbent), the meals they consumed, and the occurrence of heartburn or other symptoms. Analysis of the data, excluding the meal times, was performed using BioVIEW Analysis software (version 5.3.4; Sandhill Scientific). This software is capable of automatically evaluating parameters such as reflux frequency for liquid, gas, and mixtures of the two, as well as liquid pH and the symptom indices (SI) of these parameters, with a high degree of reliability[13]. SI was defined as positive if the proportion of the symptoms due to reflux accounted for ⩾ 50% of the overall symptoms in the 24 h period[14]. After the automatic analysis, an expert visually confirmed the results. In the group with clear intraesophageal prolongation of the esophageal acid reflux exposure time, based on the proportion of the 24-h intraesophageal pH monitoring when the pH was < 4 and the DeMeester scores[15], the reflux of non-acidic material (*i.e.*, liquid or gas with pH ⩾ 4) was evaluated based on the relationship with SI (subgroups with SI ⩾ 50% and with SI < 50%). This method was chosen as the relationship between symptoms and liquid/gas reflux materials is difficult to evaluate using reflux frequencies and time ratios. In this study, reflux materials consisting of a mixture of liquid and gas was treated as liquid reflux.

Medical interview

During the medical examination, reflux symptoms were assessed using the questionnaire for the diagnosis of reflux disease (QUEST)[16] and the frequency scale for the symptoms of GERD (FSSG)[17]. Patients also completed the gastrointestinal symptoms rating scale (GSRS) as an indicator of the other gastrointestinal symptoms that involve acid reflux, the SF-36[18] to evaluate lifestyle and health, and the Cornell Medical Index (CMI) questionnaire to evaluate for the presence of neuroticism.

Statistical analysis

Data are reported as mean ± SD, and the analysis was performed using the Kruskal-Wallis test or the χ^2 test, where appropriate. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Pathologic classification of PPI-refractory NERD

A total of 111 patients with PPI-refractory NERD were recruited and underwent intraesophageal pressure testing. Thirty-three patients were diagnosed with esophageal motility abnormalities, including achalasia (n = 4), ineffective esophageal motility (n = 8), nonspecific esophageal motility disorders (n = 13), hypertensive LES (n = 5), and nutcracker esophagus (n = 3). All patients with esophageal motility disorders were excluded, and the remaining 78 patients [40 men, 38 women; mean age: 55.5 ± 15.4 years; mean body mass index (BMI): 22.3 ± 3.1 kg/m^2] were evaluated using 24MII-pH. Twenty-two patients showed a clear intraesophageal prolongation of esophageal acid exposure time based on the proportion of the 24 h when their intraesophageal pH was < 4 and on the DeMeester scores[15]. In addition, 34 patients were classified into the group with normal esophageal acid exposure time and SI associated

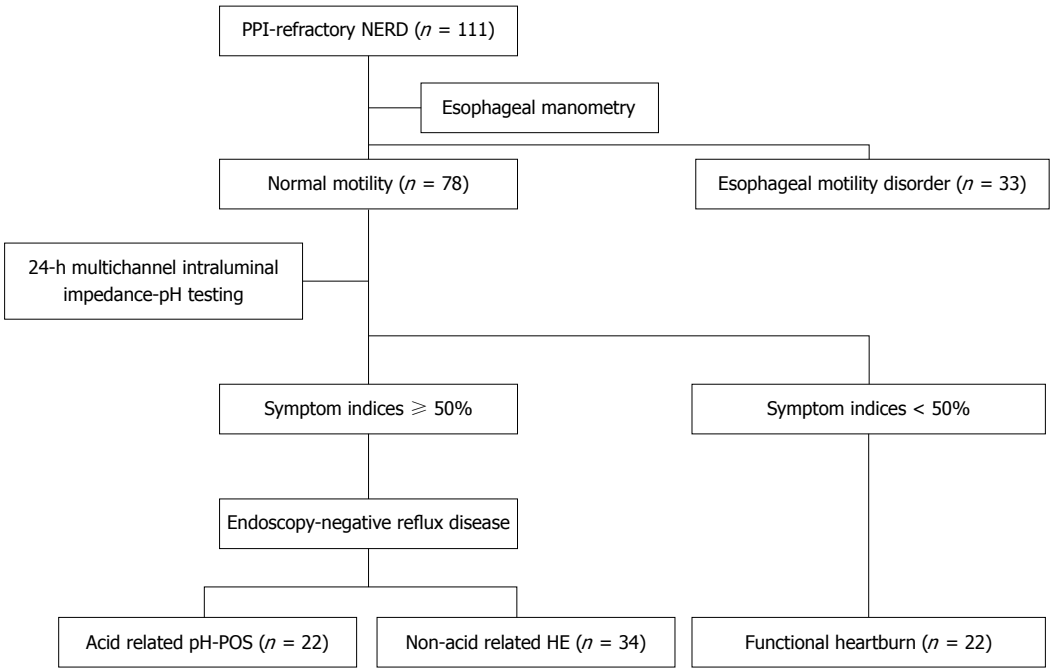

Figure 1 Based on the results obtained from intraesophageal manometry and 24-h-long intraesophageal pH/impedance monitoring, the subjects were classified into three groups according to the Rome Ⅲ criteria: acid reflux-related mechanism (*n* = 22), non-acid reflux-related mechanism (*n* = 34), and functional heartburn (*n* = 22). pH-POS: Excessive intraesophageal acid exposure time; HE: Hypersensitive esophagus (non-excessive esophageal acid exposure time and positive symptom index); FH: Functional heartburn; PPI: Proton pump inhibitor.

Table 1 Clinical characteristics

Characteristic	pH-POS (*n* = 22)	HE (*n* = 34)	FH (*n* = 22)	*P* value
Age, yr	54.8 ± 3.9 (26–80)	54.9 ± 2.6 (21–86)	57.7 ± 2.7 (29–76)	0.3
Sex, male:female	14:08	17:17	9:13	0.3
Body mass index, kg/m^2	21.9 ± 3.5	22.0 ± 3.0	21.3 ± 2.7	0.6
Drinking	8 (36.3)	16 (47.0)	8 (36.3)	0.4
Smoking	7 (31.8)	10 (29.4)	5 (22.7)	0.7
Neuroticism (Ⅲ or Ⅳ)[1]	4 (22.7)	8 (23.5)	5 (22.7)	0.8

Data are expressed as mean ± SD (range), or as *n* (%), unless otherwise indicated; [1]Neuroticism was defined using the Cornel Medical Index. *P*-values were obtained using the Kruskal-Wallis or χ^2 tests. FH: Functional heartburn; HE: Hypersensitive esophagus (non-excessive esophageal acid exposure time and positive symptom index); pH-POS: Excessive intraesophageal acid exposure time.

with ⩾ 50% non-acid reflux, and 22 patients were classified into the group in which reflux contributed to < 50% of the symptoms. Based on this classification, patients with PPI-refractory NERD were classified into 4 subgroups according to whether their symptom onset was associated with: (1) esophageal motility abnormality; (2) acid reflux; (3) non-acid reflux; or (4) no reflux (Figure 1).

Based on the results of the esophageal function testing, the three subgroups, except for those with esophageal motility abnormality, were classed as: the pH-positive group (pH-POS) with acid reflux, the hypersensitive esophagus group (HE) group with no acid, and the FH group with no reflux. The pH-POS group contained 22 patients (14 men, 8 women; mean age: 54.8 ± 3.9 years; BMI: 22.9 ± 3.5 kg/m^2), the HE group contained 34 patients (17 men, 17 women; mean age: 54.9 ± 2.6 years; BMI: 22.0 ± 3.0 kg/m^2), and the FH group contained 22 patients (9 men, 13 women; mean age: 57.7 ± 2.7 years; BMI: 21.3 ± 2.7 kg/m^2). When these groups were compared, no differences in sex, age, or BMI were observed, and there was no causal relationship with alcohol or tobacco use (Table 1). The results of the symptom evaluations did not differ significantly between the FSSG and QUEST questionnaires, and there were no differences in any of the GSRS scores (Table 2). The SF-36 scores fell below the normal population values on all subscales, although no intergroup differences were observed. On the CMI health questionnaire, scores of grades Ⅲ and Ⅳ indicate neuroticism. Four patients in the pH-POS group scored grades Ⅲ or Ⅳ, compared to 8 patients in the HE group and 5 patients in the FH group; these differences were not statistically significant (Table 1).

The distal and proximal numbers of gastro-esophageal reflux episodes (total and acid) were significantly higher in the pH-POS group than those in the HE and FH groups, although there was no difference between the HE and FH groups (Table 3).

DISCUSSION

The Japanese Society of Gastroenterology GERD Diagnosis Guideline[19], which was published in 2009, provides one flowchart for using PPI as a first-line

Table 2 Questionnaire scores

Questionnaire	pH-POS (n = 22)	HE (n = 34)	FH (n = 22)	P value[1]
FSSG score (median)	16.4 ± 7.2	18.4 ± 9.6	19.5 ± 10.3	0.6
QUEST score (median)	5.8 ± 4.7	5.1 ± 4.5	4.2 ± 4.2	0.5
GSRS scale				
Over all	2.5 ± 0.6	2.5 ± 0.8	2.2 ± 1.0	0.5
Acid reflux	3.4 ± 1.6	3.2 ± 1.5	3.0 ± 1.4	0.7
Abdominal pain	2.5 ± 1.1	2.4 ± 1.3	2.3 ± 1.3	0.7
Indigestion	2.9 ± 1.8	2.8 ± 1.1	2.5 ± 1.4	0.5
Diarrhea	1.6 ± 0.7	1.9 ± 1.2	1.7 ± 0.1	0.8
Constipation	2.3 ± 1.4	2.4 ± 1.2	2.0 ± 1.1	0.3
SF-36 scale				
Physical Functioning	46.5 ± 11.1	45.1 ± 16.2	44.7 ± 16.1	0.9
Role Physical	40.5 ± 13.2	41.0 ± 15.4	40.4 ± 15.1	0.9
Bodily Pain	43.6 ± 9.1	44.1 ± 10.1	43.5 ± 11.8	0.9
General Health	38.9 ± 9.8	42.1 ± 6.3	43.1 ± 10.5	0.5
Vitality	46.0 ± 10.6	45.5 ± 9.5	45.4 ± 11.1	0.9
Social Functioning	44.8 ± 10.6	39.2 ± 13.1	44.6 ± 11.2	0.2
Role Emotional	41.9 ± 14.3	42.5 ± 14.3	44.6 ± 14.2	0.8
Mental Health	43.9 ± 10.5	42.2 ± 11.0	41.8 ± 13.8	0.9

[1]Determined by the Kruskal-Wallis test; Data are expressed as mean ± SD unless otherwise indicated. FH: Functional heartburn; FSSG: Frequency scale for the symptoms of gastroesophageal reflux disease; GSRS: Gastrointestinal symptoms rating scale; HE: Hypersensitive esophagus (non-excessive esophageal acid exposure time and positive symptom index); pH-POS: Excessive intraesophageal acid exposure time; QUEST: Questionnaire for the diagnosis of reflux disease.

therapy and another flowchart for using endoscopy as the initial phase of treatment for patients with symptoms indicative of GERD. Therefore, early treatment can be started for all patients, even at facilities where endoscopy is unavailable. In both cases, a pathophysiologic evaluation using 24-h pH monitoring at a specialist facility is recommended only if the symptoms persist after the PPI treatment. In Japan, pH monitoring and esophageal motility function tests are not widely used in clinical practice. As a result, cases in which no obvious organic lesions or mucosal damage are detected during endoscopy are typically classified as NERD, as ENRD if the symptoms respond to PPI treatment, or as FH if PPI treatment does not improve the symptoms. Thus, patients with PPI-refractory NERD are often treated as having FH. Therefore, to identify the characteristics of Japanese patients with FH, it is appropriate to target the broadly defined group of patients with FH who are characterized by a diagnosis of NERD based on endoscopy and lack of symptom response to PPI treatment.

One hundred and eleven patients with PPI-refractory NERD were grouped into those with esophageal motility abnormalities (n = 33), patients with ENRD and whose symptoms were related to some form of reflux (pH-POS and HE, n = 56), and patients with FH based on the Rome Ⅲ criteria (n = 22). Regarding the ENRD pathophysiology, 22 patients exhibited insufficient suppression of acid secretion, despite taking a standard dose of PPI for 8 wk, and 34 patients had weak acid reflux or non-acidic reflux, which is considered a hypothetical diagnosis in the Rome Ⅲ criteria. Previous studies have reported that patients with abnormal gastroesophageal reflux, as confirmed by pH monitoring and including GERD patients, are typically men and often have a high BMI[9,20-22]. Furthermore, a comparison of patients with FH and those with ENRD and some form of reflux-related symptoms (based on 24MII-pH monitoring) reported that ENRD was more common among men and was associated with a high BMI[23], whereas FH was more common in younger patients and among women[24,25]. Other studies have reported that age and lifestyle habits (*i.e.*, alcohol or tobacco use) are not specific to either FH or ENRD[9,23,26]. The present study found no significant differences between the ENRD (pH-POS and HE) and FH groups when sex, age, BMI, and lifestyle habits (alcohol and tobacco use) were compared. The reason for this discrepancy remains unclear, although it may be related to the fact that BMI is lower in Japan than in the Western countries where the previous studies were conducted. In addition, a study investigating symptoms using questionnaires and 24MII-pH monitoring reported stronger reflux symptoms than heartburn symptoms in these groups[20], whereas a study that used pH monitoring to evaluate the occurrence rate of reflux symptoms reported a higher rate in patients with ENRD than in those with FH[25]. However, the present study found no significant intergroup differences in the symptoms or their occurrence rates using the FSSG and QUEST questionnaires. This discrepancy highlights the difficulty encountered when attempting to differentiate between ENRD and FH based on symptoms.

ENRD is associated with a marked deterioration in quality of life[27] and has a high rate of overlap with other functional gastrointestinal disorders[28]. In GERD, mild heartburn at least twice per week has a considerable effect on quality of life, whereas similar criteria for the frequency and severity of symptoms in FH have not yet been identified[29]. In the present study, the mean SF-36 scores in these groups fell below the normal population values on all subscales, although no significant intergroup differences were observed. This indicates that patients who have FH experience a similar deterioration in their quality of life compared to patients who have ENRD (pH-POS and HE). FH often occurs in tandem with symptoms that are normally considered to be indicative of dyspepsia, such as nausea, abdominal bloating, and early satiation[30,31]. In the study of 200 patients with NERD by Savarino *et al*, a questionnaire given to the 54 patients who were diagnosed with FH revealed that the prevalence of postprandial bloating, early satiation, and nausea was significantly higher in patients with FH, whereas in a study of 68 patients with NERD by Capasso *et al*[32], a significantly higher score for indigestion in functional dyspepsia (FD) symptoms was found among 25 patients with no abnormal acid reflux according to pH monitoring. In

Table 3 Twenty-four-hour intraesophageal pH-multichannel intraluminal impedance testing

Variable	pH-POS (n = 22)	HE (n = 34)	FH (n = 22)	P value[1]
% acid exposure time	16.1 ± 15.3[b]	1.4 ± 2.4	0.7 ± 0.8	< 0.01
Number of reflux episodes	77.8 ± 40.6[b]	54.6 ± 30.3	33.9 ± 20.8	< 0.01
Acid reflux (pH < 4)	34.1 ± 23.7[b]	11.2 ± 11.1	7.1 ± 6.3	< 0.01
Non-acid reflux (pH ≥ 4)	41.0 ± 35.1	37.7 ± 32.2	23.8 ± 17.3	0.2
Number of proximal reflux episodes	34.4 ± 26.7[b]	22.2 ± 12.5	14.1 ± 9.0	< 0.01
% of proximal reflux Episodes	42.5 ± 14.3	42.5 ± 13.3	41.4 ± 14.3	0.9
% of gastric acid exposure time	66.1 ± 20.5	50.6 ± 30.9	49.9 ± 28.7	0.1

[1]Determined by the Kruskal-Wallis test; [b]P < 0.01 *vs* HE and FH. FH: Functional heartburn; HE: Hypersensitive esophagus (non-excessive esophageal acid exposure time and positive symptom index); pH-POS: Excessive intraesophageal acid exposure time.

the patients with GSRS, a high degree of overlap in the gastrointestinal symptoms that were unrelated to acid was expected in the FH group, although no significant difference was observed between these groups. This difference might be explained by whether the subjects were or were not receiving PPI treatment. Patients with reflux esophagitis and who were treated with a PPI (pantoprazole) for up to 16 wk showed improvements in their reflux, FD, and irritable bowel syndrome symptoms[33]. However, when those patients were monitored for up to 6 mo after stopping the PPI treatment, their reflux symptoms returned, although their FD and irritable bowel syndrome symptoms did not. Similarly, the subjects in the present study had received PPI treatment at the standard oral dose for at least 8 wk, and it is possible that it suppressed the non-acid reflux dyspepsia symptoms.

In clinical practice, the psychologic profile of patients with reflux symptoms is characterized by higher scores for anxiety and depression than those of patients with no reflux[34]. Similarly, patients with heartburn that is poorly correlated with acid reflux during pH monitoring have higher scores for anxiety and hysteria than those in whom heartburn onset is correlated with acid reflux[35]. Therefore, many cases of FH are interpreted as esophageal neurosis. However, the present analysis of the CMI health questionnaire responses found no significant intergroup differences in the number of patients who were diagnosed with an emotional disturbance, and the scores were similar in the FH and ENRD (pH-POS and HE) groups. This finding suggests that the contribution of emotional factors to the putative factors that affect FH is not as high as was previously thought.

Savarino *et al*[23] reported that the increased number of weakly acidic reflux episodes and the higher rate of proximal reflux are the main causes of symptoms in HE patients who were evaluated with 24MII-pH. However, the data in the present indicate that the total reflux and proximal reflux time of the acid contents are significantly higher in the pH-POS group than in the HE and FH groups, which do not differ. This difference may be related to the presence or absence of PPI treatment. The present analysis was undertaken to evaluate the clinical practical situation of NERD, and to identify its pathophysiology, while avoiding the acid-related effects.

This study has several limitations. First, there may have been a case selection bias, as the subjects were patients with PPI-refractory NERD who were expertly examined at a specialist facility. Furthermore, the sample size was small, as the study was limited to one facility. Second, the subjects were patients who were receiving PPI at the standard dose set within the context of healthcare covered by Japanese national health insurance. Therefore, caution is required when comparing the present data with data from other countries, particularly regarding PPI dose and the duration of administration.

Despite this study's limitations, the pathophysiologic classification of patients with PPI-refractory NERD, who are routinely encountered in clinical practice, was achieved by testing their esophageal function. In addition, the pathology of FH in Japanese patients, based on the Rome Ⅲ criteria, was clarified through a comparison of patients with NERD who were divided into FH and ENRD (pH-POS and HE) groups. Furthermore, this study was conducted while the patients were receiving a continuous course of PPI at the standard doses to reflect the current Japanese clinical practice and to more clearly define the pathophysiology of FH, while avoiding the acid-related effects. A recently published review has mentioned that the use of 24MII-pH is the only functional method to reliably perform sub-classification of the complex population of patients with NERD. In addition, this technique is recommended to clearly separate the subsets of patients with real reflux disease from the subset with FH[36]. Our study supports this idea, and indicates that NERD is a markedly heterogeneous condition from the pathophysiologic and clinical points of view, and that it should be correctly classified by esophageal function testing to provide adequate relief from the related symptom. For example, patients with FH should be treated with non-PPI and non-reflux inhibitor medication, such as with pain modulators[36]. Our data might contribute to developing a therapeutic strategy for patients with PPI-refractory NERD.

In conclusion, Japanese patients with PPI-refractory NERD, who are typically treated as having FH in

clinical practice, exhibit a mix of several pathologies and low rates of rigorously defined FD. The FH and PPI-refractory ENRD groups showed no differences in their clinical characteristics, such as their background, symptoms, and neuroticism, and it was difficult to differentiate between these groups. However, both manometry and MII-pH testing should be used to differentiate between patients with PPI-refractory ENRD and those with FH. Further studies with larger samples are needed to validate the diagnostic criteria for FH based on the Rome Ⅲ criteria.

COMMENTS

Background

The Rome Ⅲ criteria for functional heartburn (FH) suggest various possible factors that are involved in these mechanisms, and position FH as being primarily defined by a normal esophageal acid exposure time, with no relationship between symptoms and reflux, and no response to proton pump inhibitor (PPI) treatment. However, in Japanese clinical practice, PPI-refractory non-erosive reflux disease (NERD) is often treated as FH, and the pathophysiology of these diseases has not been adequately investigated.

Research frontiers

This study was performed to elucidate the pathophysiology of FH in Japanese patients, and found that there were no differences between the clinical characteristics of FH and PPI-refractory endoscopy-negative reflux disease (ENRD) patients. It is difficult to differentiate these conditions, and therefore, both manometry and 24-h *multichannel intraluminal* impedance-pH (24MII-pH) testing should be used to obtain an accurate diagnosis.

Innovations and breakthroughs

Japanese patients with PPI-refractory NERD, who are typically treated as FH in clinical practice, demonstrate the combination of several pathologies and low rates of rigorously defined FH. The FH and PPI-refractory ENRD groups showed no differences in their clinical characteristics, such as their background, symptoms, and neuroticism, and it was difficult to differentiate between the two groups. Those data would contribute to develop a therapeutic strategy for patients with PPI-refractory NERD.

Applications

This is thre first prospective study of patients with PPI-refractory NERD, which is routinely diagnosed as FH, to investigate the pathophysiology of FH and to define rigorous diagnostic Rome Ⅲ criteria for FH in Japanese patients. However, there may have been a case selection bias, and the sample size was small, as the study was limited to one facility. Further studies with larger samples are needed to validate the diagnostic criteria for FH based on the Rome Ⅲ.

Terminology

24MII-pH, which is different from conventional pH monitoring, can discriminate between the physical characteristics of the refluxate (liquid, gas or mixed) and non-acidic gastroesophageal reflux. NERD is characterized by the absence of esophageal mucosal damage seen in upper gastrointestinal endoscopy, despite the presence of typical symptoms of gastroesophageal reflux such as heartburn and acid reflux. According to the latest Rome Ⅲ criteria, FH is defined by burning retrosternal pain or discomfort occurred for the past 3 mo with symptom onset ⩾ 6 mo previously. The absence of gastroesophageal reflux and the absence of histopathologically defined esophageal motility disorders is also required.

Peer-review

This study was carried out to assess the pathophysiologic alterations of patients with FH and to compare them to those of the other subgroups of patients with NERD. The study is prospective and well done and an adequate number of patients were recruited.

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ORIGINAL ARTICLE

Prospective Study

Usefulness of two-point Dixon fat-water separation technique in gadoxetic acid-enhanced liver magnetic resonance imaging

Ying Ding, Sheng-Xiang Rao, Cai-Zhong Chen, Ren-Chen Li, Meng-Su Zeng

Ying Ding, Sheng-Xiang Rao, Cai-Zhong Chen, Ren-Chen Li, Meng-Su Zeng, Department of Radiology, Zhongshan Hospital of Fudan University, Shanghai Institute of Medical Imaging, Shanghai 200032, China

Author contributions: Rao SX and Zeng MS designed the study; Ding Y and Chen CZ performed the research; Li RC and Rao SX analyzed the data; Ding Y wrote the paper; and Ding Y revised the manuscript for final submission.

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Ethics approval: The study was reviewed and approved by the Zhongshan Hospital Fudan University Institutional Review Board.

Clinical trial registration: This study is registered at Zhongshan Hospital Fudan University. The registration identification number is 2010-17.

Informed consent: The requirement for informed consent was waived for our study.

Conflict-of-interest: No potential conflicts of interest relevant to this article are reported.

Data sharing: No additional data are available.

Correspondence to: Sheng-Xiang Rao, MD, PhD, Department of Radiology, Zhongshan Hospital of Fudan University, Shanghai Institution of Medical Imaging, 180 Fenglin Road, Xuhui District, Shanghai 200032, China. raoxray@126.com
Telephone: +86-21-64041990
Fax: +86-21-64041990

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Abstract

AIM: To compare differences between volumetric interpolated breath-hold examination (VIBE) using two-point Dixon fat-water separation (Dixon-VIBE) and chemically selective fat saturation (FS-VIBE) with magnetic resonance imaging examination.

METHODS: Forty-nine patients were included, who were scanned with two VIBE sequences (Dixon-VIBE and FS-VIBE) in hepatobiliary phase after gadoxetic acid administration. Subjective evaluations including sharpness of tumor, sharpness of vessels, strength and homogeneity of fat suppression, and artifacts that were scored using a 4-point scale. The liver-to-lesion contrast was also calculated and compared.

RESULTS: Dixon-VIBE with water reconstruction had significantly higher subjective scores than FS-VIBE in strength and homogeneity of fat suppression (< 0.0001) but lower scores in sharpness of tumor (P < 0.0001), sharpness of vessels (P = 0.0001), and artifacts (P = 0.034). The liver-to-lesion contrast on Dixon-VIBE images was significantly lower than that on FS-VIBE (16.6% ± 9.4% vs 23.9% ± 12.1%, P = 0.0001).

CONCLUSION: Dixon-VIBE provides stronger and more homogenous fat suppression than FS-VIBE, while has lower clarity of focal liver lesions in hepatobiliary phase after gadoxetic acid administration.

Key words: Dixon-volumetric interpolated breath-hold examination; Fat saturation-volumetric interpolated breath-hold examination; Gadoxetic acid; Volumetric interpolated breath-hold examination; Magnetic

resonance imaging

Core tip: The role of three dimensional gradient echo sequence with volumetric interpolated breath-hold examination (VIBE) by using chemically selective fat-saturation for abdominal magnetic resonance (MR) imaging is well established and it is now part of the standard clinical work-up, especially for dynamic contrast-enhanced liver MR imaging. The Dixon technique has been improved extensively in the aspects of phase errors, noise and artifacts. There are no reports yet on the potential value of two-point Dixon fat-water separation technique for image quality and focal liver lesions in hepatobiliary phase of gadoxetic acid-enhanced MR imaging. Therefore, we compare the image quality and liver-to-lesion contrast in hapatobiliary phase between VIBE using two-point Dixon fat-water separation and chemically selective fat saturation.

Ding Y, Rao SX, Chen CZ, Li RC, Zeng MS. Usefulness of two-point Dixon fat-water separation technique in gadoxetic acid-enhanced liver magnetic resonance imaging. *World J Gastroenterol* 2015; 21(16): 5017-5022 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/5017.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.5017

INTRODUCTION

Gadoxetic acid is a hepatocyte-specific contrast agent for magnetic resonance (MR) imaging of the liver[1-4]. The major value of gadoxetic acid compared with other gadolinium-based agents is that it can be taken up by normal functional hepatocytes and excreted into the bile duct. Many studies have showed that gadoxetic acid-enhanced imaging can increase the detection of focal liver lesions, especially for small liver lesions[5-7], as well as improve the confidence for characterization of liver lesions[8-10]. The role of three dimensional (3D) gradient echo sequence with volumetric interpolated breath-hold examination (VIBE) by using chemically selective fat-saturation for abdominal MR imaging is well established and it is now part of the standard clinical work-up, especially for dynamic contrast-enhanced liver MR imaging[11-13]. Chemically selective fat-saturation imaging reduces the potential degradation of image quality resulting from motion-related artifacts, helps increase image contrast resolution and highlights lesions such as contrast-enhancing tissue, edema, and blood products by eliminating the high-intensity signal of fat[11,14]. However, this technique is susceptible to B_0 and B_1 inhomogeneities, which remains a challenge, particularly for imaging off-center, with large field of view, or anatomies with strong susceptibility effects[15,16]. Studies are therefore focusing on the potential added benefit of other methods for fat suppression like Dixon fat-water separation technique[17-21]. The Dixon technique, described for water and fat imaging in 1984[22-24], has been improved extensively in the aspects of phase errors, noise and artifacts. In addition, this technique is routinely used to find intracytoplasmic lipid (also referred to as microscopic fat)[25], as well as to have the potential for detection and grading of liver iron[26], based on comparison of signal intensity on in-phase and opposed-phase images. Ragan *et al*[19] demonstrated that 2-point Dixon fat separation with water reconstruction provided more reliable and homogenous fat suppression than chemical saturation in phantoms and mouse MR imaging. Previous studies also reported the similar results for abdominal and pelvic MR imaging in humans[21-23]. However, Rosenkrantz *et al*[23] showed that the contrast between focal liver lesions and liver parenchyma was slightly lower for Dixon imaging with water reconstruction than that for chemical saturation. To our knowledge, there are no reports yet on the potential value of two-point Dixon fat-water separation technique for image quality and focal liver lesions in hepatobiliary phase of gadoxetic acid-enhanced MR imaging.

The aim of this study was to compare the image quality and liver-to-lesion contrast in hapatobiliary phase between VIBE using two-point Dixon fat-water separation (Dixon-VIBE) and chemically selective fat saturation (FS-VIBE).

MATERIALS AND METHODS

Patients

This study was approved by Institutional Review Board of our hospital. The requirement for informed consent was waived for our study. This study was performed at our department between December 2012 and July 2013. Inclusion criteria for patients consisted of (1) undergoing liver MR imaging with injection of gadoxetic acid; (2) detectable focal liver lesions; (3) breath-holding capacity for at least 18 s; and (4) normal liver function. Clinical data were obtained from medical records.

MR imaging

Liver examinations were performed on a 1.5-T MR system with a phased-array coil (Magneto Aera, Siemens Medical Solution, Erlangen, Germany). In routine liver MR protocol at our department, early dynamic contrast-enhanced MR imaging and hepatobiliary phase at 20 min were performed using FS-VIBE besides conventional MRI sequences. Dixon-VIBE was also routinely performed before gadoxetic acid administration. Besides conventional MR imaging sequences, Dixon-VIBE was performed for the hepatobiliary phase before FS-VIBE (no more than 2 min). The sequence parameters are displayed in Table 1. A parallel imaging technique

Table 1 Sequence parameters

	Dixon-VIBE	FS-VIBE
Repetition time (ms)	8.88	3.47
Echo time (ms)	2.39/4.77	1.36
Section thickness (mm)	3.5	3.5
Field of view (mm)	380-400 × 300-324	380-400 × 300-324
Flip angle (degree)	10	10
Matrix	320 × 240	320 × 195
Bandwidth (Hz/pixel)	430/490	400
Acquisition time (s)	18	14

FS: Chemically selective fat saturation; VIBE: Volumetric interpolated breath-hold examination.

Table 2 mean ± SD for subjective image scores for Dixon fat-water separation and chemically selective fat saturation

Parameter	FS-VIBE	Dixon-VIBE	*P* value
Sharpness of tumor	3.22 ± 0.47	2.78 ± 0.47	< 0.0001
Sharpness of vessels	3.20 ± 0.46	2.73 ± 0.49	0.0001
Strength and homogeneity of fat suppression	2.98 ± 0.25	3.6 ± 0.53	< 0.0001
Artifacts	2.98 ± 0.25	2.86 ± 0.35	0.0340

FS: Chemically selective fat saturation; VIBE: Volumetric interpolated breath-hold examination.

(R factor of 2) was performed with generalized autocalibrating partially parallel acquisition for the 2 sequences. Four image sets were obtained by two-point Dixon-VIBE: an in-phase image, an out-of-phase image, a fat-reconstruction image, and a water-reconstruction image, with the water-reconstruction image being analogous to the standard FS-VIBE sequence. In all patients, 0.025 mmol/kg body weight of gadoxetic acid (Primovist; BayerSchering Pharma AG, Berlin, Germany) was injected manually at about 1 mL/s, as recommended, by one investigator through a 20-gange intravenous catheter placed in a cubical or cephalic vein. Immediately afterwards, a 20 mL saline flush was administered at the same injection rate.

Image evaluation

MR imaging data were evaluated in the hospital's Picture Archiving and Communication System. All MR images were assessed by a single reader (Ren-chen Li) with 5 years of experience in reading abdominal MR images. The reader was blinded to clinical data of the patients. For each patient, qualitative and quantitative analyses were performed on the largest available lesion.

Qualitative analysis

The reader reviewed Dixon-VIBE and FS-VIBE sequences on 2 separate dates with 4-8 wk separating interpretation of the 2 sequences. For each data set, the reader scored the following parameters: sharpness of tumor, sharpness of vessels, strength and homogeneity of fat suppression, and artifacts, which were scored using the following 4-point scale: 4, excellent quality; 3, good quality, not impairing diagnostic performance; 2, fair quality, somewhat impairing diagnostic performance; 1, poor quality, impairing diagnostic performance.

Quantitative analysis

The signal intensities (SIs) of focal liver lesions were measured on Dixon-VIBE with water reconstruction and FS-VIBE images in hepatobiliary phase. The reader manually traced the lesion boundary by placing free hand region of interest at the largest axial slice. The SI of liver parenchyma surrounding the tumor was also measured avoiding large vessels and artifacts. The liver-to-lesion contrast (%) was calculated according to the Michelson contrast formula[18]: $100 \times (SI_{liver} - SI_{lesion})/(SI_{liver} + SI_{lesion})$, where SI_{liver} is SI of liver parenchyma and SI_{lesion} is SI of focal liver lesions.

Statistical analysis

The statistical methods of this study were reviewed by Ren-chen Li from Zhongshan Hospital Fudan University. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS version 18.0, SPSS, Chicago, IL, United States). The liver-to-lesion contrast was pared using a paired *t*-test when data were normally distributed or a Wilcoxon signed ranked test when data were not normally distributed. A Wilcoxon signed ranked test was also used to compare quality factors. Differences with a *P*-value less than 0.05 were considered statistically significant.

RESULTS

Patient characteristics

A total of 49 consecutive patients meeting the inclusion criteria were included in our study. Of the 49 patients, 41 were male and 8 were female (media age, 51 years; range, 26-84). Forty-one target lesions were confirmed by surgical pathologic assessment (33 hepatocellular carcinomas, 2 cholangiocaicinomas, 4 metastases from colorectal carcinoma, and 2 hemangiomas) and the remaining 8 lesions were hemangiomas which were confirmed by imaging findings combined with clinical data. Cirrhosis was diagnosed in 20 patients by pathologic assessment. The median size of the lesions was 15 mm (range, 5-70 mm); 12 (24.5%) were ⩽ 10 mm, 24 (49.0%) were between 10 and 20 mm and 13 (26.5%) were > 20 mm.

Dixon-VIBE vs FS-VIBE

All the target lesions were detected as hypointense lesions on both Dixon-VIBE and FS-VIBE. The mean values for subjective scores (sharpness of tumor, sharpness of vessels, homogeneity of fat suppression, and artifacts) are shown in Table 2, Figures 1 and 2, and the results of liver-to-lesion contrast are displayed

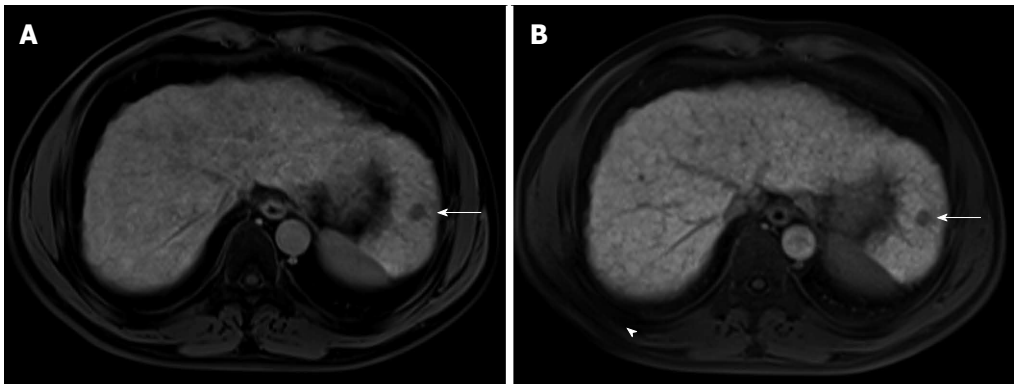

Figure 1 A 54-year-old patient with hepatocellular carcinoma. The fat suppression is stronger and more uniform with Dixon-volumetric interpolated breath-hold examination (VIBE) (A) than chemically selective fat saturation-VIBE (FS-VIBE) (B), the slight signal loss is seen with FS-VIBE (arrowhead, B), and the score was 4 for Dixon-VIBE and 3 for FS-VIBE. However, the hepatic veins are clearer with FS-VIBE. The tumor is well depicted with each sequence (arrow).

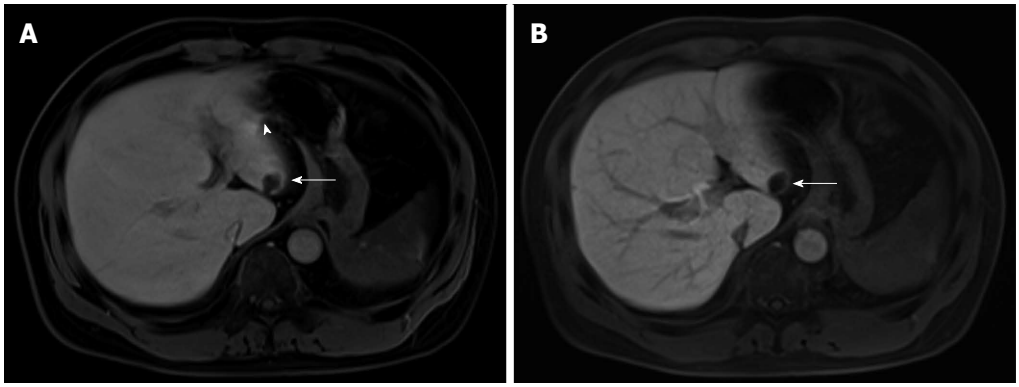

Figure 2 A 68-year-old patient. The border of tumor (arrow) and intrahepatic portal vein are sharper with chemically selective fat saturation-volumetric interpolated breath-hold examination (FS-VIBE) (B) than Dixon-VIBE (A), and the score was 3 for Dixon-VIBE and 4 for FS-VIBE. Artifact from the air in the stomach is seen with Dixon-VIBE (arrowhead, A). The suppression of subcutaneous fat is stronger with Dixon-VIBE. VIBE: Volumetric interpolated breath-hold examination; FS: Fat saturation

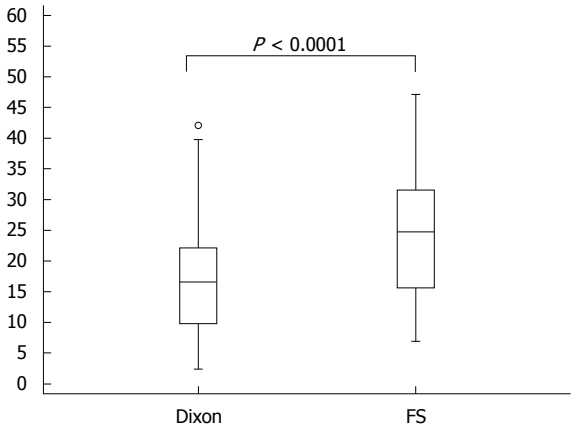

Figure 3 Box-and-whisker plots show median and interquartile ranges for liver-to-lesion contrast in hapatobiliary phase for chemically selective fat saturation-volumetric interpolated breath-hold examination and 2-point Dixon fat-water separation-volumetric interpolated breath-hold examination.

in Figure 3. Dixon-VIBE with water reconstruction had significantly higher subjective scores than FS-VIBE in strength and homogeneity of fat suppression (< 0.0001) but lower scores in sharpness of tumor (P < 0.0001), sharpness of vessels (P = 0.0001), and artifacts (P = 0.034). The liver-to-lesion contrast on Dixon-VIBE images was significantly lower than that on FS-VIBE (16.6% ± 9.4% *vs* 23.9% ± 12.1%, P = 0.0001).

DISCUSSION

The aim of this study was to compare Dixon-VIBE with conventional FS-VIBE sequence in gadoxetic acid-enhanced liver MR imaging. Our results indicated that Dixon-VIBE with water reconstruction demonstrated significantly stronger and more homogenous fat suppression but had lower clarity of hepatic vessels and lesions and increased artifacts, when compared with conventional FS-VIBE in hepatobiliary phase.

Inhomogeneous and incomplete fat suppression especially for the area near the edges of surface coils could degrade the image quality of conventional FS-VIBE for abdominal MR imaging, while Dixon methods are insensitive to B_1 inhomogeneities and provide robust water-fat separation[16-18]. Our study showed that Dixon-VIBE with water reconstruction yielded fat suppression with superior quality than conventional FS-VIBE, which was in keeping with the results of previous studies[21-23]. We further found the improvement for

homogeneous fat suppression adjacent to the lung and chest/abdominal wall by Dixon-VIBE with water reconstruction. This might have potential value for visualization of the uptake of gadoxetic acid by hepatocytes in the hepatobiliary phase. Unfortunately, our study failed to demonstrate that Dixon-VIBE with water reconstruction had the superiority for visualization of focal liver lesions with significantly lower subjective scores and liver-to-lesion contrast. The possible reason might be that Dixon fat separation techniques avoid the potentially severe suppression artifacts due to B_0 inhomogeneities and frequency offsets among separate volumes of interest, including inadvertent suppression of the water signal, which can reduce image contrast[19,20]. This results also might be in part affected by earlier acquisition for Dixon-VIBE compared with FS-VIBE in our study. In theory, the peak liver parenchymal enhancement was observed to occur at 20 min after administration of gadoxetic acid. However, in our study there was only no more than 2 min between the two scans. A previous study also reported the lower, without reaching significant difference, liver-to-lesion contrast for Dixon-VIBE than FS-VIBE at about 3-min delayed gadolinium-enhanced liver MR imaging[27]. Our poor results for the increased artifacts on Dixon-VIBE may be due to the B_0 inhomogeneities and relative longer acquisition time. Some researchers[28-30] modified the two-point Dixon method by acquiring a third image (three-point Dixon) that was used to compensate for B0 inhomogeneities, which have not been assessed for high resolution 3D VIBE breath-hold post-contrast liver MR imaging.

There are some limitations to our study. First, we did not change the routine default parameters for the 2 sequences, which were different in some parameters. Second, as above-mentioned we performed Dixon-VIBE a little earlier ($\leqslant$ 2 min) than FS-VIBE, which might affect the calculation of the liver-to-lesion contrast. Finally, we did not evaluate the detection of the lesions. However, all the tumors (ranging from 5 mm to 70 mm) could be found in both sequences, which are consistent with the results of no difference for detection between the 2 sequences in the previous study[23].

In conclusion, Dixon-VIBE provides stronger and more homogenous fat suppression than FS-VIBE, while has lower clarity of focal liver lesions in hepatobiliary phase after gadoxetic acid administration. Dixon-VIBE has potential value when incomplete fat suppression is achieved by the FS-VIBE sequence.

COMMENTS

Background

The role of 3D gradient echo sequence with volumetric interpolated breath-hold examination (VIBE) by using chemically selective fat-saturation for abdominal magnetic resonance (MR) imaging is well established and it is now part of the standard clinical work-up, especially for dynamic contrast-enhanced liver MR imaging. Chemically selective fat-saturation imaging reduces the potential degradation of image quality resulting from motion-related artifacts, helps increase image contrast resolution and highlights lesions such as contrast-enhancing tissue, edema, and blood products by eliminating the high-intensity signal of fat. However, this technique is susceptible to B0 and B1 inhomogeneities, which remains a challenge, particularly for imaging off-center, with large field of view, or anatomies with strong susceptibility effects. Studies are therefore focusing on the potential added benefit of other methods for fat suppression like Dixon fat-water separation technique.

Research frontiers

The Dixon technique has been improved extensively in the aspects of phase errors, noise and artifacts. In addition, this technique is routinely used to find intracytoplasmic lipid (also referred to as microscopic fat), as well as to have the potential for detection and grading of liver iron, based on comparison of signal intensity on in-phase and opposed-phase images.

Innovations and breakthroughs

Ragan *et al* demonstrated that 2-point Dixon fat separation with water reconstruction provided more reliable and homogenous fat suppression than chemical saturation in phantoms and mouse MR imaging. Previous studies also reported the similar results for abdominal and pelvic MR imaging in humans. However, Rosenkrantz *et al* showed that the contrast between focal liver lesions and liver parenchyma was slightly lower for Dixon imaging with water reconstruction than that for chemical saturation. To our knowledge, there are no reports yet on the potential value of two-point Dixon fat-water separation technique for image quality and focal liver lesions in hepatobiliary phase of gadoxetic acid-enhanced MR imaging. Therefore, the authors compared the image quality and liver-to-lesion contrast in hepatobiliary phase between VIBE using two-point Dixon fat-water separation (Dixon-VIBE) and chemically selective fat saturation (FS-VIBE).

Applications

Dixon-VIBE provides stronger and more homogenous fat suppression than FS-VIBE, while has lower clarity of focal liver lesions in hepatobiliary phase after gadoxetic acid administration. Dixon-VIBE has potential value when incomplete fat suppression is achieved by the FS-VIBE sequence.

Terminology

Dixon-VIBE provides stronger and more homogenous fat suppression than FS-VIBE, while has lower clarity of focal liver lesions in hepatobiliary phase after gadoxetic acid administration.

Peer-review

The paper describes the comparison between VIBE using two-point Dixon-VIBE and chemically selective FS-VIBE in gadoxetic acid-enhanced liver MR imaging. Authors demonstrates that Dixon-VIBE provides stronger and more homogenous fat suppression than FS-VIBE, while has lower clarity of focal liver lesions in hepatobiliary phase after gadoxetic acid administration. The paper is scientifically accurate and complete. Many parameters (sharpness of tumor, sharpness of vessels, strength and homogeneity of fat suppression, artifacts and liver-to-lesion contrast) have been investigated to explore the differences between Dixon-VIBE and conventional FS-VIBE sequence.

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ORIGINAL ARTICLE

Randomized Controlled Trial

Irsogladine maleate and rabeprazole in non-erosive reflux disease: A double-blind, placebo-controlled study

Takayoshi Suzuki, Masashi Matsushima, Aya Masui, Shingo Tsuda, Jin Imai, Jun Nakamura, Yoko Tsukune, Tetsufumi Uchida, Hiroki Yuhara, Muneki Igarashi, Jun Koike, Tetsuya Mine

Takayoshi Suzuki, Masashi Matsushima, Aya Masui, Shingo Tsuda, Jin Imai, Jun Nakamura, Yoko Tsukune, Tetsufumi Uchida, Hiroki Yuhara, Muneki Igarashi, Jun Koike, Tetsuya Mine, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan

Author contributions: Suzuki T and Matsushima M designed the research; Suzuki T and Masui A analyzed the data; Suzuki T, Matsushima M, Tsuda S, Imai J, Nakamura J, Tsukune Y, Uchida T, Yuhara H, Igarashi M, Koike J and Mine T performed the research; and Suzuki T wrote the paper.

Ethics approval: The study was reviewed and approved by the Tokai University's Ethics Committee. This study was performed in accordance with the principles laid down in the Declaration of Helsinki.

Clinical trial registration: This study was registered in the UMIN Clinical Trials Registry. The registration identification number is UMIN000015731.

Informed consent: All study participants provided informed written consent prior to study enrollment.

Conflict-of-interest: The authors have no conflicts of interest to declare.

Data sharing: All of the participants gave their informed consent for data sharing.

Correspondence to: Dr. Takayoshi Suzuki, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan. takayosh@is.icc.u-tokai.ac.jp
Telephone: +81-463-931121
Fax: +81-463-937134

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Abstract

AIM: To evaluate the efficacy of adding irsogladine maleate (IM) to proton-pump inhibitor (PPI) therapy in non-erosive reflux disease (NERD) treatment.

METHODS: One hundred patients with NERD were recruited and randomized to receive rabeprazole plus IM (group I) or rabeprazole plus placebo (group P). The efficacy of the treatment was assessed using the Frequency Scale for the Symptoms of Gastroesophageal Reflux Disease (FSSG) and the short form (SF)-36 quality of life questionnaires after four weeks of treatment. We also assessed whether patients with NERD with minimal changes (grade M) had different responses to the therapies compared with patients who did not have minimal changes (grade N).

RESULTS: Group I and group P showed significant improvements in their FSSG scores after the treatment (from 17.9 ± 7.9 to 9.0 ± 7.6, and from 17.7 ± 7.3 to 11.2 ± 7.9, respectively, P = 0.0001), but there was no statistically significant difference between the FSSG scores in group I and those in group P. Subgroup analysis showed that significant improvements in the FSSG scores occurred in the patients in group I who had NERD grade N (modified Los Angeles classification) (7.8 ± 7.4 *vs* 12.5 ± 9.8, P = 0.041). The SF-36 scores for patients with NERD grade N who had received IM and rabeprazole were significantly improved in relation to their vitality and mental health scores.

CONCLUSION: The addition of IM to rabeprazole significantly improves gastroesophageal reflux disease

symptoms and the quality of the lives of patients with NERD grade N.

Key words: Irsogladine maleate; Rabeprazole; Non-erosive reflux disease; Randomized controlled trial; Frequency scale for the symptoms of gastroesophageal reflux disease; Quality of life

Core tip: Proton pump inhibitor (PPI) therapy is considered the mainstay for the treatment of non-erosive reflux disease (NERD). However, it is less effective in NERD than it is in reflux esophagitis. This study evaluated the efficacy of adding irsogladine maleate (IM) to PPI therapy in the treatment of NERD. Adding IM to rabeprazole improved both the gastroesophageal reflux disease symptoms and the quality of the lives of patients with NERD showing no endoscopic abnormalities, a categorization that was based on the modified Los Angeles classification.

Suzuki T, Matsushima M, Masui A, Tsuda S, Imai J, Nakamura J, Tsukune Y, Uchida T, Yuhara H, Igarashi M, Koike J, Mine T. Irsogladine maleate and rabeprazole in non-erosive reflux disease: A double-blind, placebo-controlled study. *World J Gastroenterol* 2015; 21(16): 5023-5031 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/5023.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.5023

INTRODUCTION

Gastroesophageal reflux disease (GERD) is a chronic disorder characterized by unpleasant reflux symptoms that include heartburn, sore throat, belching, nausea, voice changes, chest pain, cough, and acid regurgitation[1]. GERD includes both reflux esophagitis (RE) and non-erosive reflux disease (NERD). According to the Montreal consensus conference[2], RE is characterized by mucosal damage within the esophagus that can be visualized during endoscopy, whereas mucosal breaks are not seen in NERD. Endoscopic examinations indicate that about two-thirds of patients with GERD symptoms have no erosive changes[3]. The detailed endoscopic findings from patients with NERD can be divided into two groups that can be defined using a modification of the Los Angeles classification, with one group showing no endoscopic abnormalities (grade N), and the other group showing minimal endoscopic changes, for example, whitish or reddish edematous changes (grade M)[4]. However, little is known about clinical utility of endoscopic classification in NERD.

Most GERD patients are treated with proton pump inhibitors (PPI) to control their symptoms. Reports have suggested that compared with patients with RE, those with NERD had lower response rates to acid suppression therapy, and about half of the patients with NERD did not show adequate improvements in their reflux symptoms when a standard dose of PPI was administered[3,5]. Nevertheless, the initial management of NERD is the same as that of RE, and it comprises PPI mono-therapy.

However, recent studies revealed that these endoscopy-negative patients with reflux symptoms include different pathophysiological mechanisms. On the basis of esophageal 24-h multichannel intraluminal impedance combined with pH measurements, NERD patients can be classified into three groups, as follows: (1) patients with abnormal distal esophageal acid exposure; (2) patients with normal distal esophageal acid exposure and positive symptom associations for either acid and/or non-acid reflux (hypersensitive esophagus); and (3) patients with normal distal esophageal acid exposure and negative symptom associations (functional heartburn)[6,7]. Furthermore, a meta-analysis emphasized that well-defined NERD patients, including those in the aforementioned groups (1) and (2), have responses to PPI therapy that are similar to those seen in patients with RE, and that patients with functional heartburn do not respond to PPI therapy[8]. However, NERD that is diagnosed on the basis of endoscopic findings alone cannot exclude some patients who have functional heartburn.

Although the pathophysiology of NERD remains unclear, electron microscopy and light microscopy have revealed esophageal mucosal dilated intercellular spaces (DIS) in patients with NERD. Exposure to excessive amounts of acid could cause the development of DIS, which correlates well with the symptom of heartburn, and DIS could be useful markers for the breaks in the epithelial barrier that reflect an increase in paracellular permeability[9,10].

Irsogladine maleate (IM) activates gap junctional intercellular communication, and it has been widely used as an anti-ulcer treatment in Japan, Korea, and China; however, little is known about its effects on patients with NERD. This study aimed to evaluate the efficacy of adding IM to PPI therapy in the treatment of patients with NERD in a prospective, randomized, double-blind, placebo-controlled trial.

MATERIALS AND METHODS

Patients who presented to Tokai University Hospital from August 2008 to July 2012 with persistent GERD symptoms were enrolled to participate in the study if they did not have mucosal breaks in their esophagi, and if they had Frequency Scale for the Symptoms of GERD (FSSG) scores of $\geqslant$ 8. The FSSG is used to diagnose GERD and to determine the efficacy of GERD treatment. The FSSG questionnaire consists of seven questions that relate to reflux (questions 1, 4, 6, 7, 9, 10, 12) and five questions that relate to acid-related dysmotility symptoms (questions 2, 3, 5, 8, 11) that were generated by Kusano *et al*[11], and it was used in several clinical studies of GERD[11-13].

Table 1 Clinical features of patients receiving rabeprazole plus irsogladine maleate (group I) or rabeprazole plus placebo (group P)

	Group I (n = 49)	Group P (n = 48)	P value
Age (yr), mean ± SD	51.1 ± 16.4	51.1 ± 14.8	0.77
Sex, male/female	13/36	19/29	0.17
BMI (kg/m^2)	22.4 ± 4.3	21.8 ± 3.1	0.59
Height (cm)	159 ± 7.3	161.5 ± 8.7	0.24
Weight (kg), mean ± SD	57.1 ± 12.2	56.7 ± 8.6	0.63
NERD grade (N/M)	21/28	20/28	0.91
Hiatal hernia (+/-)	11/38	10/38	0.85
Drinking (+/-)	16/33	20/28	0.36
Smoking (+/-)	2/47	9/39	0.05

NERD: Non-erosive reflux disease; BMI: Body mass index.

Patients were excluded from the study if they were younger than 20 years, were allergic to rabeprazole or IM, had any level of esophagitis determined by gastrointestinal endoscopy, were pregnant, were taking atazanavir sulfate, or anti-coagulant, anti-platelet, or pro-kinetic agents, had a history of gastrointestinal malignancy, peptic ulcers, or gastrointestinal tract surgery, or if they had used any PPI therapy within four weeks of enrolling in the study.

After providing written informed consent, the patients were asked to complete a short form (SF)-36 quality of life (QOL) questionnaire. They were randomized by an independent investigator who used a random number generated by a computer to assign the patients to one of two groups, as follows: those who were to receive rabeprazole and IM (group I) and those who were to receive rabeprazole and placebo (group P). At the end of the four-week study period, the patients were asked to complete post-treatment FSSG and SF-36 QOL questionnaires. The FSSG results were considered in terms of the total score and each symptom score. Patients were asked whether they had experienced any adverse events at the end of the study.

The primary endpoint of this study was an improvement in the FSSG score, and determining whether patients with NERD with minimal changes (grade M) had different response to the therapies compared with those without minimal changes (grade N). The study's secondary endpoints included the SF-36 scores and the occurrence of side effects. The university's ethics committee approved the study protocol. This study was performed in accordance with the principles laid down in the Declaration of Helsinki. The study was registered in the UMIN Clinical Trials Registry (Registry ID Number: UMIN000015731).

The sample size was estimated based on the statistical analysis of the improvements in the FSSG scores in each group. Previous data have shown that about half of the patients with NERD have adequate improvements in their reflux symptoms when they are treated with a standard PPI dose. In this study, the predicted improvements in the FSSG scores were estimated to be 75% in group I and 50% in group P. As a result, 45 patients were required in each group to detect a significant difference between the groups with an α value of 0.05 and a power (1-β) of 0.8.

Statistical analysis

We compared the two groups in relation to the proportions of the sexes, smoking habits, the presence of hiatal hernias, alcohol consumption, and the endoscopic grades using the χ^2 test. Continuous variables are presented as the means ± SD. Differences in the continuous variables, including age, the FSSG scores, and the body mass indices, were analyzed using the Mann-Whitney *U*-test. A P value of < 0.05 was considered statistically significant. The SF-36 QOL questionnaire scores were evaluated using the Wilcoxon signed-rank test.

RESULTS

One hundred patients with NERD were randomized to one of the two groups, but three patients were excluded from the study because some of their data were missing. Consequently, 97 patients were included in the analysis. Group I comprised 49 individuals, which included 13 men, and their mean ± SD age was 51.1 ± 16.4 years. Group P comprised 48 individuals, which included 19 men, and their mean ± SD age was 51.1 ± 14.8 years. The baseline characteristics of the patients were comparable (Table 1).

The mean ± SD pre-treatment FSSG scores for group I and group P were 17.9 ± 7.9 and 17.7 ± 7.3 years, respectively. The mean ± SD post-treatment FSSG scores for group I and group P were 9.0 ± 7.6 and 11.2 ± 7.9 years, respectively. Although significant improvements in the FSSG scores were observed within both groups after treatment (P = 0.0001), there was no statistically significant difference between the groups with respect to the FSSG scores after four weeks of treatment (P = 0.15) (Figure 1). Of the seven subscale scores for reflux, the scores for question 10 (Do you get bitter liquid coming up into your throat?) and question 12 (Do you get heartburn if you bend over?) of the FSSG were significantly lower in group I compared with group P. There were no significant differences in the five subscale scores for acid-related dysmotility symptoms between the groups (Figure 2). Next, we assessed whether patients with NERD who had minimal changes (grade M) had different responses to the therapies compared with those without minimal changes (grade N). The clinical characteristics of the patients assigned to the modified Los Angeles classification grades N and M are shown in Table 2. The data indicate that the characteristics associated with NERD grade N were a shorter stature and a lower frequency of esophageal hiatal hernias. Subgroup analysis showed that the FSSG scores for the patients with NERD grade N in

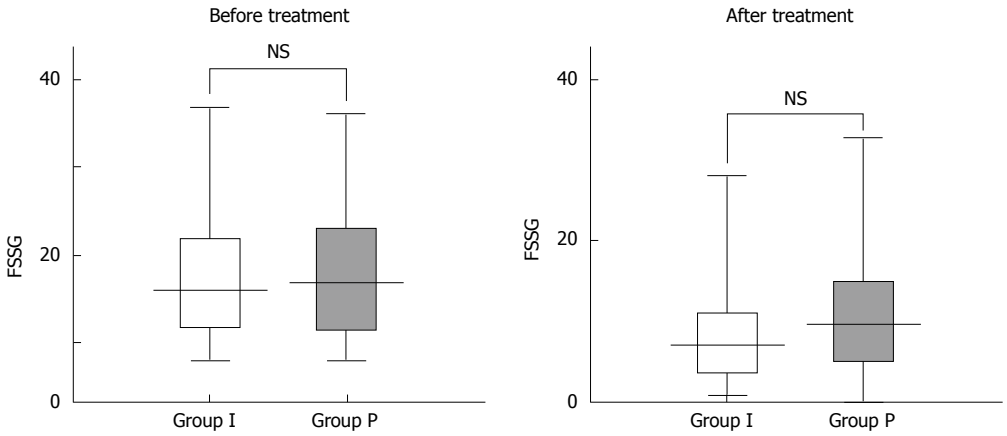

Figure 1 Total Frequency Scale for the Symptoms of Gastroesophageal reflux disease scores for groups I and P before and after four weeks of treatment. Values are expressed as the mean ± SD. FSSG: Frequency Scale for the Symptoms of Gastroesophageal reflux disease; NS: Not significant.

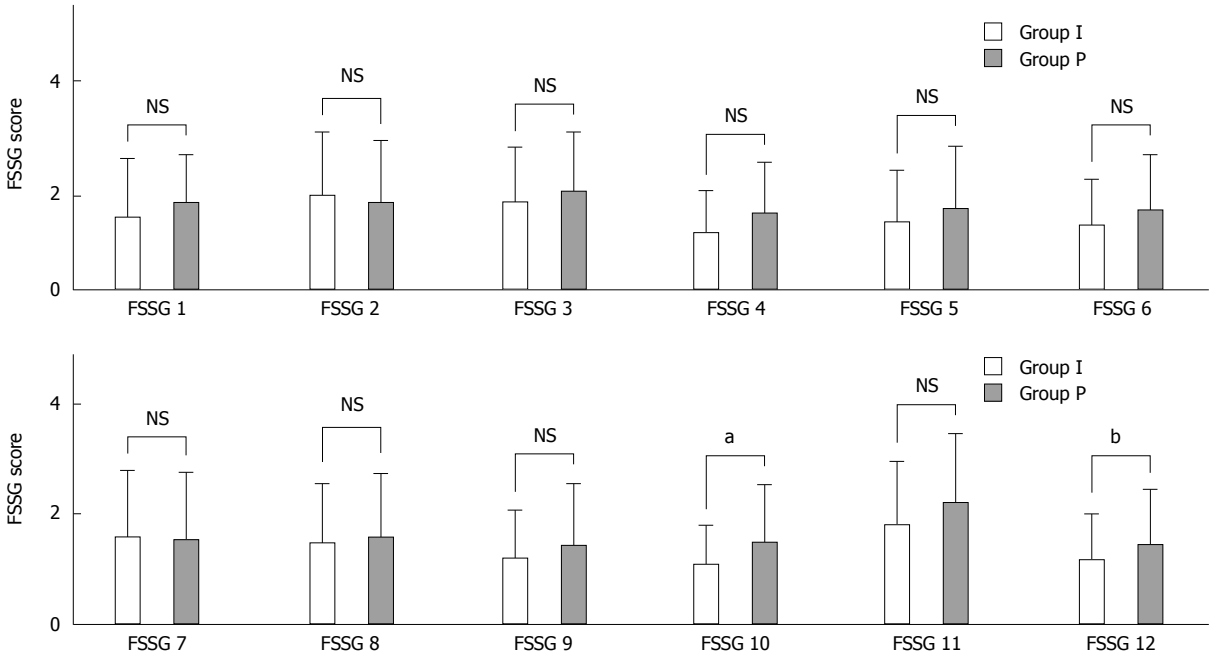

Figure 2 Changes in the Frequency Scale for the Symptoms of Gastroesophageal reflux disease scores for groups I and P after four weeks of treatment. Values are expressed as the mean ± standard deviations. Significant differences were found between the groups. [a]*P* = 0.03 and [b]*P* = 0.01. FSSG: Frequency Scale for the Symptoms of Gastroesophageal reflux disease.

Table 2 Clinical features of non-erosive reflux disease Grade N and M

	Grade N (*n* = 56)	Grade M (*n* = 41)	*P* value
Age (yr), mean ± SD	49.8 ± 16.7	53.3 ± 13.7	0.32
Sex, male/female	14/42	18/23	0.05
BMI (kg/m^2)	22.5 ± 4.4	21.5 ± 2.6	0.44
Height (cm)	158.6 ± 7.3	162.9 ± 8.3	0.02
Weight (kg)	56.7 ± 12.1	57.2 ± 8.4	0.50
Hiatal hernia (+/-)	7/49	14/27	0.02
Drinking (+/-)	17/39	18/23	0.10
Smoking (+/-)	7/49	8/23	0.30

BMI: Body mass index.

group I were significantly lower than the FSSG scores for the patients with NERD grade N in group P after four weeks of treatment (7.8 ± 7.4 *vs* 12.5 ± 9.8, *P* = 0.041), but there was no statistically significant difference between the study groups in relation to the FSSG scores for patients with NERD grade M (Figure 3).

The SF-36 scores for group I significantly improved in relation to general health, vitality, and mental health after treatment, whereas the SF-36 scores for group P improved in relation to general health and mental health after treatment (Figure 4). Subgroup analysis showed that the SF-36 scores for patients with NERD grade N in group I significantly improved in relation

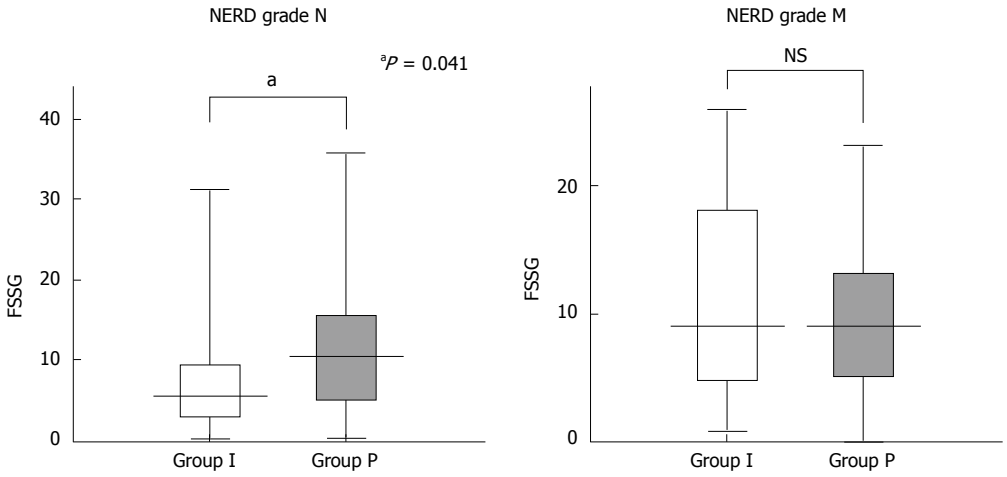

Figure 3 Comparisons of the total Frequency Scale for the Symptoms of Gastroesophageal reflux disease scores between the study groups in relation to the non-erosive reflux disease grades N and M after four weeks of treatment. Values are expressed as the mean ± SD. FSSG: Frequency Scale for the Symptoms of Gastroesophageal reflux disease; NS: Not significant; NERD: Non-erosive reflux disease.

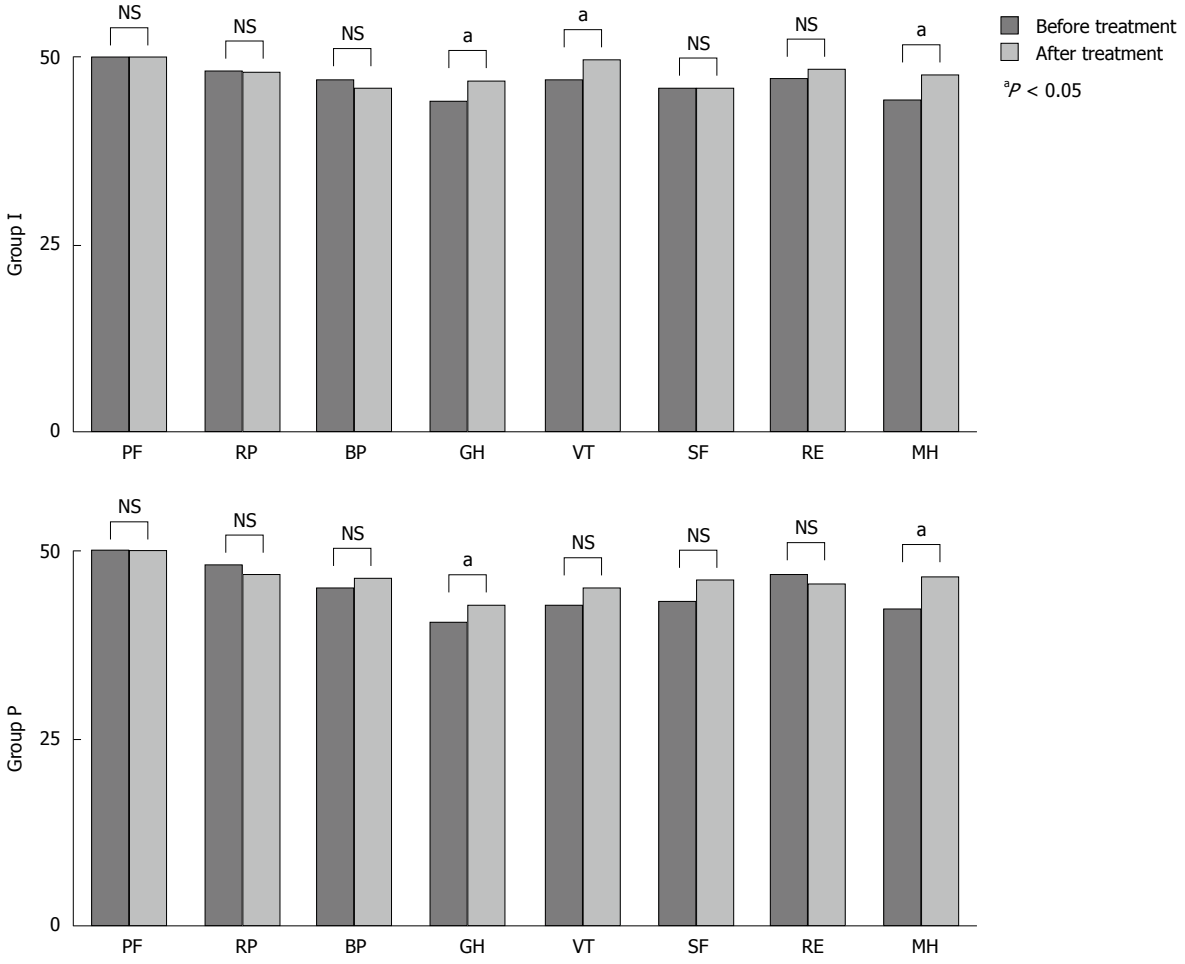

Figure 4 Quality of life assessment of patients in groups I and P before and after treatment using the Short Form-36 questionnaire. PF: Physical functioning; RP: Role limitation-physical; BP: bodily pain; GH: General health; VT: Vitality; SF: Social functioning; RE: Role limitation-emotional; MH: Mental health; NS: Not significant.

to vitality and mental health after treatment, but there were no statistically significant differences in the SF-36 scores for patients with NERD grade M in group I after treatment (Figure 5). We did not detect any improvements in the SF-36 scores in group P after treatment, with the exception of the mental health scores for the patients with NERD grade M.

Patient compliance was determined by counting

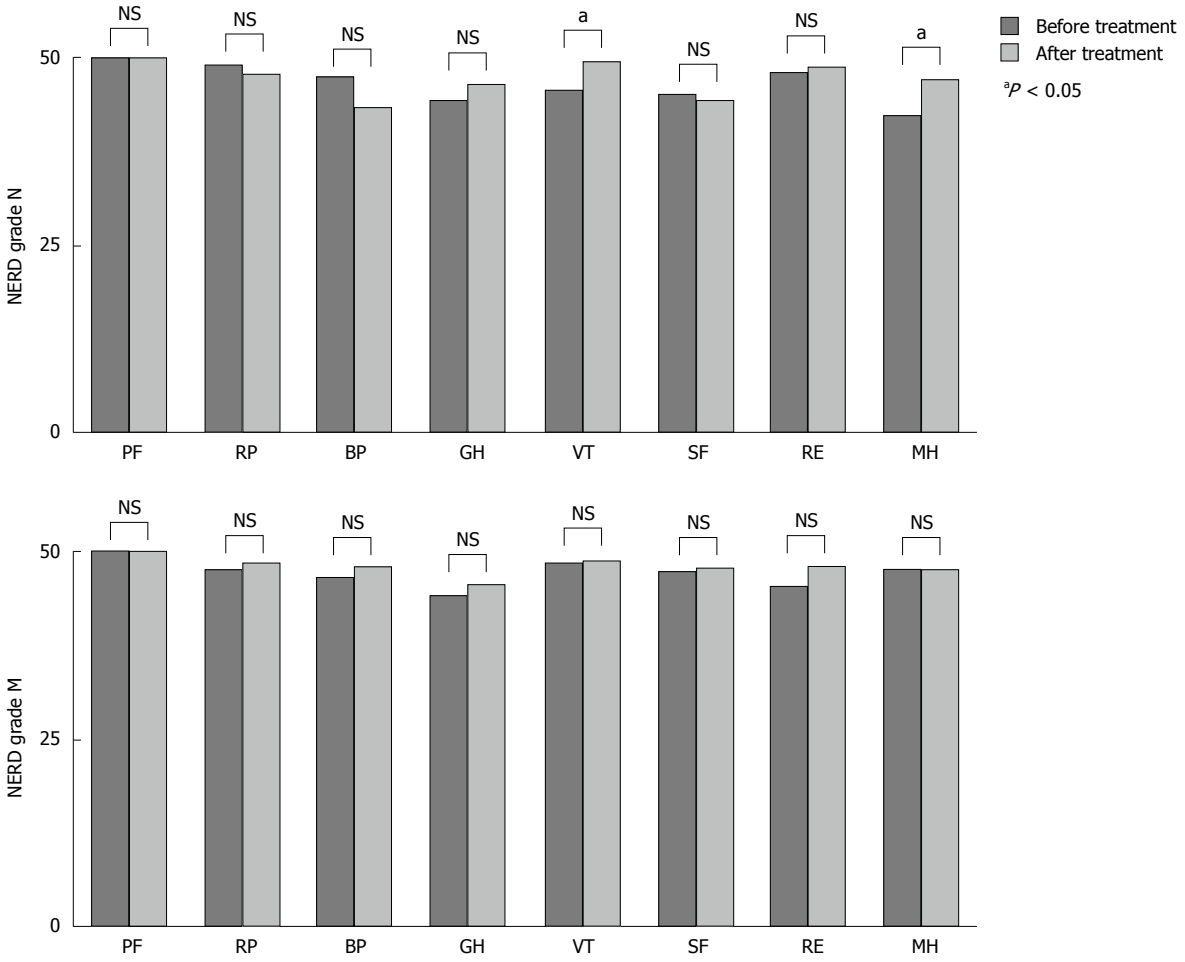

Figure 5 Quality of life assessment of patients in groups I and P before and after treatment using the Short Form-36 questionnaire for non-erosive reflux disease grades N and M. NERD: Non-erosive reflux disease; PF: Physical functioning; RP: Role limitation-physical; BP: bodily pain; GH: General health; VT: Vitality; SF: Social functioning; RE: Role limitation-emotional; MH: Mental health; NS: Not significant.

the remaining units of medication at the end of the four-week study period. All of the patients took more than 90% of the prescribed medication. No serious adverse events occurred, and none of the participants terminated the study because of adverse events.

DISCUSSION

The inhibition of acid secretion within the stomach using PPI or histamine 2 receptor blockers is the main approach for the treatment of NERD[14-17]. However, approximately half of the patients with NERD are resistant to therapy[18]. Many factors are associated with the pathogenesis of acid-suppression therapy-refractory NERD, including gas or bile acid reflux, mucosal hypersensitivity, delayed gastric emptying, and psychological disorders[18-21]. An appropriate therapeutic approach for patients with NERD has yet to be established if they fail to respond to acid suppression therapy. Therefore, determining the efficacy of therapeutic agents added to PPI therapy in patients with NERD has been anticipated for a long time. Sakata *et al*[12] reported that rikkunshito, which acts as a prokinetic agent and improves gastric empting and gastric accommodation, improved GERD symptoms in patients with PPI-refractory NERD. However, adding mosapride, which is another prokinetic agent, to PPI therapy did not significantly improve GERD symptoms compared with PPI therapy administered alone to patients with NERD[22]. Rebamipide, a cytoprotective anti-ulcer agent, also failed to effectively control reflux symptoms in patients with PPI-refractory NERD[23]. In the present study, we evaluated the efficacy of adding IM to rabeprazole in the treatment of patients with NERD in a prospective, randomized, double-blind, placebo-controlled trial. The impact of the therapy on the quality of the patients' lives was evaluated as a secondary endpoint.

The results showed that using the PPI alone and in combination with IM significantly improved the FSSG scores after four weeks of treatment (P = 0.0001); however, there was no statistically significant difference between the groups. These data suggest that controlling acid secretion in the stomach is quite important for the treatment of NERD. Comparisons of the groups in relation to each FSSG score showed that the scores for two reflux-related questions significantly improved in the patients who received combination

therapy compared with those who received mono-therapy, which suggests that IM may play a greater preventive role in relation to reflux compared with acid-related dysmotility symptoms. These results appear to be reasonable, given that IM could affect the epithelial cell connections or communications in the esophagus.

The Montreal consensus conference defined NERD as the presence of troublesome reflux-associated symptoms in the absence of mucosal breaks on endoscopy[2]. Recently, the endoscopic findings associated with NERD were classified into two groups, grades N and M. However, the clinical differences between the groups are not clear. In this study, the group of patients classified as having NERD grade N was characterized by shorter statures and lower frequencies of esophageal hiatal hernias. Joh *et al*[24] reported that NERD grade M patients tended to show a higher frequency of esophageal hiatal hernias, and they suggested that the minimal changes are most likely attributable to gastric acid reflux, which was based on their ambulatory 24-h esophageal monitoring data. Our study showed that in patients with NERD grade N, the addition of IM to PPI therapy significantly improved the FSSG scores compared with PPI mono-therapy, but this was not seen in patients with NERD grade M. These data suggest that factors that affect intercellular communication as well as reflux acid may be associated with the development of NERD grade N, whereas gastric acid reflux may play a major role in the development of NERD grade M.

IM facilitates intercellular communication through the gap junctions in the gastrointestinal tract, including the esophagus. Oyamada *et al*[25] demonstrated the existence of connexins 26 and 43 in human esophageal tissue. Several investigators have suggested that connexin-mediated intercellular communication could affect the formation of functional tight junctions[26-28]. A microscopic study found that esophageal mucosal DIS are present in patients with NERD, they are produced as a consequence of exposure to excess acid, and that esophageal mucosal DIS are associated with the symptom of heartburn[6]. A more recent study has shown that hypersensitive esophagus, which is associated with normal acid exposure and positive symptom associations for either acid and/or non-acid reflux, was related to DIS as well as basal cell hyperplasia and papillae elongation[29]. Taken together, these data suggest that the facilitation of gap junctional intercellular communication by IM may relieve NERD symptoms by ameliorating esophageal mucosal hypersensitivity to non-acid reflux[7,29,30]. We could easily speculate based on the current study's results that NERD grade N includes more patients with hypersensitive esophagi than NERD grade M. Further investigations are required to elucidate the precise therapeutic mechanisms of IM.

Patients with GERD symptoms tend to have a lower QOL compared with the general population[31]. Recently, the SF-36 questionnaire has been used to screen for GERD and to assess QOL before and after treatment[32]. In the present study, the patients who received combination therapy showed significant improvements in three subscale scores, namely, general health, vitality, and mental health, whereas patients who received mono-therapy only showed improvements in two subscale scores. Subgroup analysis showed significant improvements in the vitality and mental health scores for patients with NERD grade N who received combination therapy, whereas no statistically significant improvements were observed for the patients with NERD grade M who received combination therapy. Given that the QOL data corresponded with the improvements in the GERD symptoms after the patients had been treated with IM and PPI, these results are acceptable.

Our study has some limitations that are described next. The main limitation of this study relates to the fact that NERD was diagnosed on the basis of the endoscopic findings alone. An accurate and reliable definition of NERD is needed to appropriately inform the discussion about the treatment of NERD. The second limitation relates to the study being a single-center investigation with a relatively small sample size. Hence, further trials employing larger numbers of subjects are necessary to verify our findings, and in these trials the pathological mechanisms underlying NERD should be investigated. The third limitation associated with this study relates to the controversy that surrounds the diagnostic and clinical relevance of the modified Los Angeles Classification for NERD. The fourth limitation of this study relates to the effects that IM metabolites or drug interactions among IM, rabeprazole, and other medicines might have had on the results of this study.

In conclusion, this is the first randomized controlled trial that has demonstrated that adding IM to PPI therapy improves patients' GERD symptoms as well as the quality of their lives, particularly in NERD grade N patients. We consider that combination therapy may be an appropriate therapeutic option for patients with NERD.

COMMENTS

Background

Proton pump inhibitor (PPI) therapy is considered the mainstay for the treatment of non-erosive reflux disease (NERD). Some reports have suggested that compared with patients with reflux esophagitis, those with NERD have lower response rates to acid suppression therapy. However, a recent meta-analysis emphasized that well-defined NERD patients, including those with abnormal distal esophageal acid exposure and those with hypersensitive esophagi, have responses to PPI therapy that are similar to those seen in patients with RE.

Innovations and breakthroughs

Although the pathophysiology of NERD remains unclear, electron microscopy and light microscopy have demonstrated the presence of esophageal mucosal dilated intercellular spaces (DIS) in patients with NERD. Irsogladine maleate (IM) activates gap junctional intercellular communication and affects the mucosal

DIS; however, little is known about the effects of IM on patients with NERD.

Applications

This double-blind, placebo-controlled study showed that adding IM to rabeprazole improved gastroesophageal reflux disease (GERD) symptoms and the quality of the lives of patients categorized as having NERD who did not have minimal changes (grade N), which was based on the modified Los Angeles classification.

Peer-review

This prospective, double-blind, placebo-controlled trial was designed to evaluate the efficacy of adding IM to PPI therapy in endoscopy-negative Japanese patients with reflux symptoms. The study's main finding was that there was no statistically significant difference between group I (IM + PPI) and group P (placebo + PPI) with respect to the Frequency Scale for the Symptoms of GERD (FSSG) scores. Moreover, a subgroup analysis showed that significant improvements in the FSSG scores had occurred in the patients in group I who had NERD grade N, which was defined based on endoscopic assessments.

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ORIGINAL ARTICLE

Randomized Clinical Trial

Efficacy of moxifloxacin-based sequential therapy for first-line eradication of *Helicobacter pylori* infection in gastrointestinal disease

Jae Jin Hwang, Dong Ho Lee, Ae-Ra Lee, Hyuk Yoon, Cheol Min Shin, Young Soo Park, Nayoung Kim

Jae Jin Hwang, Dong Ho Lee, Ae-Ra Lee, Hyuk Yoon, Cheol Min Shin, Young Soo Park, Nayoung Kim, Department of Internal Medicine, Seoul National University Bundang Hospital, Seongnam 463-707, Gyeonggi-do, South Korea

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Correspondence to: Dong Ho Lee, MD, Department of Internal Medicine, Seoul National University Bundang Hospital, No. 300 Gumi-dong, Bundang-gu, Seongnam 463-707, Gyeonggi-do, South Korea. dhljohn@yahoo.co.kr
Telephone: + 82-31-7877006
Fax: + 82-31-7874051

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Abstract

AIM: To evaluate the efficacy of 14-d moxifloxacin-based sequential therapy as first-line eradication treatment of *Helicobacter pylori* (*H. pylori*) infection.

METHODS: From December 2013 to August 2014, 161 patients with confirmed *H. pylori* infection randomly received 14 d of moxifloxacin-based sequential group (MOX-ST group, n = 80) or clarithromycin-based sequential group (CLA-ST group, n = 81) therapy. *H. pylori* infection was defined on the basis of at least one of the following three tests: a positive ^{13}C-urea breath test; histologic evidence of *H. pylori* by modified Giemsa staining; or a positive rapid urease test (CLOtest; Delta West, Bentley, Australia) by gastric mucosal biopsy. Successful eradication therapy for *H. pylori* infection was defined as a negative ^{13}C-urea breath test four weeks after the end of eradication treatment. Compliance was defined as good when drug intake was at least 85%. *H. pylori* eradication rates, patient compliance with drug treatment, adverse event rates, and factors influencing the efficacy of eradication therapy were evaluated.

RESULTS: The eradication rates by intention-to-treat analysis were 91.3% (73/80; 95%CI: 86.2%-95.4%) in the MOX-ST group and 71.6% (58/81; 95%CI: 65.8%-77.4%) in the CLA-ST group (P = 0.014). The eradication rates by per-protocol analysis were 93.6% (73/78; 95%CI: 89.1%-98.1%) in the MOX-ST group and 75.3% (58/77; 95%CI: 69.4%-81.8%) in the CLA-ST group (P = 0.022). Compliance was 100% in both groups. The adverse event rates were 12.8% (10/78) and 24.6% (19/77) in the MOX-ST and CLA-ST group, respectively (P = 0.038). Most of the adverse events were mild-to-moderate in intensity; there was none serious enough to cause discontinuation of treatment

in either group. In multivariate analysis, advanced age (≥ 60 years) was a significant independent factor related to the eradication failure in the CLA-ST group (adjusted OR = 2.13, 95%CI: 1.97-2.29, P = 0.004), whereas there was no significance in the MOX-ST group.

CONCLUSION: The 14-d moxifloxacin-based sequential therapy is effective. Moreover, it shows excellent patient compliance and safety compared to the 14-d clarithromycin-based sequential therapy.

Key words: *Helicobacter pylori*; First-line eradication treatment; Moxifloxacin; Sequential therapy; Eradication rate

Core tip: This is the first study to evaluate the efficacy of 14-d moxifloxacin-based sequential therapy compared to that of 14-d clarithromycin-based sequential therapy as a first-line eradication treatment of *Helicobacter pylori* infection. Our study showed that the moxifloxacin-based therapy is effective and shows excellent patient compliance and safety compared with the clarithromycin-based sequential therapy. The high eradication rate, excellent compliance, and safety of the moxifloxacin-based sequential therapy suggest its suitability as an alternative to standard triple therapy.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is the single most important factor causing chronic atrophic gastritis, peptic ulcer disease, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphoma[1]. The eradication of *H. pylori* infection effectively reduces the incidence of peptic ulcer and gastric cancer and prevents their recurrence[2]. The most important first-line treatment for eradication of *H. pylori* is currently the standard triple therapy comprising a proton pump inhibitor (PPI), clarithromycin, and either amoxicillin or metronidazole[3,4]. Although many studies have indicated that this therapy is highly effective, the reported eradication rates vary between 70% and 95%[5,6] and have shown a tendency to decrease due to increasing antibiotic resistance[7,8]. Therefore, more effective alternative regimens are needed.

Many alternative, first-line treatment regimens have been studied. Sequential therapy is one alternative regimen, which consists of a PPI and amoxicillin for the first seven days, followed by a PPI plus metronidazole and clarithromycin for another seven days[9]. This regimen is currently recommended as an alternative first-line treatment for *H. pylori* infection in European guidelines[3]. In Korea, a region with relatively high antibiotic resistance, the efficacy of sequential therapy has been reported in several randomized controlled trials, including our previous prospective study[10-12]. These studies initially indicated sequential therapy to be effective, but recent studies have shown less satisfactory results. The main causes of sequential therapy failure are patient non-compliance and antibiotic resistance[13]. Non-compliance is due mainly to patients' complicated schedules[14]. Another key element of treatment failure is bacterial resistance to clarithromycin. Resistance to clarithromycin is relatively high in Korea[15,16] and plays an role in diminishing the effect of sequential therapy[13].

Recently, changing the antibiotic agents that are included in the eradication regimen to improve *H. pylori* eradication therapy efficacy has been studied. The reason for changing antibiotic agents is to overcome resistance to clarithromycin. Among several candidates for new antibiotic agents, moxifloxacin has received attention. Compared with other fluoroquinolones, moxifloxacin has a low incidence of adverse events and small interactions with other drugs. Therefore, we hypothesized that 14-d moxifloxacin-based sequential therapy might increase *H. pylori* eradication as compared to clarithromycin-based sequential therapy in an area with high clarithromycin resistance. A head-to-head comparison between moxifloxacin and clarithromycin regimens has not been addressed in the literature yet.

The aim of the present study was to compare the *H. pylori* eradication rates, patient compliance, and adverse events between first-line moxifloxacin-based sequential therapy and clarithromycin-based sequential therapy.

MATERIALS AND METHODS

Patient selection

This study was conducted at Seoul National University Bundang Hospital between December 2013 and August 2014. A total of 161 patients with *H. pylori* infection were enrolled in this prospective, open-labeled, randomized pilot study. *H. pylori* infection was defined on the basis of at least one of the following three tests: (1) a positive ^{13}C-urea breath test (^{13}C-UBT); (2) histologic evidence of *H. pylori* by modified Giemsa staining in the lesser and greater curvature of the body and antrum of the stomach; or (3) a positive rapid urease test (CLOtest; Delta West, Bentley, Australia) by gastric mucosal biopsy from the lesser curvature of the body and antrum of the stomach. Patients were excluded if they had received PPIs, H_2 receptor

antagonists, or antibiotics in the previous four weeks, or if they had used non-steroidal antiinflammatory drugs or steroids in the two weeks prior to the ^{13}C-UBT. The other exclusion criteria were age below 18 years, previous gastric surgery, or endoscopic mucosal dissection for gastric cancer, advanced gastric cancer, severe current disease (hepatic, renal, respiratory, or cardiovascular), pregnancy, and any condition thought to be associated with poor compliance (*e.g.*, alcoholism or drug addiction).

Study design

This prospective, open-labeled, single-center, randomized pilot study compared 14-d moxifloxacin-based sequential therapy with 14-d clarithromycin-based sequential therapy as a first-line eradication treatment of *H. pylori* infection. All enrolled participants filled in a questionnaire on demographic information, history of comorbidities, body mass index (BMI), smoking habit, and alcohol consumption. Each participant underwent esophagogastroduodenoscopy to confirm clinical diagnosis (such as gastritis or peptic ulcer disease) and to conduct a biopsy for *H. pylori* infection, colonization, atrophic changes, and intestinal metaplasia.

The 161 participants were randomly assigned to one of the two treatment groups using a computer-generated numeric sequence. The 14-d moxifloxacin-based sequential therapy group (MOX-ST group, n = 80) received 20 mg rabeprazole and 1 g amoxicillin twice daily for the first week, followed by 20 mg rabeprazole twice daily, 500 mg metronidazole twice daily, and moxifloxacin 400 mg once daily for the remaining week. Participants in the 14-d clarithromycin-based sequential therapy group (CLA-ST group, n = 81) received 20 mg rabeprazole and 1 g amoxicillin twice daily for the first week, followed by 20 mg rabeprazole, 500 mg metronidazole, and clarithromycin 500 mg twice daily for the remaining one week.

Patient compliance was evaluated by remnant pill counting and direct questions from a physician 1 wk after completion of the treatment. Compliance was defined as good when less than 15% of the pills were unconsumed at remnant pill counting. At the same time, all of the patients were asked about adverse events. Successful eradication therapy for *H. pylori* infection was defined as a negative ^{13}C-UBT test four weeks after the cessation of eradication treatment. The study protocol was approved by the Ethics Committee at Seoul National University Bundang Hospital (IRB number: B-1409/268-103).

^{13}C-Urea breath test

Before the ^{13}C-UBT, the patients were instructed to stop taking medications (*i.e.*, antibiotics for 4 wk, or PPIs for 2 wk) that could affect the result, and fasted for a minimum of 4 h. After patients cleaned their oral cavities by gargling, a pre-dose breath sample was obtained. Then, 100 mg of ^{13}C-urea powder (UBiTkit™; Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) was dissolved in 100 mL of water and was administered orally, and an additional breath sample was obtained. Breath samples were taken with special breath collection bags while patients were in the sitting position, both before drug administration (baseline) and 20 min after the powder medication. The samples were analyzed using an isotope-selective, non-dispersive infrared spectrometer (UBiT-IR 300®; Otsuka Pharmaceutical Co. Ltd, Tokyo, Japan).

Statistical analysis

The primary and secondary outcomes of the present study were *H. pylori* eradication rates and treatment-related adverse events, respectively. The eradication rates were determined by intention-to-treat (ITT) and per-protocol (PP) analyses. ITT analysis compared the treatment groups, including all patients as originally allocated; the PP analysis compared the treatment groups, including only those patients who had completed the treatment as originally allocated. Mean standard deviations were calculated for quantitative variables. Student's t test was used to evaluate the continuous variables, and χ^2 test and Fisher's exact test were utilized to assess the non-continuous variables. Additionally, univariate and multivariate analyses were conducted to assess the effects of factors on the eradication rate. All statistical analyses were performed using the Predictive Analytics Software 20.0 version for Windows (SPSS Inc., IBM, Chicago, IL, United States). A P value of less than 0.05 was defined as clinically significant.

RESULTS

Characteristics of patients

A schematic diagram of the study is provided in Figure 1. A total of 161 patients with *H. pylori* infection were randomly allocated to the MOX-ST group or the CLA-ST group by 1:1. Of the 161 patients, 155 (96.2%) completed their allocated regimens. The remaining six patients (3.8%) were excluded from study analysis. Therefore, 78 MOX-ST patients and 77 CLA-ST patients were included in the PP analysis. The enrolled patients' baseline demographic and clinical characteristics did not statistically differ between the two groups (Table 1).

Helicobacter pylori eradication rates

Table 2 shows the rates of eradication of *H. pylori* infection according to the ITT and PP analyses. The overall ITT eradication rate was 81.3% (131/161). The final ITT eradication rates were 91.3% [73/80; 95%CI: 86.2-95.4%] in the MOX-ST group and 71.6% (58/81; 95%CI: 65.8-77.4%) in the CLA-ST group (P = 0.014; Table 2). The overall PP eradication rate was 84.5% (131/155), and the final PP eradication

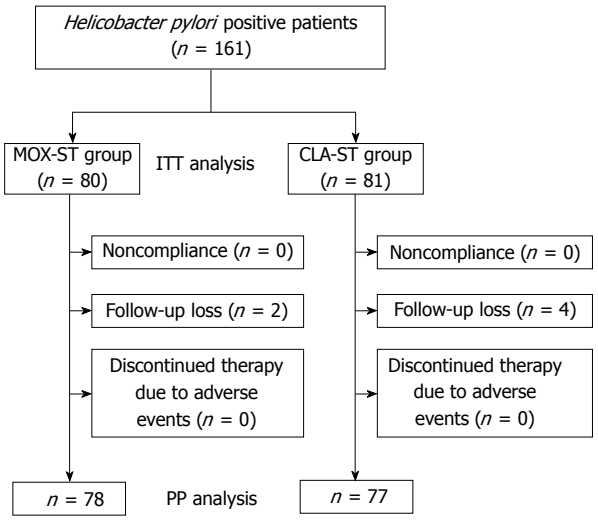

Figure 1 Flow schematic of the study included in intention-to-treat and per-protocol analyses. MOX-ST: 14-d moxifloxacin-based sequential therapy; CLA-ST: 14-d clarithromycin-based sequential therapy; ITT: Intention-to-treat; PP: Per-protocol.

rates were 93.6% (73/78; 95%CI: 89.1%-98.1%) in the MOX-ST group and 75.3% (58/77; 95%CI: 69.4%-81.8%) in the CLA-ST group (P = 0.022; Table 2). The *H. pylori*-eradication rates in the MOX-ST group were significantly higher than those in the CLA-ST group, according to both the ITT (P = 0.014) and the PP analysis (P = 0.022).

Clinical factors influencing H. pylori eradication

To evaluate the clinical factors influencing the efficacy of *H. pylori* eradication, univariate analyses were performed (as listed in Table 3). In the CLA-ST group, the eradication rates of participants over 60 years of age was significantly lower than those of participants under 60 years of age (P = 0.002; Table 3). Other factors in the CLA-ST group did not affect the eradication response. No factors in the MOX-ST group affected the eradication response. The multivariate analysis revealed that age greater than 60 years [adjusted OR = 2.13, 95%CI: 1.97-2.29, P = 0.004] was an independent factor predictive of eradication failure in the CLA-ST group.

Adverse events and compliance

Table 4 lists the adverse events that occurred in the two groups. Adverse events occurred for 10 of 78 patients (12.8%) in the MOX-ST group and for 19 of 77 patients (24.6%) in the CLA-ST group. The difference was statistically significant (P = 0.038). The most common adverse events were bloating/dyspepsia (4/78, 5.1%) and taste distortion (3/78, 3.8%) in the MOX-ST group and epigastric discomfort (5/77, 6.4%) and taste distortion (5/77, 6.4%) in the CLA-ST group. These differences were not statistically significant (P > 0.05). Most of the adverse events were mild-to-moderate in intensity; there was none serious enough to cause discontinuation of treatment in either group. The treatment compliance (as defined as taking at least 85% of scheduled medication doses) was 100% in both groups (Table 4).

Table 1 Demographic and clinical data at baseline (intention-to-treat population) *n* (%)

	MOX-ST	CLA-ST	*P* value
Included in ITT analysis	80	81	NS
Age (yr), mean ± SD	59.3 ± 13.1	59.4 ± 13.1	0.235
Gender (male)	34 (42.5)	33 (40.7)	0.773
BMI (kg/m^2), mean ± SD	22.9 ± 2.2	22.7 ± 2.9	0.352
Current smoker	11 (13.7)	10 (12.3)	0.385
Alcohol drinking	13 (16.2)	9 (11.1)	0.351
Diabetes	5 (6.2)	8 (9.8)	0.125
Hypertension	19 (23.7)	23 (28.3)	0.407
Previous history of peptic ulcer	12 (15.0)	9 (11.1)	0.348
Endoscopic diagnosis			0.624
Gastritis	70 (87.6)	70 (86.6)	
Gastric ulcer	4 (5.0)	5 (6.1)	
Duodenal ulcer	1 (1.2)	1 (1.2)	
Gastric and duodenal ulcer	1 (1.2)	0 (0.0)	
Adenoma	4 (5.0)	4 (4.9)	
Dysplasia	0 (0.0)	1 (1.2)	
Positive CLOtest	59 (73.7)	62 (76.5)	0.955
H. pylori colonization			0.588
Negative	3 (3.7)	5 (6.1)	
Mild	34 (42.5)	36 (44.4)	
Moderate	32 (40.0)	24 (29.6)	
Marked	11 (13.8)	16 (19.9)	
Atrophic change	8 (10.0)	2 (2.4)	0.087
Intestinal metaplasia	10 (12.5)	13 (16.0)	0.761
Drop out	2 (2.5)	4 (4.9)	0.113
Noncompliance	0 (0.0)	0 (0.0)	
Follow-up loss	2 (2.5)	4 (4.9)	
Discontinued therapy due to adverse events	0 (0.0)	0 (0.0)	

MOX-ST: 14-d moxifloxacin-based sequential therapy; CLA-ST: 14-d clarithromycin-based sequential therapy; ITT: Intention-to-treat; SD: Standard deviation; BMI: Body mass index; CLOtest: Rapid urease test; *H. pylori*: *Helicobacter pylori*; NS: Not significant; BMI: Body mass index.

Table 2 *Helicobacter pylori* eradication rates

	MOX-ST	CLA-ST	*P* value
ITT analysis			
Eradication rate	91.3% (73/80)	71.6% (58/81)	0.014
95%CI	86.2%-95.4%	65.8%-77.4%	
PP analysis			
Eradication rate	93.6% (73/78)	75.3% (58/77)	0.022
95%CI	89.1%-98.1%	69.4%-81.8%	

ITT: Intention-to-treat; PP: Per-protocol; CI: Confidence interval; MOX-ST: 14-d moxifloxacin-based sequential therapy; CLA-ST: 14-d clarithromycin-based sequential therapy.

DISCUSSION

To our knowledge, this is the first study that evaluated the efficacy of 14-d moxifloxacin-based sequential therapy compared with 14-d clarithromycin-based sequential therapy as a first-line eradication treatment

Table 3 Univariate analysis of clinical factors influencing the efficacy

	MOX-ST		CLA-ST	
	Eradication rate	*P* value	Eradication rate	*P* value
Age (yr)		0.436		0.002
< 60	97.2% (35/36)		81.1% (30/37)	
⩾ 60	90.4% (38/42)		70.0% (28/40)	
Gender		0.383		0.622
Male	91.1% (31/34)		71.8% (23/32)	
Female	95.4% (42/44)		77.7% (35/45)	
Body mass index		0.651		0.743
< 25	95.2% (20/21)		86.9% (20/23)	
⩾ 25	92.9% (53/57)		70.3% (38/54)	
Smoking		0.585		0.377
(-)	97.0% (65/67)		77.6% (52/67)	
(+)	72.7% (8/11)		60.0% (6/10)	
Alcohol		0.417		0.082
(-)	96.9% (63/65)		77.9% (53/68)	
(+)	76.9% (10/13)		55.5% (5/9)	
Diabetes		0.706		0.107
(-)	94.5% (70/74)		76.8% (53/69)	
(+)	75.0% (3/4)		62.5% (5/8)	
Hypertension		0.322		0.096
(-)	91.6% (55/60)		79.6% (43/54)	
(+)	100.0% (18/18)		65.2% (15/23)	
History of ulcer		0.454		0.828
(-)	92.5% (62/67)		75.0% (51/68)	
(+)	100.0% (11/11)		77.7% (7/9)	
Presence of ulcer		0.352		0.076
(-)	92.6% (63/68)		78.7% (52/66)	
(+)	100.0% (10/10)		54.5% (6/11)	
Positive CLOtest		0.259		0.374
(-)	76.1% (16/21)		63.1% (12/19)	
(+)	100.0% (57/57)		79.3% (46/58)	
Atrophic change		0.111		0.071
(-)	95.7% (67/70)		76.0% (57/75)	
(+)	75.0% (6/8)		50.0% (1/2)	
Intestinal metaplasia		0.270		0.322
(-)	95.5% (65/68)		76.5% (49/64)	
(+)	80.0% (8/10)		69.2% (9/13)	
Bacterial density		0.296		0.507
None	66.7% (2/3)		60.0% (3/5)	
Mild	90.6% (29/32)		67.6% (23/34)	
Moderate	100.0% (32/32)		78.2% (18/23)	
Marked	90.9% (10/11)		93.3% (14/15)	
Compliance		NS		NS
Poor	0.0% (0/0)		0.0% (0/0)	
Good	93.6% (73/78)		75.3% (58/77)	
Adverse events		0.493		0.494
(-)	92.6% (63/68)		77.6% (45/58)	
(+)	100.0% (10/10)		68.4% (13/19)	

MOX-ST: 14-d moxifloxacin-based sequential therapy; CLA-ST: 14-d clarithromycin-based sequential therapy; CLOtest: Rapid urease test; NS: Not significant.

of *H. pylori* infection. In this study, eradication rates in the MOX-ST group (ITT: 91.3%; PP: 93.6%) were higher than those in the CLA-ST group (71.6%/75.3%), with statistically significant differences ($P < 0.05$). These results represented statistically significant differences: namely, markedly higher eradication rates for the MOX-ST group ($P < 0.05$). Moreover, the total adverse-event rate for the MOX-ST group was 12.8% (10/78), which was significantly lower than that for the CLA-ST group (24.6%, 19/77), with statistically

Table 4 Adverse events and compliance *n* (%)

Adverse events	MOX-ST (*n* = 78)	CLA-ST (*n* = 77)	*P* value
Bloating/dyspepsia	4 (5.1)	4 (5.3)	0.383
Taste distortion	3 (3.8)	5 (6.4)	0.316
Epigastric discomfort	2 (2.6)	5 (6.4)	0.296
Nausea	1 (1.3)	1 (1.3)	0.505
Abdominal pain	0 (0.0)	1 (1.3)	0.309
Diarrhea	0 (0.0)	3 (3.9)	0.075
Constipation	0 (0.0)	0 (0.0)	NS
Total	10 (12.8)	19 (24.6)	0.038
Compliance, *n* (%)	78 (100.0)	77 (100.0)	NS

MOX-ST: 14-d moxifloxacin-based sequential therapy; CLA-ST: 14-dclarithromycin-based sequential therapy; NS: Not significant.

differences ($P = 0.038$). The drug compliance was 100% in both groups. Thus, our study showed the 14-d moxifloxacin-based sequential therapy is effective and shows excellent compliance and safety compared with the 14-d clarithromycin-based sequential therapy.

In the clarithromycin-based sequential therapy, the key theoretical basis is the effect of amoxicillin on the bacterial cell wall. Amoxicillin administered in the first half of the regimen damages cell wall of *H. pylori*; this is thought to overcome antibiotic resistance and increase eradication rate by two mechanisms. First, damage to the cell wall damage may ease the penetration of subsequent antibiotics into the *H. pylori* strain. Second, the damaged cell wall cannot develop efflux channels for clarithromycin[17,18]. Several large, multicenter studies have reported high eradication rates with clarithromycin-based sequential therapy[11,19,20]. An earlier Korean study on clarithromycin-based sequential therapy performed in 2008 and 2009 reported a high eradication rate (85.9% by ITT analysis and 92.6% by PP analysis)[21]. However, subsequent studies performed in our institution suggest efficacy of clarithromycin-based sequential therapy is decreasing in Korea. The eradication rate of clarithromycin-based sequential therapy was 79.3% by ITT analysis and 81.9% by PP analysis in 2009 and 2010[10], 75.6% (ITT) and 76.8% (PP) in 2011 and 2012[22], and 71.6% (ITT) and 75.3% (PP) in 2013 and 2014 in these study. These findings imply that resistance to antibiotics in *H. pylori* treatment is increasing, and that clarithromycin-based sequential therapy might already be suboptimal in areas with high prevalence of clarithromycin resistance.

A recent meta-analysis evaluating *H. pylori* strains in Western populations found fluoroquinolone-resistance prevalence in less than 5.0%[15]. Fluoroquinolone resistance in Japan is 15%[23]. In Gyeonggi Province, Korea, the rates of resistance were 5.0% for levofloxacin and moxifloxacin, 5.0% for amoxicillin, 16.7% for clarithromycin, 34.3% for metronidazole, and 8.0% for tetracycline[8]. These results might be related to different patterns of regional and institutional fluoroquinolone use[24]. This explains why a moxifloxacin-based triple

regimen achieved successful eradication in 84%-87% of cases (by PP analysis), as compared with the markedly lower rates recorded for levofloxacin-based triple regimens elsewhere in Asia[25-28]. These results suggest that appropriate *H. pylori*-eradication therapies should be continually adjusted according to local bacterial resistance patterns. Therefore, we could explain that the reason moxifloxacin-based sequential therapy is more effective than clarithromycin-based sequential therapy in Korea is the low resistance to moxifloxacin compared with clarithromycin.

Our study showed that advanced age (≥ 60 years) was a significant independent factor related to the eradication failure in the CLA-ST group, whereas there was no significance in the MOX-ST group in multivariate analysis. Other studies have also reported that advanced age was associated with treatment failure in *H. pylori* eradication therapy[29,30]. However, the mechanisms by which advanced age interfere with eradication remain unclear. Immunity degradation, which is one of the physiological changes of the human body by aging, may be associated with poor treatment response[31]. Further studies are needed to investigate the mechanisms underlying the association between advanced age and poor response to eradication treatment.

The most common adverse events of moxifloxacin are gastrointestinal disturbances, such as diarrhea and nausea. In the present study, the most common adverse events were taste distortion, epigastric discomfort, and abdominal bloating. The total adverse-event rate for the 14-d moxifloxacin-based sequential treatment was 12.8% (10/78), which was significantly lower than that for the 14-d clarithromycin-based sequential treatment (24.6%, 19/77). In both groups, the adverse events were mild to moderate; none was serious enough to require discontinuation or interfered with regular life.

This study has several limitations. First, this study was a single-center pilot study with a relatively small sample size. Larger prospective studies will be needed to confirm our results in regions where different patterns of resistant are present. However, we think our exploratory study would be a good reference for clinicians and researchers to help design new studies on this subject. Second, we could not investigate the antibiotic resistance in each patient. However, this was a pilot study comparing alternative first-line regimens in a Korean population. Moreover, selection bias is ruled out by randomized allocation of the participants, so that the prevalence of primary antibiotic resistance is expected to be equally distributed among the therapeutic groups.

In conclusion, 14-d moxifloxacin-based sequential therapy is a more effective first-line eradication treatment than 14-d clarithromycin-based sequential therapy for *H. pylori* infection. The high eradication rate, excellent patient compliance, and safety of the moxifloxacin-based therapy suggest its suitability as an alternative to standard triple therapy. Further large prospective studies are required to determine the broad application of this regimen in comparison with currently approved first-line therapies.

COMMENTS

Background

A recent meta-analysis reported that the efficacy of sequential therapy for *Helicobacter pylori* (*H. pylori*) infection is modest in Asia, exemplifying the need to find a better regimen.

Research frontiers

The potential role of moxifloxacin as an antibiotic agent useful for eradication treatment in *H. pylori* infection has been suggested by a few animal and human studies.

Innovations and breakthroughs

This is the first randomized controlled study to evaluate the efficacy of 14-d moxifloxacin-based sequential therapy (as compared with 14-d clarithromycin-based sequential therapy) as a first-line eradication treatment of *H. pylori* infection. The high eradication rate, excellent compliance, and safety of the 14-d moxifloxacin-based sequential therapy suggest its suitability as an alternative to the standard triple therapy.

Applications

This pilot study's design and findings could be used to determine sample size for a larger, prospective study aiming to test the efficacy of moxifloxacin-based sequential therapy for *H. pylori* eradication.

Terminology

H. pylori is found in the stomach and is linked to the development of gastritis, peptic ulcers, and stomach cancer. To prevent recurrence in patients with these diseases, it is necessary to eradicate *H. pylori* infection.

Peer-review

This study presents a topic of interest in clinical practice, not often considered in literature. Methods and study population are adequate, and conclusions are reasonable and of possible practical use. This article presents an important issue. This is the first study to compare the efficacy of 14-d moxifloxacin-based sequential therapy with that of 14-d clarithromycin-based sequential therapy.

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Hepatitis B virus preS1 deletion is related to viral replication increase and disease progression

Seoung-Ae Lee, Ki-Jeong Kim, Hong Kim, Won-Hyuk Choi, Yu-Sub Won, Bum-Joon Kim

Seoung-Ae Lee, Hong Kim, You-Sub Won, Bum-Joon Kim, Department of Biomedical Sciences, Microbiology and Immunology, and Liver Research Institute, Seoul National University College of Medicine, Seoul 110-799, South Korea
Ki-Jeong Kim, Department of Microbiology, School of Medicine, Chung-Ang University, Seoul 110-799, South Korea
Won-Hyuk Choi, Department of Internal Medicine, Konkuk University, Seoul 110-799, South Korea
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Correspondence to: Bum-Joon Kim, Professor, Department of Biomedical Sciences, Microbiology and Immunology, and Liver Research Institute, Seoul National University College of Medicine, Seoul 110-799, South Korea. kbumjoon@snu.ac.kr
Telephone: +82-2-7408316
Fax: +82-2-7430881
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Abstract

AIM: To investigate the clinical implications of hepatitis B virus (HBV) preS1 deletion.

METHODS: We developed a fluorescence resonance energy transfer-based real-time polymerase chain reaction (RT-PCR) that can detect four genotypes (wild type, 15-bp, 18-bp and 21-bp deletion). The PCR method was used in two cohorts of Korean chronic HBV subjects with genotype C infections. Cohort Ⅰ included 292 chronic HBV subjects randomly selected from Cheju National University Hospital (Jeju, South Korea) or Seoul National University Hospital (Seoul, South Korea), and cohort Ⅱ included 90 consecutive chronic HBV carriers recruited from Konkuk University Hospital (Seoul, South Korea); the cohort Ⅱ patients did not have hepatocellular carcinoma or liver cirrhosis.

RESULTS: The method proposed in this study identified 341 of 382 samples (89.3%). Deletion variants were identified in 100 (29.3%) of the 341 detected samples. In both cohorts, the subjects with deletions had a significantly higher Hepatitis B virus e antigen (HBeAg)-positive seroprevalence [cohort Ⅰ, wild (51.0%) *vs* deletion (75.0%), $P < 0.001$; cohort Ⅱ, wild (69.2%) *vs* deletion (92.9%), $P = 0.002$] and higher HBV DNA levels [cohort Ⅰ, wild (797.7 pg/mL) *vs* deletion (1678.9 pg/mL), $P = 0.013$; cohort Ⅱ, wild (8.3×10^8 copies/mL) *vs* deletion (2.2×10^9 copies/mL), $P = 0.049$], compared to subjects with wild type HBV.

CONCLUSION: HBV genotype C preS1 deletion may affect disease progression in chronic HBV subjects through an extended duration of HBeAg seropositive status and increased HBV replications.

Key words: Hepatitis B virus; PreS1 start codon deletion; Hepatitis B virus e antigen; Hepatocellular carcinoma;

Genotype C

Core tip: Our data indicate that the hepatitis B virus (HBV) genotype C preS1 deletion might significantly contribute to disease progression in chronic HBV subjects through an extended duration of Hepatitis B virus e antigen seropositive status and increased HBV replication. This study provides novel insight into the greater infectivity and virulence of genotype C compared with other genotypes. In addition, the fluorescence resonance energy transfer-based real-time polymerase chain reaction used to detect the preS1 deletion shows promise for the earlier prediction of the risk of liver disease progression in chronic HBV subjects.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global health problem, and more than 350 million people are chronic carriers of the virus[1]. The infection is associated with a wide spectrum of clinical manifestations ranging from acute or fulminant hepatitis to various forms of chronic infection, including asymptomatic carrier, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC)[2]. Despite the recent significant decline in HBV chronic subjects thanks to a successful HBsAg vaccination program, South Korea is still recognized as an endemic area of HBV infection[3]. It has also been reported that genotype C2 is exclusively prevalent in South Korea[4], which is known to be more prone to mutations and is related to more severe liver diseases, as well as having a lower antiviral response compared with genotype B[5]. Furthermore, the high prevalence of basal core promoter (BCP) double mutations and the presence of distinct immune responses against HBV proteins in the Korean population could lead to the generation of distinct HBV variants that are not (or rarely) encountered in other areas[6-15] and that result in distinct clinical manifestations in Korean chronic HBV subjects[16].

The preS proteins might have a pivotal function in virus assembly and attachment to hepatocytes[17,18]. Infections with preS/S HBV variants, particularly deletion variants, correlated with the most progressive forms of liver disease and HCC. Both preS1 and preS2 mutations reportedly contribute to hepatocarcinogenesis by inducing an endoplasmic reticulum (ER) stress pathway that is caused by the accumulation of large hepatitis B surface (LHBs) proteins in the ER or by altering the transactivating capacity[19-21].

Recently, we introduced two types of preS1 mutations that are related to the progression of liver diseases: W4P/R[11] and preS1 start codon deletion[14]. The former proved to be exclusively detected in males and also proved to be related to liver disease progression in chronic HBV subjects with genotype C infections, according to our fluorescence resonance energy transfer (FRET)-based real-time polymerase chain reaction (RT-PCR) method[11]. The latter mutation proved to be prevalent in chronic HBV subjects with genotype C, according to our previous molecular epidemiologic study based on a direct sequencing protocol[14]. However, the clinical implications of HBV preS1 mutations have rarely been investigated. Furthermore, the direct sequencing protocol has a limitation that could underestimate the presence of quasispecies variants in a subject, which could interfere with the genuine interpretation of specific mutations from clinical and virological perspectives. To overcome this drawback, FRET-based RT-PCR could be used for molecular epidemiologic purposes. The FRET-based RT-PCR allows not only the simultaneous identification of coexisting quasispecies but also the direct identification of target mutations from primary specimens, such as serum through melting curve analyses of the amplification product[22,23].

Therefore, the present study aims to achieve the following goals: (1) to develop a FRET-based RT-PCR method to detect the HBV preS1 start codon deletion; and (2) to determine the clinical implications of this mutation using the developed methods on DNA from the sera of genotype C-infected chronic HBV subjects.

MATERIALS AND METHODS

Subjects

In an effort to further support the clinical implications of preS1 deletion, our developed RT-PCR method was applied to two patient cohorts. Cohort Ⅰ included 292 subjects randomly selected from the chronic HBV subjects who visited Cheju National University Hospital (Jeju, Korea) in 2003 or Seoul National University Hospital (Seoul, Korea) in 2005. The cohort Ⅰ study protocol was approved by the Institutional Review Board of Seoul National University Hospital (C-1007-021-322). In cohort Ⅱ, 90 consecutive chronic HBsAg carriers without liver cirrhosis or HCC were recruited from January 2012 to March 2012 at Konkuk University Hospital (Seoul, Korea). The cohort Ⅱ study protocol was approved by the Institutional Review Board of Konkuk University Hospital (KUH-1010544). The clinical details of cohorts Ⅰ and Ⅱ are presented in Table 1.

The HBV DNA sera levels for cohort Ⅰ and cohort Ⅱ were determined using different methods. In cohort Ⅰ,

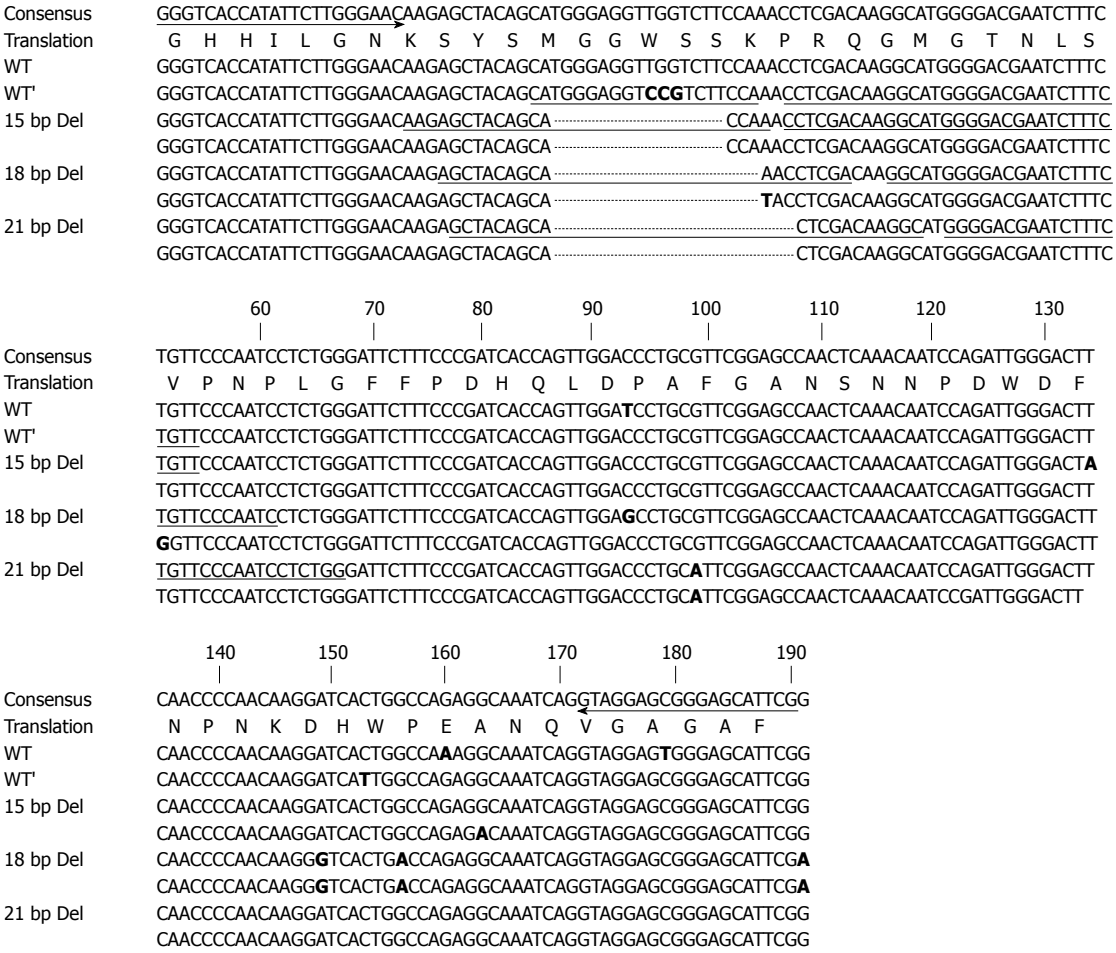

Figure 1 Primer and probe positions designed to detect hepatitis B virus pre-S1 gene deletion sequence variants at the start codon of preS1. Four types of probes for detection of wild type and three deletion types (15-bp, 18-bp and 21-bp deletions) were used to screen the deletion variants. The arrows indicate the primer positions. The underlining indicates the probe positions. The numbers indicate the nucleotide position on the S gene sequence. The boldface bases indicate different bases from those of the consensus sequence. The amino acid sequence is shown as the one-letter amino acid symbol. WT: Wild type; WT': W4P mutation; 15, 18, 21bp Del: 15, 18, 21 base pair deletion.

Table 1 Clinical data of the subjects in cohort Ⅰ (292 subjects) and cohort Ⅱ (90 subjects) *n* (%)

Clinical factor	Cohort Ⅰ	Cohort Ⅱ
Patient No.	292	90
Age in years, mean ± SD	46.8 ± 16.0	37.8 ± 9.6
Male	225 (77.1)	46 (51.1)
HBeAg-positive	162 (55.5)	58 (64.4)
Liver disease No.	C : CH : LC : HCC	C : CH
	66 : 33 : 67 : 126	36 : 54
ALT [IU/liter (mean ± SD)]	77.7 ± 153.4	117.4 ± 226.5
Median of HBV-DNA (range)	979.4 (0-6000)[1]	1.3×10^9
		$(100\text{-}15.1 \times 10^9)$[2]

[1]Median of HBV-DNA: pg/mL; [2]Median of HBV-DNA: copies/mL. C: Carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B virus e antigen; ALT: Alanine amino-transferase; IU: International unit.

the serum HBV DNA levels were determined using the Digene Hybrid Capture Ⅱ assay (Digene Diagnostic Inc., Gaithersburg, MD, United States), which has a lower limit of detection of 0.5 pg/mL. In cohort Ⅱ, the serum HBV DNA levels were assessed using the COBAS Amplicor PCR assay, which has a lower limit of detection of 300 copies/mL (Roche Molecular Systems, Branchburg, NJ, United States).

Primer and probe design for RT-PCR

Figure 1 and Table 2 present the primers designed to amplify the HBV preS1 gene fragment and the probes designed to identify the deletion sequence variants at the start codon, respectively. A total of 99 cloned HBV genes were aligned using SeqMan™ Ⅱ software (DNASTAR). A primer pair for amplification of the HBV preS1 gene fragment was designed using Oligo V 6.5 (Molecular Biology Insights), which produced a 203-224 bp amplicon. We used four pairs of hybridization probes (an anchor probe and a sensor probe; HybProbe) to detect wild type at channel 640 and three types of deletion polymorphisms (15-bp deletion, 18-bp deletion, and 21-bp deletion) at three separate channels (670, 610 and 640, respectively) using the LightCycler 2.0 RT-PCR system. The same preS1 primer set and wild type probe previously developed for the W4P/R mutation detection[11] were

Table 2 Primers and HybProbes developed to identify hepatitis B virus preS1 start codon deletion variants by real-time polymerase chain reaction

	Name	Sequence (5'→3')	T_m (℃)[1]	Target sequence	Channel
Forward primer	HBV_sF	GGGTCACCATATTCTTGGGAAC	62.5	203 - 224 bp of S gene	
Reverse primer	HBV_sR	CGAATGCTCCCRCTCCTAC	60.5 or 63.3	203 - 224 bp of S gene	
Anchor probe	WT_A	ACAGAAAGATTCGTCCCCATGCCTTGTCGAGG -FL[2]	72.1		
Sensor probe	WT_S	LC Red640[3]-TGGAAGACGGACCTCCCATG-PH	63.4	Wild type S gene	640
Anchor probe	18D_A	GATTGGGAACAGAAAGATTCGTCCCCATGCC-FL	70.3		
Sensor probe	18D_S	LC Red610[4]-TCGAGGTTTGCTGTAGCT-PH[5]	59.9	18 bp deletion	610
Anchor probe	21D_A	CCAGAGGATTGGGAACAGAAAGATTCGTCCCC-FL	70.5		
Sensor probe	21D_S	LC Red640-GCCTTGTCGAGTGCTGTAGC -PH	64.7	21 bp deletion	640
Anchor probe	15D_A	ACAGAAAGATTCGTCCCCATGCCTTGTCGAGG -FL	73.2		
Sensor probe	15D_S	LC Red670[6]-TTGGTGCTGTAGCTCTT-PH	57	15 bp deletion	670
Anchor probe	preS1_A	GATCCTTGTTGGGGTTGAAGTCCC-FL	65.8		
Sensor probe	preS1_S	LC Red610[4]-ATCTGGATTGTTTGAGTTGGCT-PH	62.4	PreS1	610

[1]Temperature was calculated using LC PDS software (version 2.0); [2]FL: Fluorescein; [3]LC Red640: Light Cycler dye Red640; [4]LC Red610: Light Cycler dye Red610; [5]PH: Phosphate; [6]LC Red670: Light Cycler dye Red670. Tm: Melting temperature; 15D, 18D, 21D_A: 15, 18, 21 base pair deletion anchor probe; 15D, 18D, 21D_S: 15, 18, 21 base pair deletion sensor probe; S gene: Surface region gene.

used. The remaining probes, which were used to detect three types of deletion variants, were designed in this study.

RT-PCR

A LightCycler 2.0 system was used, and its four detection channels were calibrated for color compensation and activated for the experiment. The LC Faststart DNA Master HP kit (Roche Diagnostic, Basel, Switzerland) was used to prepare the master mix, according to the kit protocol. A 10 μL reaction mixture was prepared for each sample and each target variant, as follows: 1 μL Taq buffer (containing dNTP mix and 10 mmol/L $MgCl_2$), an additional 2 mmol/L $MgCl_2$, 0.75 μmol/L forward primer (HBV_sF), 0.35 μmol/L reverse primer (HBV_sR), and a pair of 0.18 μmol/L HybProbes for the target variant (Table 2). Two separate reactions were performed for each sample: one to detect the wild type virus at channel 640, and the other to detect the deletion variants at channels 610, 640, and 670. For the relative quantification of the deletion variants *vs* the wild type in a subject, the melting temperature height values of the deletion variants *vs* wild type of 72 subjects with deletions in cohort Ⅰ were calculated and compared with subjects with severe types of disease (HCC and liver cirrhosis) and mild types of disease (carriers and chronic hepatitis). The values of each deletion probe were normalized to those of the wild type probe.

Direct sequencing

To verify the fidelity of the developed FRET based RT-PCR, 23 samples from cohort Ⅱ, 10 samples identified as mixed infections of both wild type and deletion variants and 13 samples identified as wild type-only infections, according to the RT-PCR assay, were subject to direct sequencing analyses. PCR amplification was performed using the same primer pair used for RT-PCR. Direct sequencing was used with the forward primer.

Statistical analysis

All detection items in this study were repeated at least three times, and the results were expressed as percentages, the mean ± SD, or medians (range). Differences between categorical variables were analyzed using Fisher's exact test or the χ^2 test. For continuous variables, Student's *t*-test was used when the data showed a normal distribution, while the Mann-Whitney *U* test was used when the data were not normally distributed. SPSS version 21.0 software (Professional Statistic, Chicago, IL, United States) was used for all statistical analyses and a *P* value of < 0.05 (two-tailed) was considered statistically significant.

Ethics statement

This retrospective study was reviewed and approved by the Institutional Review Boards (IRBs) at Seoul National University Hospital (IRB Grant No. C-1007-021-322) for cohort Ⅰ and at Konkuk University Hospital (IRB Grant No. KUH-1010544) for cohort Ⅱ. The patients' medical records were anonymized and de-identified prior to the analyses. Experiments were mainly based on viral DNA extracted from isolates; therefore, the research study was conducted without informed consent. Informed consent waivers were granted by the IRBs.

RESULTS

Determination of the melting temperature for FRET-based RT-PCR to detect the deletion variants

Application of the developed FRET-based RT-PCR to the wild type and three deletion types of the control plasmid DNAs cloned from the subjects demonstrated that separation between the wild type and variants

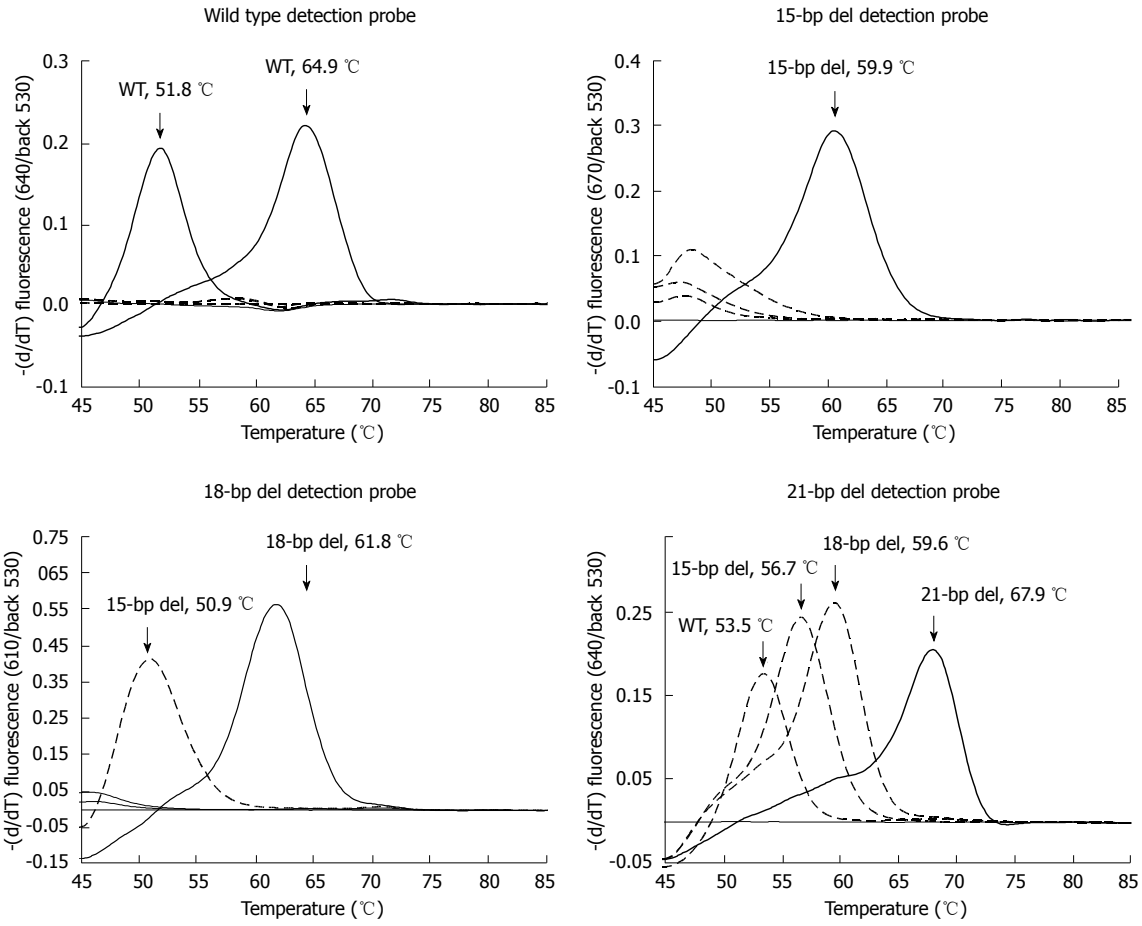

Figure 2 Real-time polymerase chain reaction melting curve analyses that identified the hepatitis B virus *preS1* deletion variants (WT, 15-bp del, 18-bp del, or 21-bp del) with deletions at the start codon of preS1 with cloned positive control plasmids and non-template controls. The T_ms were determined using duplicate runs and are presented as average and standard deviation values. The Y-axis is the negative differential of fluorescence over temperature at the detection channel and is normalized using background fluorescence at channel 530. PCR: Polymerase chain reaction; HBV: Hepatitis B virus; WT: Wild type; 15, 18, 21-bp del: 15, 18, 21-base pair deletion; HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; CH: Chronic hepatitis; C: Carrier.

Table 3 Sensitivity of fluorescence resonance energy transfer-based real-time polymerase chain reaction assay, according to the subjects' clinical statuses *n* (%)

Diseases	Number of tests positive/total	
	Cohort Ⅰ	Cohort Ⅱ
HCC	109/125 (87.2)	
LC	59/69 (85.5)	
CH	33/33 (100)	50/54 (92.6)
C	63/66 (95.5)	27/36 (75)
Total	264/292 (90.4)	77/90 (85.6)

HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; CH: Chronic hepatitis; C: Carrier.

was possible, in addition to separation between the three different types of variants (15-bp deletion, 18-bp deletion, and 21-bp deletion). The deletion variants exhibited distinct melting temperatures according to the respective probe. As demonstrated in the previous report[11], the wild type formed two distinct melting peaks at Ch. 640, one at 52.0 ± 0.2 ℃ [no mutation in codon 4 of preS1 (TGG)] and another at 65.0 ± 0.1 ℃ [CCG in codon 4 of preS1], which did not exhibit distinguishable melting peaks from the other variants. The 15-bp deletion variant clone formed a single distinct melting peak at 59.7 ± 0.1 ℃ at Ch. 670, which distinguished it from other cross-reacting melting peaks of the other variants. The 18-bp deletion variant clone formed a distinct melting peak at 67.8 ± 0.1 ℃ at Ch. 610, which exhibited a cross, as well as a clearly distinguishable 15-bp deletion peak and no detectable peaks of the other variants. The 21-bp deletion variant clone formed a distinct melting peak at the highest temperature (67.8 ± 0.2 ℃ at Ch. 640), with a cross and clearly distinguishable melting peaks from the other variants (Figure 2).

Application of FRET-based RT-PCR to serum DNA from chronic Korean subjects in the two patient cohorts

In this study, to correct the bias that results from the time and location of sample collection, we applied the developed FRET-based RT-PCR to samples from two different cohorts: cohort Ⅰ (292 samples) and cohort Ⅱ (90 samples). Applying this PCR method to 382 DNA samples taken from Korean chronic HBV subjects from the two cohorts resulted in the successful differentiation of 341 samples (89.3%), including 264 (90.4%) of the 292 samples in cohort Ⅰ and 77

Table 4 Ten types of polymorphism detected by fluorescence resonance energy transfer-based real-time polymerase chain reaction assay and their prevalences among the 341 detected subjects

Types	*n* (%)
WT only	241 (70.7)
15 del only	3 (0.9)
18 del only	3 (0.9)
21 del only	4 (1.2)
WT + 15 del	16 (4.7)
WT + 18 del	47 (13.8)
WT + 21 del	6 (1.8)
WT + 15 del + 18 del	18 (5.3)
WT + 18 del + 21 del	2 (0.6)
WT + 15 del + 18 del + 21 del	1 (0.3)
Total	341

WT: Wild type; 15, 18, 21 del: 15, 18, 21 base pair deletion mutant; FRET-based real-time PCR: Fluorescence resonance energy transfer-based real-time polymerase chain reaction.

(85.6%) of the 90 samples in cohort Ⅱ (Table 3). Ten types of polymorphisms were found in the 341 subjects detected from cohorts Ⅰ and Ⅱ. Of the 341 subjects detected, a total of 100 subjects (29.3%) [72 (27.3%) of the 264 detected samples from cohort Ⅰ and 28 (36.4%) of the 77 samples from cohort Ⅱ] proved to have more than one of the three deletions. Of the 100 subjects with deletions, 90 (90%) simultaneously exhibited positive signals with wild type probes, which indicated the coexistence of both wild type and deletion variants in most subjects with deletions (Table 4).

Comparison of clinical data between the subjects with and without deletions

In the 264 subjects detected from cohort Ⅰ, no significant differences were found in age, gender, and alanine transaminase (ALT) level between the subjects with (192 subjects) and without (72 subjects) deletions. However, significant differences in Hepatitis B virus e antigen (HBeAg) serostatus and HBV DNA levels were found. The HBeAg-positive ratio was significantly higher in the subjects with wild type HBV than in those with deletions (51.0%, 98/192 subjects *vs* 75.0%, 54/72 subjects, $P < 0.001$). The median HBV DNA level was also significantly higher in the subjects with wild type HBV than in those with deletions (797.7 pg/mL *vs* 1678.9 pg/mL, $P = 0.013$) (Table 5).

Similar to cohort Ⅰ, a significant difference was also found among the 77 cohort Ⅱ subjects, in terms of two clinical indicators of HBeAg serostatus (69.2%, 29/49 subjects *vs* 92.9%, 26/28 subjects, $P = 0.002$) and HBV DNA levels (8.3×10^8 copies/mL *vs* 2.2×10^9 copies/mL, $P = 0.049$) between the subjects with (49 subjects) and without (28 subjects) deletions (Table 5). This trend was also found in the combined analyses of the clinical data of the subjects ($n = 341$) detected in cohorts Ⅰ and Ⅱ. A significant difference in the HBeAg serostatus (52.7%, 127/241 subjects *vs* 80.0%, 80/100 subjects, $P < 0.001$) and HBV DNA levels (4.6×10^8 copies/mL *vs* 1.4×10^9 copies/mL, $P = 0.014$) was observed between the subjects with (241 subjects) and without (100 subjects) deletions (Table 6).

Of the 100 subjects with deletions in cohorts Ⅰ and Ⅱ, the 18-bp deletion variants were the most prevalent (70 subjects, 70%), followed by the 15-bp deletion (38 subjects) and 21-bp deletion (13 subjects). Twenty-one subjects (21%) proved to have mixed deletion variants, with more than two types (Table 6).

Quantification of variants vs wild type via melting temperature height value in 72 subjects with deletions in cohort Ⅰ, according to the different clinical stages

Significant differences in the prevalence of preS1-del were not found between the subjects with different clinical stages in cohort Ⅰ [CH (27.1%, 26/96 subjects), liver cirrhosis (20.3%, 12/59 subjects) and HCC (31.2%, 34/109 subjects)]. It is possible to estimate the relative amounts of deletion variants *vs* wild type in a subject by calculating the melting temperature height values[24]. We compared the mean melting temperature height values of the variants *vs* the wild type of the 72 subjects with deletions in cohort Ⅰ, according to the different clinical stages. Among the subjects with the three types of deletion variants, those with severe types of diseases (*i.e.*, liver cirrhosis and HCC) exhibited significantly higher levels of melting temperature height values in deletion variants *vs* wild type compared with the subjects with mild types of chronic hepatitis. The latter finding suggests that increases in deletion variants in a subject may contribute to liver disease progression (Figure 3).

Detecting deletion variants: FRET-based RT-PCR compared with the direct sequencing protocol

To validate the effectiveness of FRET-based RT-PCR in detecting deletion variants, 23 samples from cohort Ⅱ (*i.e.*, 10 samples identified as mixed infection of deletion and wild type and 13 samples of wild type-only infection identified by FRET-based RT-PCR) were subjected to direct sequencing analyses. In direct sequencing data, samples that exhibited the presence of mixed peaks in the electropherogram were regarded as mixed infections with wild type and deletion variants. No difference was found between the two protocols in the detection of wild type-only infections. The 13 samples identified as wild type-only infection using RT-PCR were also identified as wild type using the direct sequencing protocol. However, a discrepancy between the two protocols was found in detecting the deletion variants. While only 7 (70%) of 10 samples were identified as mixed infection using RT-PCR, mixed infections with both wild type and deletion variants were proven using direct sequencing protocols, and mixed peaks were not found in the other three samples (Table 7). This discrepancy might have resulted from the sensitivity difference between

Table 5 Comparison of clinical data between subjects of cohort Ⅰ and Ⅱ with and without deletion variants *n* (%)

Clinical factor	Cohort Ⅰ			Cohort Ⅱ		
	Wild type (*n* = 192)	Deletion (*n* = 72)	*P* value	Wild type (*n* = 49)	Deletion (*n* = 28)	*P* value
Age in years, mean ± SD	46.3 ± 15.7	44.7 ± 17.8	0.471	35.8 ± 8.9	39.1 ± 11.1	0.186
Male	148 (77.1)	52 (72.2)	0.423	20 (40.8)	16 (57.1)	0.235
HBeAg-positive	98 (51.0)	54 (75.0)	< 0.001	29 (69.2)	26 (92.9)	0.002
Liver disease (C : CH : LC : HCC)	43 : 27 : 47 : 75	20 : 6 : 12 : 34		18 : 31	9 : 19	
ALT [IU/liter (mean ± SD)]	87.7 ± 182.4	54.4 ± 33.5	0.295	125.5 ± 271.2	120.9 ± 160.3	0.935
Median of HBV-DNA (range)	797.7 (0-6000)[1]	1678.9 (0-6000)[1]	0.013	8.3×10^8 ($100\text{-}10.1 \times 10^9$)[2]	2.2×10^9 ($2.3 \times 10^3\text{-}15.1 \times 10^9$)[2]	0.049

[1]Median level of HBV-DNA, pg/mL; [2]Median level of HBV-DNA, copies/mL. C: Carrier; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; HBeAg: Hepatitis B virus e antigen; ALT: Alanine aminotransferase.

Table 6 Comparison of the clinical data between the combined cohort Ⅰ and Ⅱ subjects with and without deletion and the prevalence of 3 types of deletion variants, according to their clinical statuses *n* (%)

	Wild type (*n* = 241)	Deletion (*n* = 100)	21 del (*n* = 13)	18 del (*n* = 70)	15 del (*n* = 38)	*P* value
Age in years, mean ± SD	44.1 ± 15.2	43.1 ± 16.3	51.6 ± 15.7	42.0 ± 15.9	40.3 ± 14.8	0.564
Male	168 (69.7)	68 (68.0)	6 (69.2)	46 (65.7)	24 (63.1)	0.797
HBeAg-positive	127 (52.7)	80 (80.0)	8 (61.5)	58 (82.9)	33 (86.8)	< 0.001
Liver disease (C : CH : LC : HCC)	61 : 58 : 47 : 75	29 : 25 : 12 : 34	4 : 3 : 0 : 8	21 : 17 : 9 : 23	12 : 13 : 5 : 8	
ALT [IU/liter (mean ± SD)]	96.8 ± 207.1	81.1 ± 111.2	63.2 ± 43.3	85.5 ± 128.3	89.0 ± 122.4	0.476

One patient from hepatocellular carcinoma (HCC) has mixed mutations of 15, 18 and 21 deletion. Three patients from HCC have mixed mutations of both 15 and 18 deletion. Two patients from liver cirrhosis (LC) have mixed mutations of both 15 and 18 deletion. Two patients from LC have mixed mutations of both 18 and 21 deletion. Six patients from chronic hepatitis (CH) have mixed mutations of both 15 and 18 deletion. Seven patients from C have mixed mutations of both 15 and 18 deletion. Median of hepatitis B virus-DNA was converted into copies/mL according to a conversion constant presented by NIH (1 pg = 280000 copies). C: Carrier; HBeAg: Hepatitis B virus e antigen; ALT: Alanine aminotransferase.

Table 7 Comparison of fluorescence resonance energy transfer-based real-time polymerase chain reaction *vs* direct sequencing protocol for detection of deletion variants *n* (%)

Direct sequencing	Result for FRET-based real-time PCR	
	Only wild type (*n* = 13)[1]	Mixed infection (*n* = 10)[2]
Only wild type	13 (100)	3
Mixed infection	0	7 (70)

[1]Thirteen samples that were identified as only wild type infection by FRET-based real-time PCR were subjected to a direct sequencing protocol; [2]The samples identified as mixed infection with both wild type and deletion variant by FRET-based real-time PCR were subjected to a direct sequencing protocol. FRET-based real-time PCR: Fluorescence resonance energy transfer-based real-time polymerase chain reaction.

the two protocols used to detect the deletion variants in the mixed samples. To address this issue, we investigated the limits of our RT- PCR method (15-bp-, 18-bp and 21-bp deletion) for detection of deletion variants mixed with various concentrations of wild type DNA. Our analysis showed that the RT-PCR could detect deletion variants even in cases of a 100-fold higher level of wild type DNA compared with deletion DNA, irrespective of the deletion types (Figure 4).

DISCUSSION

There are clinical and virological differences between the HBV genotypes. Genotype C represents most HBV infections in Asian areas, including China, South Korea, and Japan, where HBV infections are the most prevalent worldwide. Recent studies have demonstrated that patients with genotype C are more likely to develop HCC than patients with other HBV genotypes[25-27]. Furthermore, higher levels of sustained HBeAg seropositivity and serum HBV-DNA, which are known to be HCC risk factors, were found in the genotype C subjects compared with the genotype B subjects[25,28]. In a previous study, we found that the preS1 start codon deletion is related to HCC in Korean HBV subjects[14]. Interestingly, comparisons of clinical information between subjects with the preS1 deletion and preS2 deletion indicated that the preS2 deletion (as with most HBV mutations) but not the preS1 deletion is positively related to HBeAg seronegative status[14]. This finding indicates that the preS1 deletion might be generated in the HBeAg-positive phase rather than in the HBeAg-negative phase. It also indicates that the preS1 deletion might be produced by factors other than the host immune response.

Despite the presence of overt, distinct traits in genotype C infection, the underlying mechanisms capable of explaining these distinct traits remain unknown to date. Therefore, in the present study, we used the FRET-based RT-PCR method to investigate the clinical implications of preS1 deletion in Korean chronic HBV subjects with genotype C infections. Thus, we demonstrated the significant roles of preS1 start codon deletions in liver disease progression among Korean

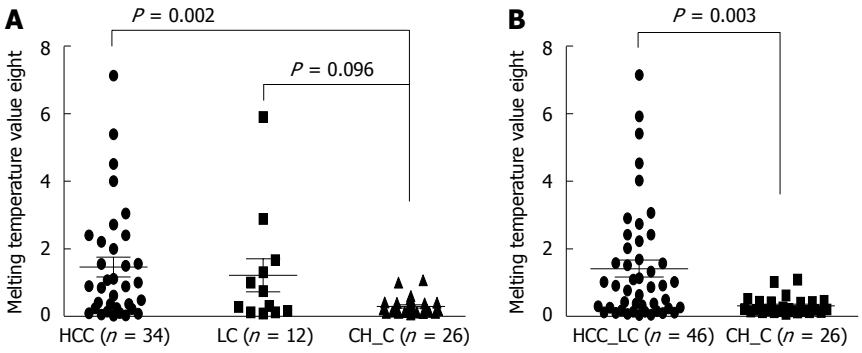

Figure 3 Quantification of deletion variants *vs* wild type virus by melting temperature height values in 72 cohort I subjects with deletions, according to their clinical statuses. The melting temperature height values of the deletion probe of each subject were normalized to the wild type probe. A: Comparison of the melting temperature height values between the subjects with hepatocellular carcinoma (HCC), liver cirrhosis (LC), and mild diseases [chronic hepatitis (CH) and carrier (C)]; B: Comparison of the melting temperature height values between subjects with severe diseases (HCC and LC) and mild disease (CH and C).

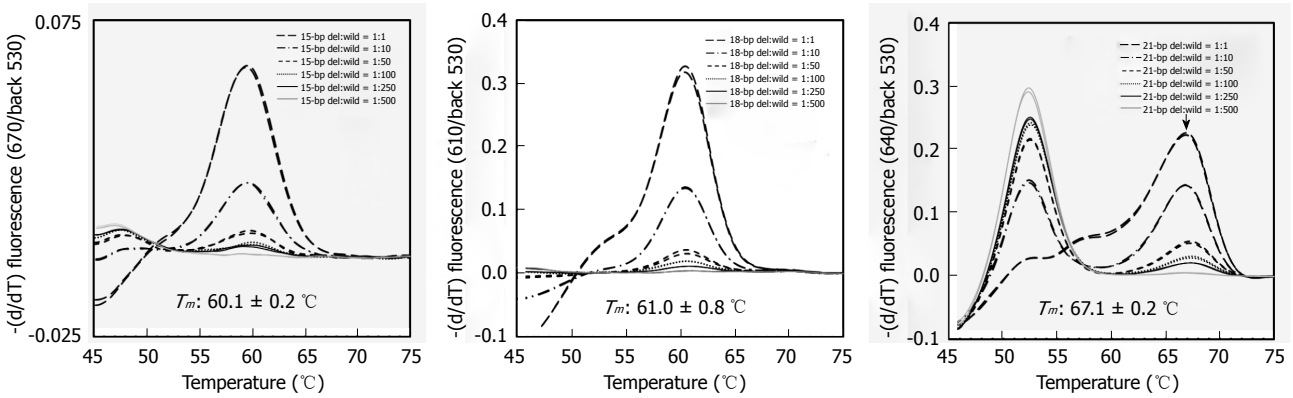

Figure 4 Detection limits of real-time polymerase chain reaction for detection of deletion variants mixed with various concentration of wild type hepatitis B virus DNA. Control plasmid DNA was used for this experiment. Wild: Wild type; 15, 18, 21-bp del: 15, 18, 21-base pair deletion.

chronic HBV subjects infected with genotype C *via* enhanced duration of HBeAg-positivity and HBV DNA replication. Our data might provide, at least in part, an explanation for the distinct traits of genotype C infections (higher levels of sustained HBeAg-positivity and HBV DNA replication) compared with other genotypes, particularly genotype B infections. Most HBV mutations found to date, including BCP double mutations and preC mutations, have been positively related to HBV persistent infections and HBeAg seronegative status[29,30]. However, this relationship is not true for the preS1 start codon deletions. To the best of our knowledge, the preS1 deletion is the first HBV mutation related to enhanced HBV replication and HBeAg-positive serostatus.

We applied the proposed FRET-based RT-PCR method to two different cohorts. The results from both cohorts were similar, which further strengthens our data. Our FRET-based RT-PCR method demonstrated that there were no significant differences in the prevalence of the preS1 deletion between subjects with different clinical stages (Tables 5 and 7), which does not align with the previous result indicating that the preS1 deletion is significantly more prevalent in HCC subjects than in subjects with mild types of liver disease, CH, and carriers[14]. The difference between the two studies could be related to differences in the applied protocols: FRET-based RT-PCR and nested-direct sequencing. Unlike the latter protocol, the former can detect deletion variants even if they exist in a small percentage compared to the wild type. Because three types of probes (for detecting the variants) existed in their respective independent channels, even the majority type cannot interfere with the detection of other probes. Using control plasmid DNAs, we discovered that our FRET-based RT- PCR could accurately detect the respective deletion type even in cases of mixed DNA showing a 100-fold higher level of wild type DNA than deletion type DNA (Figure 4). These differences can also explain the disparity in sensitivity found between our FRET-based RT-PCR and the direct sequencing protocol for the detection of deletion variants, demonstrating the superiority of the former protocol over the latter as a method for early prediction of liver disease progression. Although there were no differences in the prevalence of the preS1 deletion between the different clinical stages, a comparison of the melting temperature heights across the different clinical stages demonstrated increases in the deletion variants *vs* the wild type in subjects with

severe liver disease (Figure 3), which indicates the positive effect of the variants on disease progression.

Interestingly, the most deletion variants (90%, 90/100 variants) coexisted with the wild type in the subjects with chronic hepatitis (Table 5). This finding indicates that the preS1 deletion event might occur sequentially after a wild type infection in HBeAg-positive stages instead of a de novo horizontal infection of deletion variants. Our phylogenetic study based on the quasispecies sequences demonstrated the same coherent clustering of wild type and deletion variants in a subject (data not shown), thus supporting this notion.

In particular, irrespective of the deletion types, all preS1 start codon deletions led to 11-N terminal loss from the preS1 of the otherwise 119 amino acids, as shown in genotype D, with 108 amino acids of preS1. Although the function of N-terminal region remains unknown, its absence in the genotype D strain indicates that it is dispensable in the HBV life cycle. However, together with a previous report demonstrating that deletions leading to 11 amino acids in the preS1 5′ region were observed more frequently in HCC subjects[14], our data strongly support that the N terminal loss of 11 amino acids plays a primary role in the progression of liver disease through enhanced HBeAg production and DNA replication. Alternatively, the N terminal deletion region could lead to simultaneous deletion of the overlapped polymerase region. Therefore, the molecular mechanism for enhanced HBeAg production and DNA replication induced by deletion variants should be elucidated in a future study that focuses on the modified function of the deleted LHBs or deleted polymerase.

In conclusion, the HBV genotype C preS1 deletion has the potential to lead to disease progression in chronic HBV subjects through the extended duration of HBeAg seropositive status and increases in HBV replications. This study provides novel insight into the higher infectivity and virulence of genotype C compared with other genotypes. In addition, the FRET-based RT-PCR for detection of the preS1 deletion shows promise for the earlier prediction of the risk of liver disease progression in chronic HBV subjects.

COMMENTS

Background

The preS1 start codon deletion proved to be prevalent in chronic hepatitis B virus (HBV) subjects with genotype C using our previous molecular epidemiologic study based on a direct sequencing protocol. However, its clinical implications have rarely been investigated. In this study, the authors developed a molecular diagnostic method to detect this mutation and gained insight into its clinical implications.

Research frontiers

This study developed a novel fluorescence resonance energy transfer (FRET)-based real-time polymerase chain reaction (RT-PCR) method to detect the preS1 start codon deletion and applied it to 2 Korean chronic HBV patient cohorts.

Innovations and breakthroughs

For the first time, the authors proved that the HBV genotype C preS1 deletion might significantly contribute to disease progression in chronic HBV subjects by extending the duration of Hepatitis B virus e antigen (HBeAg) seropositive status and increasing HBV replications. This study provides novel insight into the understanding of the higher infectivity and virulence of genotype C compared with other genotypes.

Applications

The FRET-based RT-PCR method for detection of the preS1 deletion developed in this study might hold potential for the early prediction of the risk of liver disease progression in chronic HBV subjects.

Terminology

The FRET-based RT-PCR could be used for molecular epidemiologic purposes. It can permit not only the simultaneous identification of coexisting quasispecies but also the direct identification of target mutations from primary specimens, such as serum through melting curve analyses of the amplification product.

Peer-review

For the first time, the authors discovered the clinical implications of the preS1 start codon deletion *via* a molecular epidemiologic approach using FRET-based RT-PCR, which might significantly contribute to disease progression in chronic HBV subjects infected with genotype C by extending the duration of HBeAg seropositive status and increasing HBV replications. The results are interesting and may explain the higher infectivity and virulence observed in genotype C compared with other genotypes.

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SYSTEMATIC REVIEWS

Anterior rectopexy for full-thickness rectal prolapse: Technical and functional results

Jean-Luc Faucheron, Bertrand Trilling, Edouard Girard, Pierre-Yves Sage, Sandrine Barbois, Fabian Reche

Jean-Luc Faucheron, Bertrand Trilling, Edouard Girard, Pierre-Yves Sage, Sandrine Barbois, Fabian Reche, Colorectal Unit, Department of Surgery, University Hospital, 38043 Grenoble cedex, France

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Data sharing: Technical appendix and dataset are available from the corresponding author at jlfaucheron@chu-grenoble.fr. No additional data are available.

Correspondence to: Jean-Luc Faucheron, MD, Professor of Surgery, Chief, Colorectal Unit, Department of Surgery, University Hospital, TIMC-IMAG Joseph Fourier University, CS 10217, 38043 Grenoble cedex, France. jlfaucheron@chu-grenoble.fr
Telephone: +33-4-76765371
Fax: +33-4-76768780

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Abstract

AIM: To assess effectiveness, complications, recurrence rate, and recent improvements of the anterior rectopexy procedure for treatment of total rectal prolapse.

METHODS: MEDLINE, PubMed, EMBASE, and other relevant database were searched to identify studies. Randomized controlled trials, non-randomized studies and original articles in English language, with more than 10 patients who underwent laparoscopic ventral rectopexy for full-thickness rectal prolapse, with a follow-up over 3 mo were considered for the review.

RESULTS: Twelve non-randomized case series studies with 574 patients were included in the review. No surgical mortality was described. Conversion was needed in 17 cases (2.9%), most often due to difficult adhesiolysis. Twenty eight patients (4.8%) presented with major complications. Seven (1.2%) mesh-related complications were reported. Most frequent complications were urinary tract infection and urinary retention. Mean recurrence rate was 4.7% with a median follow-up of 23 mo. Improvement of constipation ranged from 3%-72% of the patients and worsening or new onset occurred in 0%-20%. Incontinence improved in 31%-84% patients who presented fecal incontinence at various stages. Evaluation of functional score was disparate between studies.

CONCLUSION: Based on the low long-term recurrence rate and favorable outcome data in terms of low *de novo* constipation rate, improvement of anal incontinence, and low complications rate, laparoscopic anterior rectopexy seems to emerge as an efficient procedure for the treatment of patients with total rectal prolapse.

Key words: Total rectal prolapse; Laparoscopy; Anterior rectopexy; Ventral rectopexy; Results; Recurrence; Systematic review

Core tip: Several procedures have been described to correct full-thickness rectal prolapse. They can be separate into abdominal procedures and perineal procedures. Laparoscopic anterior rectopexy has become the procedure of choice for the treatment of total rectal prolapse in many colorectal surgical teams. This review assesses effectiveness, morbidity, recurrence rate, and recent improvements of the technique.

Faucheron JL, Trilling B, Girard E, Sage PY, Barbois S, Reche F. Anterior rectopexy for full-thickness rectal prolapse: Technical and functional results. *World J Gastroenterol* 2015; 21(16): 5049-5055 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/5049.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.5049

INTRODUCTION

Total or complete rectal prolapse is the circumferential full-thickness protrusion of the rectal wall through the anus[1]. The cause of the disease is unknown, but anatomical disturbances are commonly found in patients with total rectal prolapse. These are a straight rectum, a lack of fascial attachments of the rectum against the sacrum, a redundant sigmoid colon, a diastasis of the levator ani, an abnormally deep Douglas pouch, and a patulous anus[2]. Full-thickness rectal prolapse can affect men and women, of any age. However, it is more common in women, reflecting the fact that obstetric injuries are its most common cause[3]. The impact on the quality of life can be very severe. Patients with total rectal prolapse present with a lump at the anal verge, typically after defecation, which may reduce spontaneously or require reduction by digital pressure. This should be distinguished from other causes of a lump, such as mucosal prolapse or hemorrhoids. Many patients report fecal incontinence which can be passive incontinence, urge incontinence, or mucus discharge (soiling). Total rectal prolapse may also cause pain, ulceration, bleeding[4], incarceration[5] and even gangrene. Patients may report a history of slow transit constipation, and/or obstructed defecation syndrome, which is typically characterized by a sensation of incomplete evacuation or of a blockage, hard stools, the need to digitate vaginally, anally, or perianally, straining, repeated (often unsuccessful) visits to the toilet, and anorectal heaviness or even pain, bringing up the problem of a past history of internal rectal prolapse.

Several procedures have been described to correct full-thickness rectal prolapse[6]. The objectives of the surgical treatment are to cure the anatomical abnormality, to cure the accompanying symptoms of incontinence, constipation, and pain, with an acceptable rate of recurrence and the lowest rate of complications.

Two approaches are generally possible to treat the patients. The perineal approach with the Delorme[7] and the Altemeier[8] procedures are less and less proposed to the patients due to the high rate of recurrences. As a result, they are only advocated for patients who are not candidates for an abdominal operation[6]. It is nowadays generally accepted that the abdominal procedures including the rectopexy to the promontory carry a lower recurrence rate and improved functional outcome and are therefore preferred over the perineal operations[9]. Since its first description by Orr in 1953, and the modification introduced by Loygues[10] in 1984, the procedure of rectopexy has evolved through years and has become the procedure of choice in case of total rectal prolapse, but also in cases of other kind of posterior pelvic floor dysfunction such as internal rectal prolapse and enterocele. The aim of this review is to assess the effectiveness, complications, recurrence rate, and recent improvements of the so-called anterior or ventral rectopexy procedure for treatment of total rectal prolapse.

MATERIALS AND METHODS

Specific guidelines outlined in the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) statement have been followed[11]. A systematic review of the literature was performed on the major electronic databases including MEDLINE, PubMed, EMBASE, and the Cochrane Central Register of Controlled Trials. Research keywords syntax was: [(total rectal prolapse) OR (full-thickness rectal prolapse)] AND [(rectopexy) OR (anterior rectopexy) OR (ventral rectopexy)] AND [laparoscopy] AND [(results) OR (technical results) OR (functional results) OR (morbidity)]. The titles and abstracts resulting from the search were screened by two reviewers independently (JLF and BT). The full text versions of the relevant articles were obtained. All references of these articles were carefully screened for any further articles that could have been not identified in the initial search.

Inclusion criteria were: randomized controlled trials, non-randomized studies and original articles in English language, with more than 10 patients, who underwent laparoscopic ventral rectopexy, for full-thickness rectal prolapse, with a follow-up over 3 mo, stating outcome measures of morbidity, functional results (constipation and fecal incontinence), or recurrence, in adult population.

Exclusion criteria were: case reports, editorials, review and meta-analysis, rectopexy associated with colonic resection, perineal approach, any kind of rectopexy for other reason than full-thickness rectal prolapse, and short-term follow-up less than 3 mo. Duplicate reports were identified and excluded from

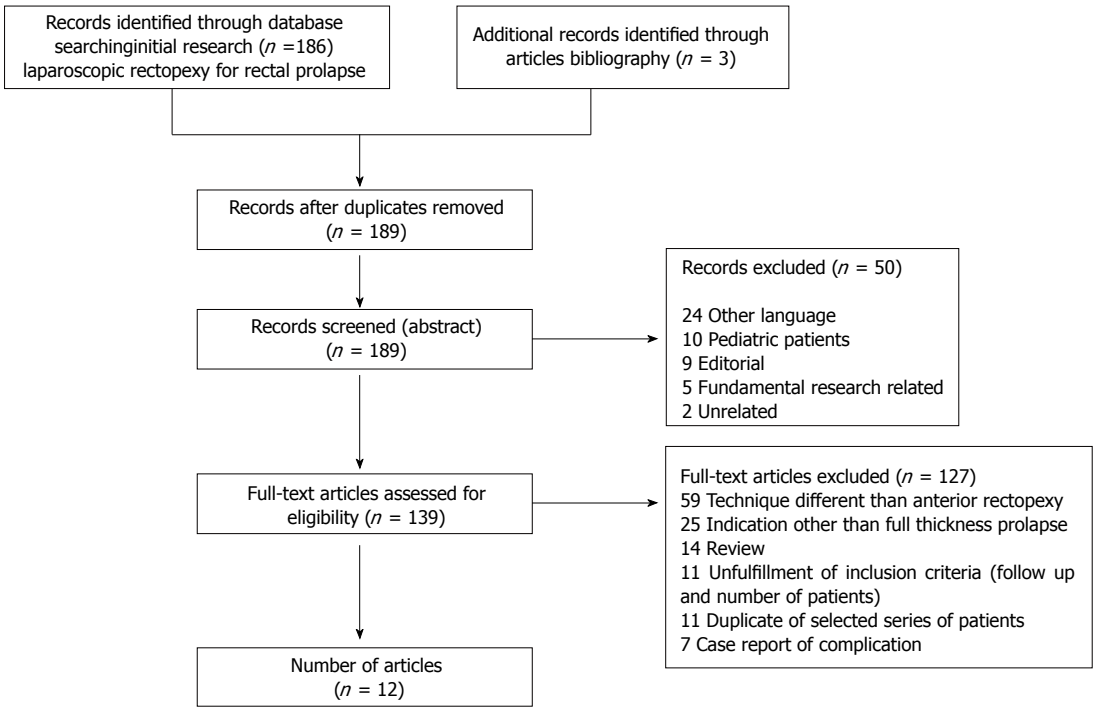

Figure 1 Selection of studies for the review.

this review. Any difference in opinion was resolved by common reading and consensus. Articles that not clearly stated indication, technique and/or outcome were excluded after common second careful reading by the two reviewers.

RESULTS

The database search identified 186 abstracts, to which 4 further papers were added from the references of the corresponding adequate articles. Following application of inclusion and exclusion criteria, 12 non-randomized case series studies with 574 patients who were treated by laparoscopic ventral rectopexy for total rectal prolapse were included in the review (Figure 1)[12-23]. A summary of included studies that gives attention to some technical issues is shown in Table 1.

Morbidity was also evaluated (Figure 2). Complications occur in 100 patients (17.4%). No surgical mortality was described. Conversion was needed in 17 cases (2.9%), most often due to difficult adhesiolysis. Twenty eight patients (4.8%) presented with major complications (Clavien Dindo $\geqslant$ Ⅲ; Figure 3). Seven mesh-related complications (1.2) were reported. Most frequent complications were urinary tract infection and urinary retention.

Mean recurrence rate was 4.7% with a median follow-up of 23 mo. Improvement of constipation range from 3%-72% of the patients and worsening or new onset occurred in 0%-20%. Incontinence improved in 31%-84% patients who presented fecal incontinence at various stages (Table 1). Evaluation of functional score was disparate between studies and it is very difficult to draw conclusions on these data.

DISCUSSION

The abdominal techniques described up to now for the treatment of total rectal prolapse differ in the approach (open versus laparoscopic), extent of rectal mobilization (anterior versus anterior and posterior versus complete mobilization), excision or simple incision of the Douglas pouch peritoneum, methods used for rectal and sacral fixation, type, size, nature and number of meshes used for the pexy, and addition or omission of a sigmoid resection. The wide range of postoperative outcomes we observed in this review can probably be explained by several parameters, one of which being the various modifications of the technique used by the authors.

All the procedures of anterior rectopexy to the promontory derived from the original open technique described by Loygue *et al*[10] in 1984. From 1994, the procedure was proposed through a laparoscopic approach[14,24,25]. The advantages of a laparoscopic rectopexy to exteriorized rectal prolapse are now well documented[14,25-29]. It has been proven as effective as open rectopexy in terms of clinical results, functional results, and recurrence rate. There are significant reductions in postoperative pain, hospital stay, recovery time, return to work, and length of scar. The laparoscopic approach is even cheaper than the open approach[30]. The laparoscopic approach has been the first manner to improve the results of anterior rectopexy.

Complete mobilization of the rectum down to

Table 1 Summary of included studies

Ref.	Year	Surgical technique	Median FU (mo)	Recurrence	Constipation (improvement-worsening/new on set)	Incontinence (improvement-worsening)	Conversion and morbidity
Formijne Jonkers *et al*[12]	2014	Laparoscopic	42	0	159%-W 25%	172%	1 conversion (injury of small bowel), 1 ileus, 2 myocardial infarctions
Germain *et al*[13]	2014	Robotic	51.8	13	167%-W 14%	182%	3 conversions (adhesion, rectal tear, presacral hemorrhage)
van Geluwe *et al*[14]	2013	Laparoscopic	25.3	4.6	171%-W 2.3%	184%	8 conversions (5 adhesiolysis, 3 bleeding), 23 UTI, 2 ileus, 9 hematomas, 6 cardiac problems, 1 bowel perforation, 1 examination under anesthesia, 1 secondary adhesiolysis, 1 strangulation, 5 mesh erosions, 5 port site
Gosselink *et al*[15]	2013	Laparoscopic	12	NS	W 8%	174%-W 11%	7 urinary retentions, 1 UTI, 1 port site infection, 1 mesh erosion
Mäkelä-Kaikkonen *et al*[16]	2013	Laparoscopic (50%) Robotic (50%)	3	2.5	W 2.5%	W 2.5%	1 conversion (bleeding), 1 vaginal perforation, 1 wound infection
Faucheron *et al*[17]	2012	Laparoscopic	75	3	NA	NA	2 brachial plexus palsy, 3 UTI, 1 ureteral lesion, 1 small bowel perforation, 1 mesh erosion
Wijffels *et al*[18]	2011	Laparoscopic	23	3	NA	NA	1 myocardial infarction, 1 small bowel obstruction, 1 wound infection, 3 port site infections, 1 UTI, 1 chest infection

NS: Not significate; NA: Not available.

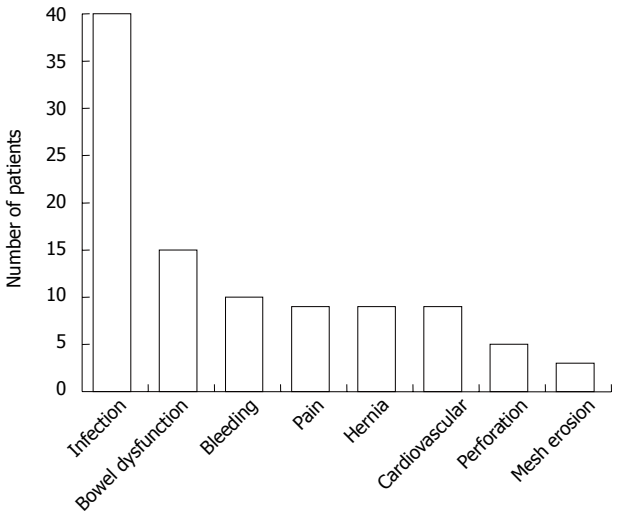

Figure 2 Type of complications. Horizontal axis for complications; Vertical axis for number of patients.

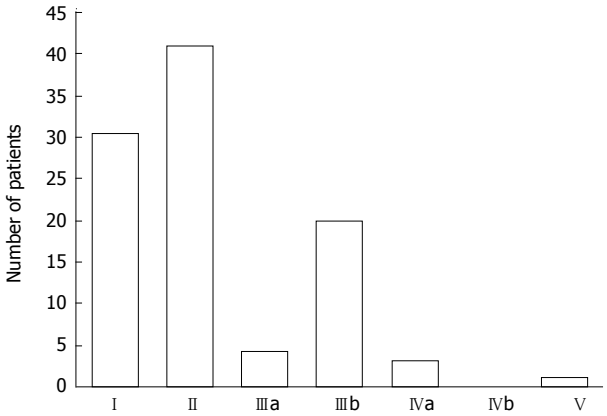

Figure 3 Severity of complications following Dindo Clavien classification. Adapted from Dindo *et al* Classification of surgical complication. *Ann Surg* 2004; 240: 205-213. Horizontal axis for Dindo Clavien grade; Vertical axis for number of patients.

the levator ani muscles, as used in the sutured rectopexy or in the posterior mesh rectopexy has been progressively abandoned by the majority of authors, due to high rate of postoperative constipation and outlet obstruction syndrome[31]. The lateral wings of the rectum contain important autonomic nerves from the pelvic plexus to the rectum[32]. The section or injury (like burning or compression) of these lateral ligaments could explain the more frequent postoperative constipation rate and the more frequent dyschezia rate observed in the surgical techniques involving posterior and posterolateral rectal mobilization, as stated by Bachoo *et al*[33] in their Cochrane review. Some cases of rectal akinesia have been published to be due to complete rectal mobilization during rectopexy[34]. The initially published anterior rectopexy, known as the Orr-Loygue procedure, involved anterior and posterior rectal mobilization to the level of the levator ani muscle, a Douglas pouch removal, and suturing of two meshes on to the anterolateral walls of the rectum and the sacral promontory[10]. In 2004, D'Hoore has described a modification that entails posterior dissection limited to exposure of the sacral promontory, no Douglas pouch excision, and suturing of a 3 centimeters wide

mesh to the ventral aspect of the low rectum[35]. At the same time, we have described another modification of the Orr-Loygue procedure (the main author learned the Orr-Loygue procedure with Parc at Saint-Antoine hospital) that involved exactly the same level of rectal dissection as the D'Hoore technique, but also a Douglas pouch peritoneum excision, the use of two thinner non absorbable meshes that were fixed on to the anterolateral part of the low rectum, and finally the closure of the peritoneum over the meshes to isolate them from the abdominal cavity and to create a shallow neopouch of Douglas[25].

Excision of the peritoneum of the pouch of Douglas was an integral part of the procedure described by Loygue and Parc, for the treatment of patients with total rectal prolapse. The rationale for that was based on the fact that in patients suffering from full-thickness rectal prolapse, one of the anatomical abnormalities is the depth of the Douglas pouch. Subsequently, the excision of the redundant peritoneal cul de sac might well decrease the risk of prolapse recurrence. In our recent published series of 175 consecutive patients with full-thickness rectal prolapse treated laparoscopically with the modification we brought to the Orr-Loygue procedure, the cumulative recurrence rate was 3% at 5 years, which is one of the lowest rate published until now[17].

Debate continues as to which type of mesh fixation is optimum for rectopexy. Up to now, there has been no randomized trial comparing the use of sutures or staples, or tacks for the fixation of the meshes to the rectum or anchorage to the sacral promontory. Laparoscopic rectopexy using mesh fixation with spiked chromium staples has been shown to be feasible and quicker than using sutures[36,37]. In our experience, the choice of the use of staples instead of sutures was based on the fact that the fixation of the meshes was safer (the size of staples avoids any protrusion inside the rectal lumen) and quicker[17]. The use of staplers might well be an improvement in the procedure of laparoscopic anterior rectopexy to the promontory.

Another debate is about which type of mesh is the best for anterior rectopexy. Smart *et al*[38] recently published a systematic review of 13 observational studies reporting outcome of 866 patients undergoing anterior rectopexy. A synthetic mesh had been used in 767 patients and a biological mesh in 99. There was no difference in terms of recurrence (3.7% *vs* 4%, $P = 0.78$) or mesh related complications (7% *vs* 0%, $P = 1.0$). Unfortunately in this review, the difference in the length of median follow-up was different from the synthetic group (up to 74 mo) and the biological group (12 mo). Given the properties and behavior of biological grafts, it is quite likely that with longer follow-up, more recurrences will become evident in patients undergoing laparoscopic ventral rectopexy with this type of implant[39]. Another important drawback of biological grafts is the higher cost when compared to synthetic mesh[40]. A very recent publication from a panel of experts suggests that biological grafts might be a better option in the following circumstances: young patients, women of reproductive age, diabetics, smokers, patients with a history of previous pelvic radiation or sepsis, inflammatory bowel disease, and in cases of intraoperative breach of the rectum or vagina, despite these authors failed to provide any data to support this[41].

In the era of robotic surgery, one could consider the use of robotic-assisted laparoscopic ventral rectopexy as an improvement for the patients presenting with a total rectal prolapse. There is evidence that robotic ventral rectopexy is a feasible and safe procedure and that bowel function may possibly be better, although the number of cases and experience of robotic ventral rectopexy are limited, the methodology of the studies is weak, and the indications for the operation are different from a study to another[16,42-45]. Robotic surgery may be more time consuming and more expensive so that Mäkelä-Kaikkonen *et al*[16] concluded they found no arguments to support the routine use of robotic assistance in rectopexy operations.

The last improvement in the management of patients presenting with full-thickness rectal prolapse might come from the length of the hospital stay. Day-case surgery has been proven efficient in selected patients, reducing the risk of in-hospital complications and cost[17,46].

In literature, there is evidence supporting effectiveness of the Altemeier procedure with similar wide range of recurrence rate depending on surgeons' experience[1,2,4,6,8,9]. Like anterior rectopexy, Altemeier procedure may offer better technical and functional results with technical modifications and ameliorations. Until prospective randomized studies comparing results between these two different approaches will clarify the issue, laparoscopic rectopexy could still not be supported as the gold standard treatment of full-thickness rectal prolapse.

Based on the low long-term recurrence rate and favorable outcome data in terms of low *de novo* constipation rate, improvement of anal incontinence, and low complications rate, laparoscopic anterior rectopexy seems to emerge as an efficient procedure for the treatment of patients with total rectal prolapse. Prospective randomized study comparing this procedure with the Altemeier procedure could answer the question: is the anterior rectopexy the best surgical option to treat full-thickness rectal prolapse?

COMMENTS

Background

Total or complete rectal prolapse is the circumferential full-thickness protrusion of the rectal wall through the anus. The cause of the disease is unknown, but anatomical disturbances are commonly found in patients with total rectal prolapse. These are a straight rectum, a lack of fascial attachments of the

rectum against the sacrum, a redundant sigmoid colon, a diastasis of the levator ani, an abnormally deep Douglas pouch, and a patulous anus.

Research frontiers

Since its first description by Orr in 1953, and the modification introduced by Loygues in 1984, the procedure of rectopexy has evolved through years and has become the procedure of choice in case of total rectal prolapse, but also in cases of other kind of posterior pelvic floor dysfunction such as internal rectal prolapse and enterocele.

Innovations and breakthroughs

To assess the effectiveness, complications, recurrence rate, and recent improvements of the so-called anterior or ventral rectopexy procedure for treatment of total rectal prolapse.

Applications

Based on the low long-term recurrence rate and favorable outcome data in terms of low *de novo* constipation rate, improvement of anal incontinence, and low complications rate, laparoscopic anterior rectopexy seems to emerge as an efficient procedure for the treatment of patients with total rectal prolapse.

Peer-review

This is a well written non comparative, longitudinal review study evaluating effectiveness, complication and recurrence rate of anterior rectopexy for full thickness rectal prolapse.

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Non-physician endoscopists: A systematic review

Maximilian Stephens, Luke F Hourigan, Mark Appleyard, George Ostapowicz, Mark Schoeman, Paul V Desmond, Jane M Andrews, Michael Bourke, David Hewitt, David A Margolin, Gerald J Holtmann

Maximilian Stephens, Luke F Hourigan, Gerald J Holtmann, Department of Gastroenterology and Hepatology, Princess Alexandra Hospital, Woolloongabba QLD 4102, Australia
Maximilian Stephens, Luke F Hourigan, Gerald J Holtmann, Faculty of Medicine and Biomedical Sciences, University of Queensland, Brisbane QLD 4072, Australia
Mark Appleyard, Department of Gastroenterology and Hepatology, RBWH, Herston QLD 4006, Australia
George Ostapowicz, Department of Gastroenterology and Hepatology, Gold Coast Hospital QLD 4215, Australia
Mark Schoeman, Jane M Andrews, Department of Gastroenterology and Hepatology, Royal Adelaide Hospital and University of Adelaide, Adelaide SA 5000, Australia
Paul V Desmond, St Vincent's Hospital, University of Melbourne, Melbourne VIC 3010, Australia
Michael Bourke, Department of Gastroenterology and Hepatology, Westmead Hospital, University of Sydney, Sydney NSW 2145, Australia
David Hewitt, School of Medicine, University of Queensland, Brisbane QLD 4072, Australia
David Hewitt, Department of Gastroenterology, Queensland Elizabeth II Jubilee Hospital, Coopers Plains, Brisbane QLD 4108, Australia
David A Margolin, Department of Colon and Rectal Surgery Ochsner Clinic Foundation, Ochsner Clinical School, University of Queensland, New Orleans LA 70121, United States
Author contributions: Holtmann GJ, Stephens M designed the research; Stephens M, Hourigan LF, Appleyard M, Ostapowicz G, Schoeman M, Desmond PV, Andrews JM, Bourke M, Hewitt D, Margolin DA, Holtmann GJ performed the research; Stephens M, Hourigan LF, Appleyard M, Ostapowicz G, Schoeman M, Desmond PV, Andrews JM, Bourke M, Hewitt D, Margolin DA, Holtmann GJ analyzed the data; Stephens M, Holtmann GJ wrote the paper.

Correspondence to: Gerald J Holtmann, MD, PhD, MBA, Professor, Department of Gastroenterology and Hepatology, Princess Alexandra Hospital, Ipswich Road, Woolloongabba, Brisbane QLD 4102, Australia. gerald_holtmann@health.qld.gov.au
Telephone: +61-7-31767792
Fax: +61-7-31765111
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Abstract

AIM: To examine the available evidence on safety, competency and cost-effectiveness of nursing staff providing gastrointestinal (GI) endoscopy services.

METHODS: The literature was searched for publications reporting nurse endoscopy using several databases and specific search terms. Studies were screened against eligibility criteria and for relevance. Initial searches yielded 74 eligible and relevant articles; 26 of these studies were primary research articles using original datasets relating to the ability of non-physician endoscopists. These publications included a total of 28883 procedures performed by non-physician endoscopists.

RESULTS: The number of publications in the field of non-specialist gastrointestinal endoscopy reached a peak between 1999 and 2001 and has decreased thereafter. 17/26 studies related to flexible sigmoidoscopies, 5 to upper GI endoscopy and 6 to colonoscopy. All studies were from metropolitan centres with nurses working under strict supervision and guidance by specialist gastroenterologists. Geographic distribution of publications showed the majority of research was conducted in the United States (43%), the United Kingdom (39%) and the Netherlands (7%). Most studies conclude that after appropriate training nurse

endoscopists safely perform procedures. However, in relation to endoscopic competency, safety or patient satisfaction, all studies had major methodological limitations. Patients were often not randomized (21/26 studies) and not appropriately controlled. In relation to cost-efficiency, nurse endoscopists were less cost-effective per procedure at year 1 when compared to services provided by physicians, due largely to the increased need for subsequent endoscopies, specialist follow-up and primary care consultations.

CONCLUSION: Contrary to general beliefs, endoscopic services provided by nurse endoscopists are not more cost effective compared to standard service models and evidence suggests the opposite. Overall significant shortcomings and biases limit the validity and generalizability of studies that have explored safety and quality of services delivered by non-medical endoscopists.

Key words: Nurse endoscopist; Cost-benefit; Service model; Patient satisfaction; Outcome parameter

Core tip: A systematic review was performed to examine the available evidence on safety, competency and cost-effectiveness of nursing staff providing gastrointestinal endoscopy services. Most studies conclude that after appropriate training nurse endoscopists safely perform procedures. Contrary to general beliefs, endoscopic services provided by nurse endoscopists are not more cost effective compared to standard service models due largely to the increased need for subsequent endoscopies, specialist follow-up and primary care consultations. The empirical evidence that supports non-physician endoscopists is limited to strictly supervised roles in larger metropolitan settings and mainly flexible sigmoidoscopy and upper endoscopy for asymptomatic or low complexity patients.

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INTRODUCTION

Nurse endoscopy training and delivery of endoscopic services was first reported in the United States more than 35 years ago for flexible sigmoidoscopy (FS)[1]. Several studies soon emerged, confirming that nursing staff with appropriate training and supervision could adequately perform endoscopic procedures such as FS. Other studies have since established that nurse endoscopists can safely perform upper endoscopies and colonoscopies. In an era where safe, yet cost-effective, policies run at the forefront of stakeholders' minds, the question of how nursing staff can undertake additional roles in the endoscopic suites is continually raised. After more than 35 years, it appears timely to review the available evidence surrounding the role of nurse endoscopists. To our knowledge, there have been no systematic reviews of the literature that have examined the full body of evidence surrounding uptake, safety, accuracy and most importantly, the cost-effectiveness of nurse endoscopists.

MATERIALS AND METHODS

Skill of endoscopy

Competent endoscopy has both procedural (manual dexterity) and cognitive aspects. Procedural skills refer to the ability of endoscopists to insert/withdraw the endoscope, navigate the alimentary tract with acceptable views and perform further actions such as biopsy, polypectomy or other interventions. Procedural (technical) competence necessitates that these actions are executed in a timely manner that exposes the patient to acceptable risk of complications. Procedural or technical skill is generally measured by direct observation of the procedure[2] alongside several quality indicators such as overall procedural time, caecal intubation time, caecal intubation rate, polyp/adenoma detection rate, depth of insertion, adequacy of views on review of video footage, rate of complications and patient satisfaction.

On the other hand, cognitive (or non-technical) skill refers to the ability of endoscopists to perform more complex tasks beyond the procedure itself. These include: (1) recognizing and interpreting gross pathology; (2) interpreting the patient's clinical picture in relation to endoscopic findings; (3) understanding the patient's current clinical risk and how this could change with/without further endoscopic treatment; (4) knowledge of any viable alternatives to endoscopic procedures that could better serve the patient; (5) recommending treatments/further investigations appropriate to the severity of pathology seen; and finally; and (6) understanding the indications and contraindications for the proposed procedure.

These cognitive elements are important during the composition of the report and any follow-up by the endoscopists, as well as during the procedure itself. By their nature, the cognitive aspects of endoscopy do not lend themselves towards easy measurement, especially when comparing competence between study groups. Assessment of endoscopists' reports by consultant gastroenterologists (gold standard) after review of video footage has been performed with some merit, but assessment is often subjective and limited to measuring sensitivity and specificity of detecting

simple gross pathology[3,4].

Physician and surgical trainees in gastroenterology and surgical specialities undergo extensive training before providing independent endoscopic services. This training addresses both the procedural and cognitive aspects of endoscopy, with procedural competency being obtained more rapidly compared to cognitive competency, which requires years of comprehensive clinical training. Indeed, many studies have shown that a high level of procedural competency for FS is typically achieved after around 100-200 supervised procedures, with higher numbers needed for colonoscopy/upper endoscopy.

This applies regardless of whether the trainees are junior doctors, advanced trainees, fellows, or non-physician personnel[5-13]. It is unlikely however, that nurses and other non-physician endoscopists routinely acquire the same level of cognitive competency as physician endoscopists or other medically qualified and trained staff. This cognitive aspect, intrinsic to the procedure, requires the ability to make a well-reasoned decision within the context of the patient's full clinical picture, to take responsibility for decisions made, to manage efficiently complications and to guide subsequent follow-up. This is a style of thinking which is fostered in physicians through years of training, yet generally not taught in nursing education, which tends to be more protocol based. On the other hand it might be argued that these cognitive skills are not required to perform a simple procedure, which requires a clearly defined but limited set of skills. However, from the perspective of the healthcare system as a whole, one needs to examine not only the actual procedure, but also all costs involved in its delivery, and this necessarily includes an evaluation of cost-effectiveness of the entire "episode of care".

This systematic review aims to assess the current evidence to support the provision of diagnostic or therapeutic endoscopic services by trained non-physicians including nurses, and the evidence regarding cost-effective delivery of services that meet patient needs. We specifically aim to assess: (1) the evidence that non-physician endoscopists can acquire the required procedural and cognitive skills to deliver endoscopic services; (2) the cost-benefit analysis of procedures performed by nurses and other non-physicians as compared to medical and surgical specialists, and (3) characterize the service models that have been used so far.

Search strategy

We devised the following Boolean search terms: nurse and endoscopist ("nurse and performed" or "delivered") or "nurse practitioner" and ("endoscopy" or "colonoscopy" or "flexible sigmoidoscopy" or "esophagogastroduodenoscopy"); ("non-medical" or "non-physician" or "non-medical" or "non-physician" or "physician assistant") and "endoscopy". We searched each of the following electronic databases using each of the above search terms individually: Cochrane Library; MEDLINE; CINAHL; Google Scholar. The most recent search was performed January 2014.

Eligibility and relevance

To be deemed eligible and relevant for inclusion in the review, studies/articles must have described or referred to non-medical personnel (*i.e.,* nurses, physicians assistants, technicians, non-medical personnel or those not practicing as a doctor) being the primary proceduralists for any form of gastrointestinal endoscopy. Non-medical personnel needed to be included in the review as there is discrepancy in the terminology in literature. Studies that were solely focused on capsule endoscopy or nurses assisting in placing percutaneous endoscopic gastrostomy tubes were excluded from the systematic search part of the review. Studies were excluded if they were not published in a peer-reviewed journal. All languages and age ranges were included. There were no date limits.

Data extraction

For all eligible studies, any entries that were identified as "comments" on other articles were removed from any analyses. The remaining articles were stratified into "primary research measuring endoscopists' performance" or "other". For each primary research study, the following information was extracted: author names and year of publication, country of origin, total number of procedures performed by nurses/non-medical endoscopists, degree of supervision of endoscopists, clinical setting, whether there was true randomization of patients, number and type of proceduralists, potential biases, outcome parameters used by the study and important outcomes. If studies referred to other pieces of research or articles that were not discovered by the systematic search, but were eligible and relevant, they were also included in the review.

Statistical analysis

The number of publications (primary research or otherwise) by year, publications by country and total non-physician procedures reported were assessed. The data were extracted from the identified primary research articles independently by two of the authors (MS and GH). These data were compared and discrepancies assessed and agreement reached when required. The review of the data also included an assessment of the overall quality of the studies. For this assessment, key criteria such as randomization, concealment of randomization, the risk of selective reporting and other relevant indicators of bias[14] and study quality were assessed with the results tabulated in keeping with the Cochrane Collaboration's Risk of Bias Assessment. A meta-analysis of the data was

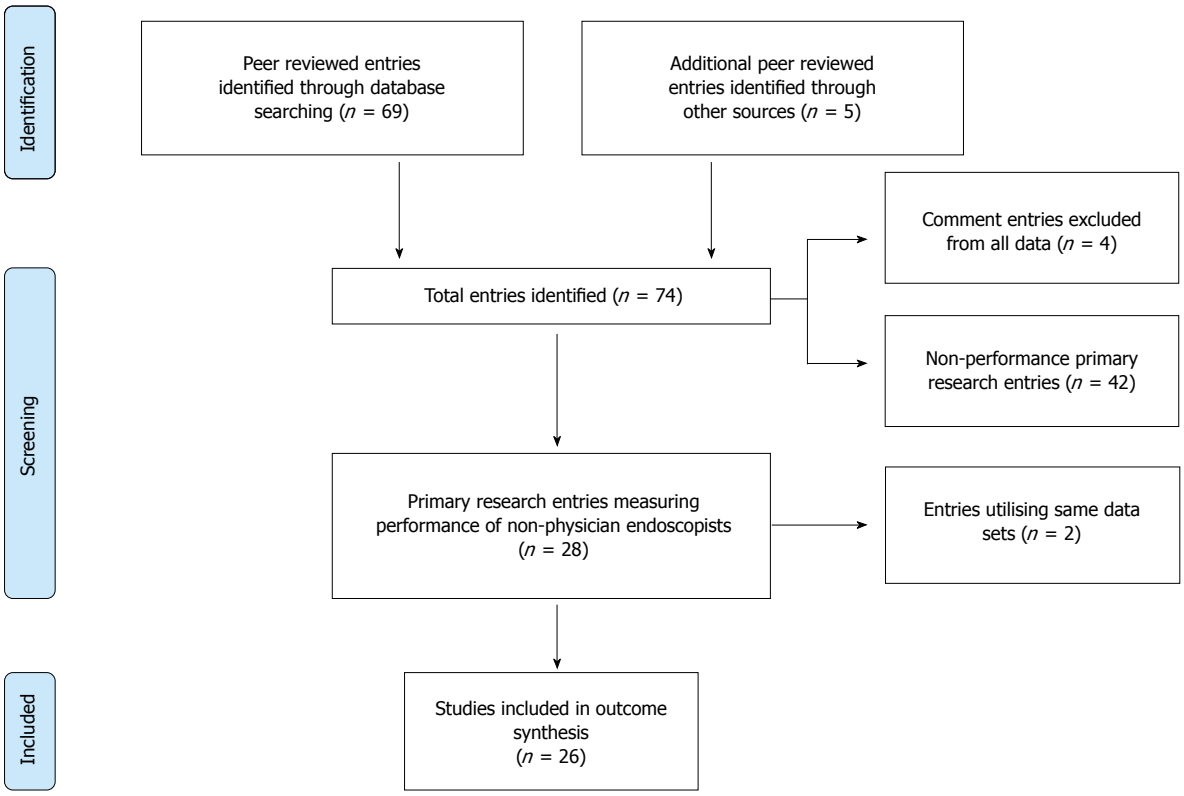

Figure 1 Flow chart of search methodology and article selection.

not possible due to the heterogeneity of the studies. Where original data were re-reported in second or third publications, only the original data were included.

RESULTS

The literature search identified 74 publications, of which 4 were identified as "comments" and removed from any further analysis. Publications were dated between March 1977 and September 2013. In these 70 studies, a total of 28883 non-physician endoscopies had been documented. Twenty eight (40%) were identified as primary research measuring endoscopists' performance with definable outcome parameters. Two studies re-reported original data and excluded leaving 26 studies suitable for further analysis (Figure 1 and Table 1). Of these primary research studies there were 17476 documented non-physician endoscopies (n = 17476).

Quality indicators

The key quality indicators of the studies are summarized in Table 2. There was a widespread lack of randomization or blinding of concealment. In addition some of these open, uncontrolled and unblinded studies[5-7,10,12,15-18] appear to represent descriptive "verifications" of training programs that were developed. Overall the quality of these studies was very limited.

Publication activity and geographic distribution

Publication activity was quantified using all 70 published papers and did not include "comment" publications. Publication activity graphed by 3-year periods (Figure 2) demonstrates a rapid increase in publications between 1990 and 2001, peaking at 15 articles for the 3-year period 1999 to 2001. Over the past 9 years, the rate has slowly decreased with fewer than 10 publications in the last 3 years. This decline is mirrored by a decline in documented procedures performed by non-physician endoscopists (Figure 3). Of note, 96% (2030/2080) of documented procedures, and 75% (3/4) of original datasets in the last 3 years have come from one (Dutch) research group. The overall geographic distribution of publication activity is demonstrated in Figure 4.

Non-physician endoscopy performance measures and comparisons, service models and cost-efficiency models

Twenty eight articles were identified in literature that measured the performance of non-physician endoscopists (Table 1). Of these, Williams *et al*[19], Richardson *et al*[20] and Williams *et al*[21] used the same datasets leaving 26 studies suitable for analysis. Seventeen out of 26 original datasets related to FSs (n = 12218), 5 to upper GI endoscopy (n = 2150) and 6 studies to colonoscopy (n = 2559). Several datasets related to 2 types of procedures. 19% of all datasets

Table 1 Summary table of all competency research regarding medical and nurse and other non-physician endoscopists

Publication	Setting	Non-physician proce-dures	Supervision	True randomization of patients?	Procedurists	Potential biases	Outcome parameters	Modality	Outcome
Rosevelt *et al*[8], 1984	Metropolitan Tertiary Centre, United States	825	Yes	No	1 NE	Patient selection bias Endoscopist selection bias Level of assistance not documented	Polyp detection rate Complications	FS	Polyp detection rate of 8.7% No complications
Schroy *et al*[4], 1988	Metropolitan Tertiary Centre, United States	100	Yes	No	1 NE	Patient selection bias Endoscopist selection bias Level of assistance not documented Concordance criteria not given	Polyp detection rate Concordance of findings with expert opinion	FS	Polyp detection rate of 36% Video review showed k = 0.72 concordance with GC Nurse sensitivity = 75%, specificity = 94%
DiSario *et al*[15], 1993	Metropolitan Tertiary Centre, United States	80	Yes	Yes	5 NEs 5 GRs	Endoscopist selection bias Level of assistance not documented 1 NE excluded due to incompetency after training Comparison of outcome groups for missed lesions not calculated	Insertion depth Identification of anatomy Complications Procedure time Missed lesions	FS	Polyp detection rate of 24% Insertion depth, complications and procedure time similar between groups NEs missed more lesions and missed more anatomy
Maule[7], 1994	Metropolitan Tertiary Centre, United States	1881	Yes	No	4 NEs 2 GCs	Endoscopist selection bias Complicated patient referred away from NEs Level of assistance not documented	Insertion depth Complications Polyp detection rate Patient satisfaction	FS	GCs had significantly deeper insertion depths Similar polyp detection rate Similar patient satisfaction
Moshakis *et al*[16], 1996	Metropolitan Tertiary Centre, United Kingdom	50	Yes	No	1 NE 1 GC	Endoscopist selection bias Patient selection bias NE was compared to GC who performed training Level of assistance not documented Method of quality scoring not given	Insertion depth "Quality and accuracy"	FS	Insertion depth, quality and accuracy were similar between comparison groups
Duthie *et al*[6], 1998	Metropolitan Tertiary Centre, United Kingdom	205	Not specified	No	1 NE	Endoscopist selection bias Patient selection bias Criteria for "successful procedure" not given	Successful procedure when compared to various other imaging modalities	FS	93% of procedures considered "successful"
Schoenfeld, Cash *et al*[17], 1999	Metropolitan Tertiary Centre, United States	114	Yes	No	1 NE 3 GFs 3 Surgical Consultants	Endoscopist selection bias Patient selection bias Level of assistance not documented	Depth of insertion Procedure time Polyp detection rate Patient satisfaction Complications	FS	Surgeons had less depth of insertion than NEs or GFs NEs had longer procedures than GFs or surgeons Polyp detection rate similar No complications
Schoenfeld, Lipscomb *et al*[10], 1999	Metropolitan Tertiary Centre, United States	151	Yes	Yes	3 NEs 4 GCs	Endoscopist selection bias Patient selection bias Level of assistance not documented High threshold for detecting difference in polyp detection rate	Polyp detection rate Depth of insertion Complications	FS	Polyp detection rates similar between groups (43%-45%) GCs had much greater depth of insertion No complications
Wallace *et al*[9], 1999	Metropolitan Tertiary Centre, United States	2323	Yes	No	1 NE 2 PAs 15 GCs	Endoscopist selection bias Patient selection bias Level of assistance not documented	Depth of insertion Polyp detection rate Complications	FS	GCs had significantly greater depths of insertion compared with NE+PAs Polyp detection rate similar between groups (23%-27%) No complications

Schoen *et al*[26], 2000	Metropolitan Tertiary Centre, United States	660	Not specified	No	1 PE 1 GC 1 MC	Endoscopist selection bias Patient selection bias Level of assistance not documented	Patient satisfaction	FS	Similar patient satisfaction between groups
Shapero *et al*[27], 2001	Metropolitan Tertiary Centre, Canada	488	No	No	2 NEs	Endoscopist selection bias Patient selection bias Level of assistance not documented	Polyp detection rate Complications Depth of insertion Procedure time	FS	Average depth of insertion 52.9 cm 8.4 min average procedure time Polyp detection rate of 15.4%
Jain *et al*[28], 2002	Metropolitan Tertiary Centre, United States	5000	No	No	Not specified No physicians	Unknown endoscopists Patient selection bias Level of assistance not documented No comparison	Polyp detection rate No complications	FS	No major complications polyp detection rate of 13.3%
Meenan *et al*[29], 2003	Metropolitan Tertiary Centre, United Kingdom	25	Yes	No	1 NE 4 GFs	Endoscopist selection bias Patient selection bias Level of assistance not documented Views by NE were limited to the esophagus	Adequacy of views obtain by radial ultrasound by endoscopy	EGD	NEs had consistently lower quality scores
Smale *et al*[30], 2003	Metropolitan Tertiary Centre, United Kingdom	1487	Yes	No	2 NEs 15 mixed medical/ surgical physicians	Endoscopist selection bias Patient selection bias Level of assistance not documented Complicated patients excluded Retrospective and prospective	Differences in sedation Patient satisfaction	EGD	No difference between groups for sedation or patient satisfaction Subjectively, nurses reporter fewer studies as normal
Wildi *et al*[22], 2003	Metropolitan Tertiary Centre, United States	40	Yes	No	1 NE 1GC	Endoscopist selection bias Patient selection bias Level of assistance not documented Method of assessment not given	Concordance of findings with GC	EGD	NE had sensitivity of 75% and specificity of 98% with GC as gold standard
Nielsen *et al*[12], 2005	Metropolitan Tertiary Centre, Denmark	69	Yes	No	2 NEs Unknown number of physicians	Endoscopist selection bias Patient selection bias Level of assistance not documented	Patient satisfaction	FS	Nurses had better patient satisfaction than physicians
Meining *et al*[3], 2007	Metropolitan Tertiary Centre, United Kingdom	190	Yes	Yes	2 NEs 1 GC 2 GRs 1 Physician not specified 1 MC	Endoscopist selection bias Patient selection bias Level of assistance not documented Subjectivey, nurses tended to focus on the entire examination whilst physicians focused on the reason for referral	Adequacy of views for entire procedure Duration of procedure Use of sedation	EGD	Nurses had twice the amount of adequate views however took twice as long on average. Nurses used sedation more frequently
Williams *et al*[19], 2006 Williams *et al*[21], 2009	Nation-wide Metropolitan Tertiary Centres, United Kingdom	957	Yes	Yes	30 NEs 67 physicians (not specified)	Significantly higher numbers of patients changed schedule from physician to nurse (due	Patient satisfaction Adequacy of views Depth of insertion Endoscopic procedures performed Duration of examination Complications	EGD	Patient satisfaction favoured nurses No difference for depth of insertion Nurses took biopsies for histology in upper endoscopy and FS more frequently than physicians. There were more normal histology findings for nurses

Richardson *et al*[20], 2009							Need for assistance Need for subsequent follow up and investigation Cost-benefit analysis		Nurses were more likely to report sedation and procedural details whilst physicians were more likely to report diagnosis and suggested treatment. Nurses took biopsies for *H. pylori* more frequently No major differences in final diagnoses frequency between 2 groups. No serious complications Similar need for assistance. Nurses had greater follow-up cost per procedure whilst physicians had greater labor costs per procedure. Physicians had greater overall costs per procedure but greater patient improvement. Physicians were 87% more likely to be cost-effective than nurse endoscopists.
Koornstra *et al*[11], 2009	Metropolitan Tertiary Centre, Netherlands	300	Yes	No	2 NEs 1 GF 1 GC	Endoscopist selection bias Patient selection bias Level of assistance not documented	Caecal intubation rate Caecal intubation time Complications Patient satisfaction	Col	Similar caecal intubation rates/times (80%-90%) between GF and NEs but much lower/longer than GC after 150 procedures. Patient satisfaction similar between GF and NEs, less than for GC. Similar complication rate.
Maslekar, Hughes *et al*[31], 2010	Metropolitan Tertiary Centre, United Kingdom	308	Not specified	No	1 NE 1 PA/T Several physicians not specified	Endoscopist selection bias Patient selection bias Level of assistance not documented NE and PA/Ts had less colonoscopies more FS	Complications Patient satisfaction	Col and FS	No difference between all 3 groups
Maslekar, Waudby *et al*[32], 2010	Metropolitan Tertiary Centre, United Kingdom	26	Yes	No	1 Surgical Registrar 1 PA/T	Endoscopist selection bias Patient selection bias Level of assistance not documented Patients needing resection excluded	Accuracy of endoscopists to gauge position in colon	FS	PA/T accuracy of 70% with Registrars accuracy of 80%, not statistically significant.
Shum *et al*[18], 2010	Metropolitan Tertiary Centre, HK	119	Yes	No	1 NE	No comparison group	Mean procedure time Depth of insertion Complications	FS	9.4 min average procedure time 53.5 cm average depth of insertion No major complications
Limoges-Gonzalez *et al*[44], 2011	Metropolitan Endoscopy Centre, United States	50	Yes	Yes	1 NE 2 GCs	Endoscopist selection bias Level of assistance not documented	Adenoma detection rate Caecal intubation rate Caecal intubation time Patient satisfaction Sedation use Complications	Col	Adenoma detection rate higher in NE (42%) than GCs (17%) All other parameters similar across both groups
de Jonge *et al*[33], 2012	Multi-metropolitan tertiary centre, Netherlands	162	Not specified	No (retrospective)	6 NEs 113 Staff not specified including GCs, GFs, surgeons, MCs	Data was retrospective review of reports	Overall caecal intubation rate Adenoma detection rate	Col	NEs and GFs and GCs found more adenomas and had greater caecal intubation rates (94%) than nongastroenterology staff, especially surgical

van Putten *et al*[34], 2012	Multi-metropolitan tertiary centre, Netherlands	1000	Yes	No	10 NEs	Endoscopist selection bias Patient selection bias	Unassisted caecal intubation rate Withdrawal time Adenoma detection rate Assistance requirements Patient satisfaction Complications	Col	Unassisted caecal intubation rate of 94% 23% of colonoscopies required assistance from GC Withdrawal time of 10 min Adenoma detection rate of 23% 1 perforation and 1 onset of atrial fibrillation 95% of patients satisfied with procedure
Massl *et al*[5], 2013	Multi-metropolitan tertiary centre, Netherlands	866	Yes	No	7 NEs 8 GFs	Endoscopist selection bias NEs had significantly lower ASA scores on patients Level of assistance not reported	Unassisted caecal intubation rate Caecal intubation time Complications Polyp detection rate	Col	Unassisted caecal intubation rate was significantly lower 77% for NE than GFs (88%). Polyp detection rate (45%), complications, withdrawal and intubation times were similar between groups. Crude cost-analysis showed a saving of €7.61 per colonoscopy where 1 GC supervises 3 NEs. Did not account for higher need for repeat colonoscopies due to incomplete procedures

Col: Colonoscopy; EGD: Esophagogastroduodenoscopy; FS: Flexible sigmoidoscopy; NE: Nurse endoscopist; GF: Gastroenterology fellow; GC: Gastroenterology consultant (attending); GR: Gastroenterology registrar/resident; PA/T: Physician's assistant or technician; MC: Medical (non-gastroenterology) consultant.

were from randomized patients (n = 1428); 64% of the original datasets were created utilizing $\leqslant$ 2 non-physician endoscopists (n = 5056). Eighty seven of the 91 (96%) non-physician endoscopists were nurse endoscopists. All studies were from centres in metropolitan regions with nurses working under strict supervision and guidance of gastroenterologists. It is important to note that all studies were conducted at tertiary hospitals or university centres in metropolitan locations. There are no data on unsupervised practice outside these closely monitored and well-defined settings.

Studies varied with regard to measures of procedural competence and outcome, however most used polyp/adenoma detection rate (FS and colonoscopy), insertion depth (FS), complications, patient satisfaction, procedure time and caecal intubation time/rate (colonoscopy). Studies attempting to measure cognitive competence were limited to Schroy *et al*[4]; Moshakis *et al*[16] and Wildi *et al*[22]. Williams *et al*[19] assessed post-procedural reports but not for content, only for the presence of specific details included on the report.

The available data do not allow a direct comparison or meta-analysis of the procedural performance of medical specialist and nurse endoscopists since the assessments were not standardized. In addition, substantial methodological limitations reduce the validity of results. For example, in studies comparing nurse and medical or surgical endoscopists, patients were seldom randomly allocated. However, based upon the data presented it appears reasonable to conclude that after appropriate training nurse endoscopists (and other non-medical staff) can perform specific endoscopic procedures under appropriate supervision by a medical or surgical specialist and within well-defined settings.

Cost-efficiency

Genuine cost-benefit analyses of non-physician endoscopists are scarce in the literature, although many articles claim non-physician endoscopists having a high likelihood of providing a cheaper per-procedure service. A crude cost-benefit analysis by Massl *et al*[5] suggested that a total saving of €7.61 (about USD10.39) per procedure could be achieved with a model of 1 senior gastroenterologist supervising 3 active nurse endoscopists. However these costs did not account for the total episode of care including follow-up procedures, follow-up consultations, histology and other factors. This is important given the likelihood that a procedure performed by a nurse endoscopist will require further follow-up for disease management. Indeed, a cost-benefit analysis from the United Kingdom took most of these factors into consideration in a national randomized National Health Service (NHS) based dataset[19-21] where 30 experienced nurse endoscopists performed both upper endoscopies and FS, but not colonoscopy (957 non-physician procedures). While physicians were more expensive per hour (£1.82/min *vs* £0.53/min), nurse endoscopists had significantly increased need for subsequent endoscopy, specialist follow-up and primary care follow-up at 1 year following their procedures. This lead to higher overall

Table 2 Assessment of the risk of bias assessments for studies included into this systematic review and key characteristics of data analysis

Publication	Sequence generation	Allocation concealment	Blinding of participants, personnel and outcome assessors	Incomplete outcome data	Selective outcome reporting	Other sources of bias	Study hypothesis and power calculation
Rosevelt[8], 1984	No randomization	No	No	Not specified	Likely, report was intended to describe a successful training program		No hypothesis, no statistics
Schroy *et al*[4], 1988	No randomization, review of videotape	No	No	Not specified	Report of an established service model. Review of videotapes Quality assurance		No statistics
DiSario[15], 1993	Computer generated randomization	Not specified	No	Not specified	Aim was to demonstrate that ".registered nurses could be trained to perform the flexible sigmoidoscopy in a similar to resident physicians'		Not powered to demonstrate equivalence, no formal power calculations
Maule[7], 1994	No randomization	Not specified	No	Not specified	The study was done to confirm that training of nurse endoscopists is feasible.		Hypothesis defined (no difference), no power calculation for equivalence study, oucome parameters not specified a priori
Moshakis *et al*[16], 1996	No randomization, no comparator	Not specified	No	Not specified	Report describes the successful training of one (1) nurse endoscopist		No hypotheses, no statistical analysis
Duthie *et al*[6], 1998	No randomization	No	No	Not specified	Evaluation of a training program that was developed and implemented by the authors (self-fulfilling prophecy)	Not evident	No hypothesis, no power calculation
Schoenfeld *et al*[17], 1999	No randomization, patients allocated to the 'first available provider'	No	No	Not specified	No evidence	Not evident	Several outcome parameters specified, but no hypothesis tested, no power calculation for equivalence.
Schoenfeld *et al*[10], 1999	Randomization of veterans referred for flexible sigmoidoscopy. Computer generated randomization	No	unknown	Not specified	Justifies the implemented clinical service model.		Several outcome parameters listed, but no specific hypothesis, power calculation provided (to identify differences, but not targeting equivalence)

Wallace *et al*[9], 1999	No randomization, nurse-coordinator assigned eligible patients to a physician or non-physician endoscopists based upon 'daily staffing assignments and patient time preference'	No	Unknown	Not specified	Justifies the implemented clinical service model.		No hypothesis stated, no power calculation
Schoen *et al*[26], 2000	No randomization	No	No	Not specified	Study targeted to demonstrate the good tolerability of flexible sigmoidoscopy	Gender distribution of patients was not equivalent across examiners, and the nurse practitioner did not have trainees working with her.	No hypothesis stated, no proper power calculation
Shapero *et al*[27], 2001	No randomization, allocation not clear	No	No	Not specified	Data justify the implemented clinical practice	Data are generated in the setting of CRC screening with flexible sigmoidoscopy, highly selective cohort.	Not done
Jain *et al*[28], 2002	No randomization	No	No	Not specified	Justification of implemented clinical practice	CRC screening utilizing flexible sigmoidoscopy, selective cohort	Not done
Meenan *et al*[29], 2003	No randomization	No	No	Not specified	Assessement of training progress		Not done
Smale *et al*[30], 2003	No randomization, part one retrospective analysis of endoscopy data base, second part prospective data collection	No	No	Not specified	Review and justification of clinical practice		Not done
Wildi *et al*[22], 2003	No randomization	No	No	Not specified	Sequential procedures Nurse endoscopist followed by physyician, potential effect of sequence.		Not done
Nielsen *et al*[12], 2005	No randomization	No	No	Not specified	Quality assurance of existing training program		Not done
Meining *et al*[3], 2007	No details in relation to the randomization process are provided. Patients unequally allocated to endoscopist or nurse	No	No	Reported but uneven numbers of 'Randomization failures (33 *vs* 0). Considerable number of patients excluded (only 367 out of 641 reported)	Review and justification of clinical practice	Primary outcome parameter was stated as "appropriate diagnosis", this outcome parameter was not reported.	Not done
Williams *et al*[19], 2006 Williams *et al*[21], 2009 Richardson *et al*[20], 2009	Randomization of patients to nurse or physician endoscopy	No	No	Properly reported	Primary outcome parameter not related to endoscopic. Measured with Gastrointestinal Symptoms Rating scale up to one year after procedure	Only patients suitable to be serviced by nurse endoscopists included. Numerically more patients from the nurse cohort were lost of follow-up without specified reasons (286 *vs* 269). A trend for more patients with weight loss in the physicians cohort, more patients in the physicians'' cohort had previously barium enema (suggesting more chronic or relapsing symptoms)	Authors make reference to required sample sizes. Total number of patients completed was below the required sample size
Koornstra *et al*[11], 2009	It is stated that patients were randomly allocated, no information is given on allocation. Proportion of inpatients lower in the nurse group. No evidence for ethic approval or consent of patients. Training of nurse and medical staff was not identical	No	No	No information provided	Multiple endpoints reported	The authors developed a training program and with their data they aimed to confirm that their training program delivered (self-fulfilling prophecy).	Not powered to verify equivalence

Maslekar *et al*[31], 2010	Patients were allocated by administrative staff into the nurse or medical specialist group.	No	No	Incomplete response data cited as reason for exlusion (48/561 excluded), no intention to treat analysis	Study justifies an implemented service model that aims to address shortage of medical specialists	The instrument was unlikely to detect group differences. Variable mixture of flexible sigmoidoiscopy and colonoscopy across groups	No power calculation
Maslekar *et al*[32], 2010	No randomization	No information given	No	Not reported	Justifies implemented service and training model	For flexible sigmoidoscopies the validity of the endoscopists impression of maximal extension was tested. A priori unlikely to identify difference.	No power calculation
Shum *et al*[18], 2010	No randomizatiom, no comparator	No	No	No information provided	Justifies the implemented training model		No
Limoges-Gonzalez *et al*[44], 2011	It is stated that patients were randomly allocated, no information is given on allocation.	No	No	No information provided	Justifies the implemented service model	Postprocedure questionnaire was administered after (at least) 30 min of recovery. Drug effects likely to blunt potential differences	No power calculation
de Jonge *et al*[33], 2012	Routine quality data were used, no randomization.	No	No	No information provided	Data were partly retrospective data, partly prospective data, no justification given.	?	No power calculation
van Putten *et al*[34], 2012	Allocation of patients by secretatial staff, no randomization	No	Not reported	Not specified	Justifies and implemented service model	Significant differences in comorbidities (more severe in the Gastroenterologists group), differences in source of referral. Outcome assessment limited to immediate salary comparisons not total costs including pathology and follow-up.	No power calculation
Massl *et al*[5], 2013	It is stated that patients allocated by administrative staff, endoscopists assigned to lists randomly based on availability	No	No	79/2025 procedures not included due to drop out of 1 nurse endoscopist for unspecified reasons	Justifies the implemented service model	Patients younger than 18 years or referred for therapeutic procedures were excluded from the nurse endoscopist group only. Drop out of nurse endoscopist not justified.	Power calculation done. Appropriate numbers achieved.

Red cell = high risk of bias, yellow cell = unclear risk of bias, green cell = low risk of bias. CRC: Colorectal cancer.

costs being calculated as £53 per procedure in the non-physician group. They also found that patients who underwent a physician procedure had on average a greater gain of 0.0153 quality adjusted life years, based on responses from patient surveys. Their final conclusion was that at a price of £30,000 per QALY, the accepted NHS value, physicians were 87% more likely to be cost-effective 1 year after upper endoscopy or FS compared to nurse endoscopists.

DISCUSSION

The role of the nurse endoscopist initially arose to meet the burgeoning demand for endoscopies in the face of a severe shortage of physician endoscopists, first in the United States and later in the United Kingdom. To this day, the demand for endoscopic procedures continues to rise, due largely to the demographic changes associated with an ageing population[23,24] and the widespread introduction of colorectal cancer screening programs[25]. Additionally, it has been suggested that non-physician endoscopists could contribute to the much-needed provision of endoscopic services in the underserved rural and remote locations. Thus this systematic review was

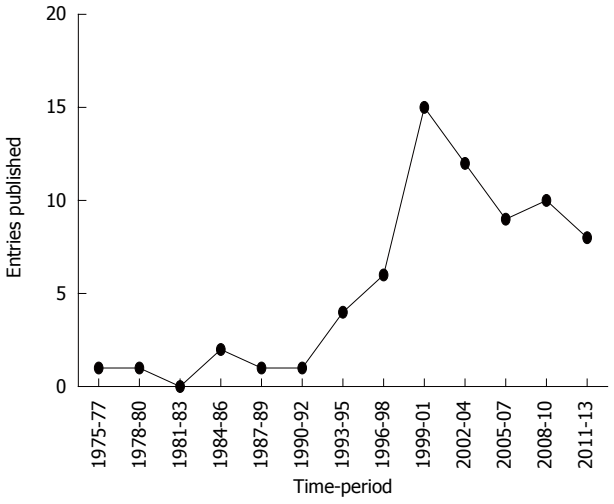

Figure 2 Number of peer reviewed publications since 1975. The number of publications peaked in 1991-2001 and continues to decline since that time.

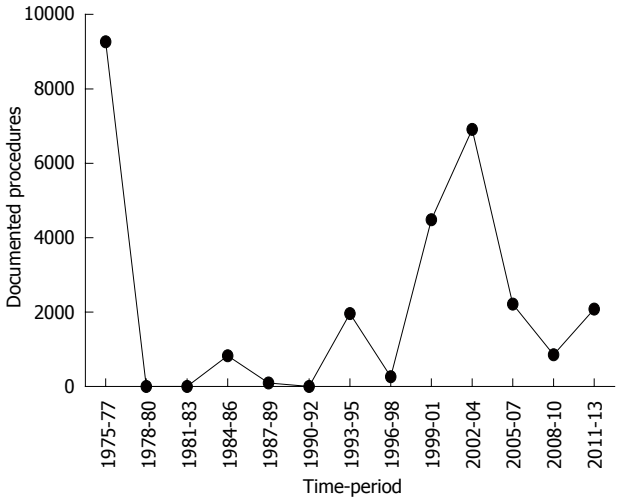

Figure 3 Number of procedures documented per 3 year time period.

conducted to assess the available evidence supporting the role of nurse endoscopists.

Our analysis suggests that under supervision by medical specialists non-physician endoscopists can perform GI endoscopy to a satisfactory standard; however this model of care does not seem to be cost efficient when compared with traditional physician service models. In addition, the studies have significant shortcomings and biases that limit their validity and generalizability. Most studies that addressed patient satisfaction and other endoscopic procedural (technical) competencies (for example, completion of procedure) were hampered by significant limitations such as lack of randomization, biased patient recruitment and allocation[4-9,11,12,16-18,22,26-34] and other methodological problems. With regard to potential general relevance of the available data, it must be noted that all studies were conducted in metropolitan centres. There are no data supporting safety and appropriateness of endoscopic services provided by non-medical endoscopists without close supervision outside of metropolitan centres. Thus, suggestions of improving endoscopic service provision at remote or rural sites by employing nurse endoscopists are unsubstantiated by any published data.

In this systematic review on non-physician endoscopy we ultimately identified 26 original datasets that were suitable for analysis. Of note, most studies were conducted prior to 2001 with a subsequent decline in total publication activity (Figure 2). Further, 96% of documented procedures performed by non-physician endoscopists in the past 3 years originated from two Dutch metropolitan tertiary centres. This apparent global decline in interest is supported by various surveys that show uptake of non-physician models is poorer than expected in the United States and United Kingdom, despite an increase in demand for endoscopies. Pathmakanthan *et al*[35] found that 42% of responding United Kingdom hospitals employed nurse endoscopists, and 90% of these were limited to esophagogastroduodenoscopies (EGDs) and FSs. However, the response rate to this survey was modest with 176 respondents from 292 (60%) surveyed hospitals. As a consequence, the results are likely to be subject to bias.

In the United States, Sharma *et al*[36] found that non-physician endoscopists performed 3.1% of EGDs, 2.2% of colonoscopies and 4% of FSs. Given that their response rate was only 2%, the true rate is likely to be much lower than this figure. The reason for the relatively limited uptake of nurse endoscopists remains unclear. However, considering that the nurse endoscopist's role was initially developed in the United States yet they perform only a small fraction of all endoscopies, it would appear that there are major flaws with the application of this service model. While reimbursement regulations may be the reason why nurse endoscopists are not contributing more substantially to service delivery, it appears that inferior cost-efficiency of nurse endoscopies is the reason for this minimal impact on service delivery.

From the data in Table 1, it is reasonable to conclude that there is evidence to support nurse endoscopists, and to a lesser extent, other non-physician endoscopists, in performing these procedures. However, there are clearly limitations. Nurses frequently had statistically significant shorter depths of insertion[7-10] but without effects on polyp or adenoma detection rates. Patient satisfaction, procedure times and complication rates were comparable when performed by physicians compared to non-physicians. It should be noted however, that complication rates of endoscopic procedures are low and are mostly related to interventions such as polypectomy. A recent large French study utilising a sample of approximately 100000 colonoscopies reported between 4.5 and 9.7 perforations per 10000 colonoscopies[37]. Complications rates for sigmoidoscopies are substantially lower[38,39]. With less than 18000 documented non-physician endoscopies

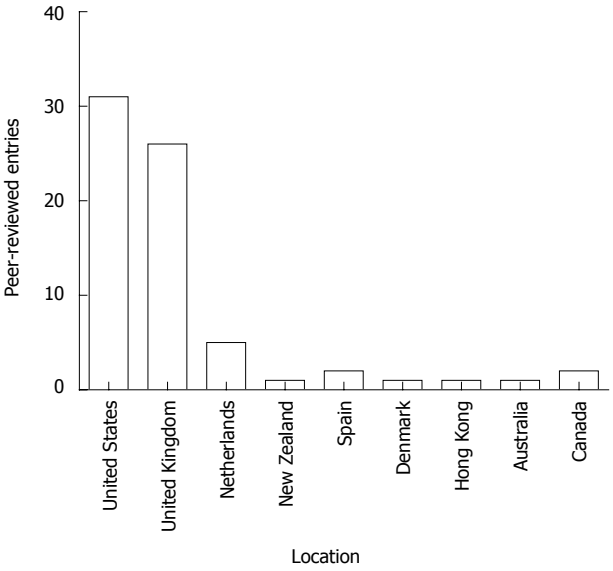

Figure 4 Geographic distribution of all publications reporting non-specialist endoscopies.

with safety data available and the majority of published studies focusing on low risk sigmoidoscopies, firm conclusions in relation to safety of endoscopic procedures done by nurse endoscopists cannot be made.

With regards to colorectal cancer prevention, colonoscopy is now the best endoscopic modality[40-43]. With regards to studies focussed on colonoscopies, there are two major Dutch studies. van Putten *et al*[34] found that nurse endoscopists could eventually reach an unassisted caecal intubation rate of 94%, however 23% of procedures still required a physician's assistance, 10 nurse endoscopists had an adenoma detection rate of 23%. There was 1 perforation from the 1000 procedures. The second Dutch study (Massl *et al*[5]) found that newly trained nurse endoscopists had a significantly lower caecal intubation rate compared to newly trained gastroenterology fellows (77% *vs* 88%, $P < 0.05$), yet procedure times and complication rates were similar. The smaller Dutch study found that nurse endoscopists and gastroenterology fellows/consultants had a similar overall caecal intubation rate, although levels of assistance were not documented[33]. The only recent non-Dutch study came from a University-based endoscopy clinic in the United States[44]. Their sole nurse endoscopist had an adenoma detection rate of 42%, *vs* 17% for 2 gastroenterology consultants. These findings were not replicated in any other colonoscopy or flexible colonoscopy study, which all demonstrated similar adenoma/polyp detection rates between non-physicians and physicians. Given the small number of patients and low number of proceduralists, this study is likely an outlier from the norm. Certainly, large trials in the United States have shown that adenoma detection rates in University-based endoscopy clinics can vary between 15.7% and 46.2%[45].

Providing endoscopic services is more than mastering the technical skills required to safely advance the scope. Lesion recognition in the context of the full clinical picture and decision making with regard to the overall management of the patient are critical components of patient care. There are no good data to support the performance of non-physician endoscopists with regard to these cognitive aspects of endoscopic procedures. If the detection of gross pathology could be perceived as a measure of cognitive competence, a study by Schroy *et al*[4] showed that nurse endoscopists performing sigmoidoscopies had a poor sensitivity (75%) while specificity (94%) was acceptable when compared with a consultant gastroenterologist's findings as the gold standard. Further evidence is required before non-physician endoscopists can be considered as being "competent" in endoscopy. Recognising relevant lesions and making clinical decisions in the context of the patient's full clinical picture is critical to deliver efficient and meaningful services.

Cost-benefit analyses are a way to assess the efficiency of services and genuine analyses on non-physician endoscopists are scarce in the literature. A crude cost-benefit analysis by Massl *et al*[5] suggests that a total saving of €7.61 per procedure could be achieved with a service model of three nurse endoscopists supervised by a gastroenterologist. However this did not account for differences in the costs of disease management including, follow-up procedures, follow-up consultations, expenditure for consumables and pathology. Indeed, nurse endoscopists take more biopsies than physicians (34.7% *vs* 26.5%, $P < 0.007$), with no additional detection of pathology[19]. Unsurprisingly, nurses had far greater levels of normal histology results than physicians ($P < 0.0001$)[19].

The sole genuine cost-benefit analysis in literature was a randomized study in the United Kingdom[19-21]. This study involved 30 nurse endoscopists performing both EGD and FS. Although the physicians' salaries were higher (£1.82/min *vs* £0.53/min), patients examined by nurse endoscopists had an increased need for subsequent endoscopies, specialist follow-up and primary care follow-up after the procedure. Thus the study concluded that at a price of £30000 per QALY, the accepted National Health Service value, physicians were more likely to be cost-effective one year after the procedure. This cost-benefit analysis did not take into account expenditure and loss of consultant time in training and supervising nurses, the increase in follow-up non-endoscopic investigations or increased expenditure for the required follow-up of biopsies. Based on the conservative estimates from the study, and the above mentioned additional unaccounted costs of nurse endoscopists, physician endoscopists appear to be more cost-effective than nurse endoscopists. While there is anecdotal evidence from individual sites that some nurse endoscopists

may work well in defined settings, the available data from larger studies does not support the assumption that the nurse endoscopy model is a more cost efficient service model.

While the salary of a nurse endoscopist might be lower as compared to a fully qualified specialist, it could be expected that services provided by nurse endoscopists are more cost efficient. However, the data point to the opposite. It is evident from the published data that the provision of endoscopic services is a multi-step process that includes a complex combination of patient-centred technical and non-technical cognitive skills. So far the assessment of endoscopic services delivered by nursing or other non-physician staff appears to be focussed on the technical aspects of procedures, rather than clinical gain of the procedures. This focus on technical/procedural aspects is appropriate to ensure safe service delivery. However, the implementation of nurse endoscopists to improve the delivery of endoscopy services is not cost-effective when compared with services delivered by specialists. This is most likely due to the cognitive aspects of service delivery. The comprehensive training of medical specialists appears to result in more cost effective service delivery.

When the model of nurse endoscopists was initially introduced in the United States and the United Kingdom the primary aim was to address the shortage of medical or surgical endoscopists. In most countries this is currently not the key problem; inappropriate funding, lack of infrastructure or issues of productivity and quality are the capacity limiting factors. Based upon the available evidence it is highly unlikely that the introduction or the increased use of nurse or other non-medical endoscopists will improve access to quality endoscopic services and improve the cost efficiency of delivery of endoscopic services.

Based upon the available anecdotal evidence and the data it appears that nurse endoscopists might be suitable to address shortages of workforce if they are imbedded into larger teams of specialists in metropolitan centres focusing on the delivery of less complex procedures such as follow-up surveillance procedures. However, before this is accepted as the appropriate setting for nurse endoscopists, the implications for training and overall productivity of services need to be properly explored, particularly in relation to colonoscopy and video capsule endoscopy where there are only limited reports on the use of non-physician endoscopists performing these procedures[46-50].

More data are obviously required. Besides the need to assess and improve the ability of nurse endoscopists to effectively contribute to the service delivery, it is necessary to explore all potential avenues that are suitable to provide cost efficient quality services to patients. This may include novel models of multi-professional teamwork, better integrated and focussed training of other professional groups such as General Practitioners for non-metropolitan (rural) settings to provide core endoscopic services to populations that are currently not properly serviced by specialists.

CONCLUSION

Most studies assessing training and performance of non-physician endoscopists have substantial methodological limitations. Studies were often uncontrolled, without random patient allocation and open to bias (selection and reporting bias). In all studies, nurse and other non-physician endoscopists worked only within a multidisciplinary team with strict supervision from a qualified physician or surgeon endoscopist.

With regard to cognitive competences in relation to the delivery of endoscopic services, nurses appear to perform less well than medical endoscopists. This emphasizes the need to work in teams with close supervision. In relation to cost-efficiency, nurse and non-physician endoscopists are probably less cost-effective than medical endoscopists. This is related to the increased need of follow-ups and reflects the cognitive component. More research is needed in this area.

All available data are from large and metropolitan centres. There is no evidence to suggest that the delivery of endoscopic services outside large metropolitan centres with several procedure rooms running in parallel benefits service delivery or is a safe option.

While nurse endoscopists may increase the capacity of endoscopic services when imbedded into larger endoscopic units, this does not appear to be a cost-efficient option as compared to traditional service models.

COMMENTS

Background

Nurse endoscopy training and delivery of endoscopic services was first reported in the United States more than 35 years ago for flexible sigmoidoscopy (FS). Several studies soon emerged, confirming that nursing staff with appropriate training and supervision could adequately perform endoscopic procedures such as FS. Other studies have since established that nurse endoscopists can safely perform upper endoscopies and colonoscopies. In an era where safe, yet cost-effective, policies run at the forefront of stakeholders' minds, the question of how nursing staff can undertake additional roles in the endoscopic suites is continually raised.

Research frontiers

There have been no systematic reviews of the literature that have examined the full body of evidence surrounding uptake, safety, accuracy and most importantly, the cost-effectiveness of nurse endoscopists.

Applications

Contrary to general beliefs, endoscopic services provided by nurse endoscopists are not more cost effective compared to standard service models and evidence suggests the opposite. Overall significant shortcomings and biases limit the validity and generalizability of studies that have explored safety and quality of services delivered by non-medical endoscopists.

Peer-review

The paper is very interesting with significant results in the field of evidence on safety, competency and cost-effectiveness of nursing staff providing gastrointestinal endoscopy services. The tables are too broad and difficult to clear. Authors need to reduce and make more informative.

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META-ANALYSIS

Value of bevacizumab in treatment of colorectal cancer: A meta-analysis

Chun-Ying Qu, Ying Zheng, Min Zhou, Yi Zhang, Feng Shen, Jia Cao, Lei-Ming Xu

Chun-Ying Qu, Min Zhou, Yi Zhang, Feng Shen, Jia Cao, Lei-Ming Xu, Department of Digestive Diseases, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China
Chun-Ying Qu, Min Zhou, Yi Zhang, Feng Shen, Jia Cao, Lei-Ming Xu, Shanghai Key Laboratory of Pediatric Gastroenterology and Nutrition, Shanghai Institute of Pediatric Research, Shanghai 200092, China
Ying Zheng, Department of Oncology, Branch of Shanghai First People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200081, China
Author contributions: Xu LM and Qu CY contributed to study conception and design; Zhou M, Zhang Y, Shen F and Cao J were responsible for acquisition and analysis of the data; Qu CY and Zheng Y drafted and revised the article; Xu LM gave the final approval of the version to be published.

Correspondence to: Lei-Ming Xu, Chief Physician, Department of Digestive Diseases, Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, 1665 Kongjiang Road, Yangpu District, Shanghai 200092, China. leiming.xu@aliyun.com
Telephone: +86-21-25077120
Fax: +86-21-25077120
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Abstract

AIM: To assess the efficacy and safety of bevacizumab in the treatment of colorectal cancer.

METHODS: All randomized controlled trials of bevacizumab for the treatment of colorectal cancer from January 2003 to June 2013 were collected by searching the Cochrane Library, PubMed, Chinese National Knowledge Infrastructure and Wanfang databases. The primary endpoint was overall survival (OS), and the secondary endpoints were progression-free survival, overall response rate and adverse events. Two reviewers extracted data independently. Statistical analyses were performed with Stata 12.0. The degree of bias was assessed using funnel plots for the effect size of OS at the primary endpoint.

RESULTS: Following the inclusion criteria and exclusion criteria, ten studies comprising 6977 cases were finally included, of which nine were considered to be of high quality (4-7 points) and one of low quality (1-3 points). Our meta-analysis revealed the efficacy of bevacizumab in patients with colorectal cancer in terms of OS (HR = 0.848, 95%CI: 0.747-0.963), progression-free survival (HR = 0.617, 95%CI: 0.530-0.719), and overall response rate (OR = 1.627, 95%CI: 1.199-2.207). Regarding safety, higher rates of grade $\geqslant$ 3 hypertension, proteinuria, bleeding, thrombosis, and gastrointestinal perforation were observed in the bevacizumab treatment group ($P < 0.05$); however, the incidence of serious toxicity was very low. There was no publication bias in the 10 reports included in this meta-analysis.

CONCLUSION: The clinical application of bevacizumab in colorectal cancer is effective with good safety.

Key words: Bevacizumab; Colorectal cancer; Clinical application; Randomized controlled trials; Meta-analysis

Core tip: Bevacizumab combined with chemotherapy can provide a significant survival advantage in patients with colorectal cancer. Despite the increased risk of hypertension, proteinuria, bleeding, thrombosis, gastrointestinal perforation and other adverse events, bevacizumab combined with chemotherapy deserves further promotion in future clinical work due to its low incidence of serious adverse events, excellent overall tolerance and good safety.

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INTRODUCTION

Bevacizumab (BV) is a monoclonal antibody specific for recombinant humanized vascular endothelial growth factor (VEGF). It binds to VEGF and prevents its interaction with VEGF receptors (VEGFR-1 and VEGFR-2), mediating inhibition of growth in a variety of tumors. In previous large-scale clinical studies, the survival benefit of bevacizumab therapy for colorectal cancer has proved controversial, and increased risk of hypertension, proteinuria, thrombosis, bleeding, and gastrointestinal perforation has been found[1-4]. Therefore, we conducted this meta-analysis of data collected on bevacizumab in the treatment of colorectal cancer to assess its efficacy and safety.

MATERIALS AND METHODS

Inclusion and exclusion criteria

Inclusion criteria were: (1) histologically confirmed colorectal cancer by pathology; (2) 0-2 points according to Eastern Cooperative Oncology Group (ECOG) score standard; (3) expected survival of more than three months; (4) older than 18 years of age; and (5) qualified hematology, liver and kidney function.

Exclusion criteria were: (1) non-randomized controlled trials; and (2) failure to comply with any of the above inclusion criteria.

Intervention measures

Bevacizumab + chemotherapy *vs* placebo (or blank control) + chemotherapy.

Retrieval strategy

Searches were performed for data recorded between January 2003 and June 2013 in the Cochrane Library, PubMed, Chinese National Knowledge Infrastructure and Wanfang databases. Additional references for the included literature were retrieved subsequently.

Keywords for retrieval were BV, colorectal cancer, randomized controlled trials and meta-analysis.

Quality evaluation of included literature

The quality of the included literature was appraised and graded according to a modified Jadad scale[5] as follows: 1-3 points, low quality; 4-7 points, high quality.

Data extraction and analysis

Hazard ratios (HRs) and odds ratios (ORs) were selected for the combined statistics of time-to-event data and combined statistics of the selected data, respectively. The heterogeneity analysis was performed before combining data (heterogeneity was quantitatively analyzed using I^2): heterogeneity was considered mild when $I^2 < 25\%$; moderate when $25\% \leqslant I^2 < 50\%$; great when $50\% \leqslant I^2 < 75\%$; and high when $75\% \leqslant I^2 < 100\%$. The combined analysis using Stata12.0 software was performed for the data with low to moderate heterogeneity; descriptive analysis was performed for the data with high heterogeneity.

The degree of bias was assessed using funnel plots for the effect size of overall survival (OS) at the primary endpoint.

RESULTS

Retrieval results

A total of 789 publications were retrieved. After reading the titles and abstracts, 681 reports of non-randomized controlled studies or those that were unrelated to our purpose were excluded. By reading the full texts of the remaining publications, eventually 10 randomized control studies[6-15] were included in accordance with the inclusion and exclusion criteria. Of these, nine reports were considered to be of high quality (4-7 points)[6,7,9-15] and one of low quality (1-3 points)[8]. The 10 studies comprised 6977 cases in total, of which 3535 were from treatment groups and 3442 from control groups. The characteristics of the included data are shown in Table 1 and the flow chart in Figure 1.

Main efficacy

Effect of bevacizumab on OS: OS data were provided in 10 studies[6-15], of which four suggested that bevacizumab prolonged OS[8,12,13,15], five suggested that bevacizumab had no effect on OS[6,7,9-11], and one study suggested that bevacizumab treatment was associated with shortened OS[14]. The combined analysis of the 10 studies suggested moderate heterogeneity (I^2 = 71.5%), and a combined HR of 0.848 (95%CI: 0.747-0.963, P = 0.011) was obtained using a random-effects model (Figure 2).

Effect of bevacizumab on progression-free survival: progression-free survival (PFS) data were provided in nine studies[7-15], of which eight suggested that

Table 1 Baseline characteristics of the included studies

Ref.	No. of cases (control group/treatment group)	Bevacizumab treatment regimen	Modified Jadad score
Kabbinavar *et al*[9]	104 (36/68)	5 mg/kg or 10 mg/kg every 14 d	4
Kabbinavar *et al*[10]	209 (105/104)	5 mg/kg every 14 d	5
Tebbutt *et al*[11]	312 (156/156)	7.5 mg/kg every 21 d	4
Stathopoulos *et al*[6]	224 (108/114)	7.5 mg/kg every 21 d	4
Saltz *et al*[7]	1400 (701/699)	5 mg/kg every 14 d or 7.5 mg/kg every 21 d	6
Hurwitz *et al*[12]	813 (411/402)	5 mg/kg every 21 d	4
Giantonio *et al*[8]	577 (291/286)	10 mg/kg every 14 d	3
Guan *et al*[13]	214 (72/142)	5 mg/kg every 14 d	4
de Gramont *et al*[14]	2306 (1151/1155)	5 mg/kg every 14 d	4
Bennouna *et al*[15]	820 (411/409)	5 mg/kg every 14 d or 7.5 mg/kg every 14 d	5

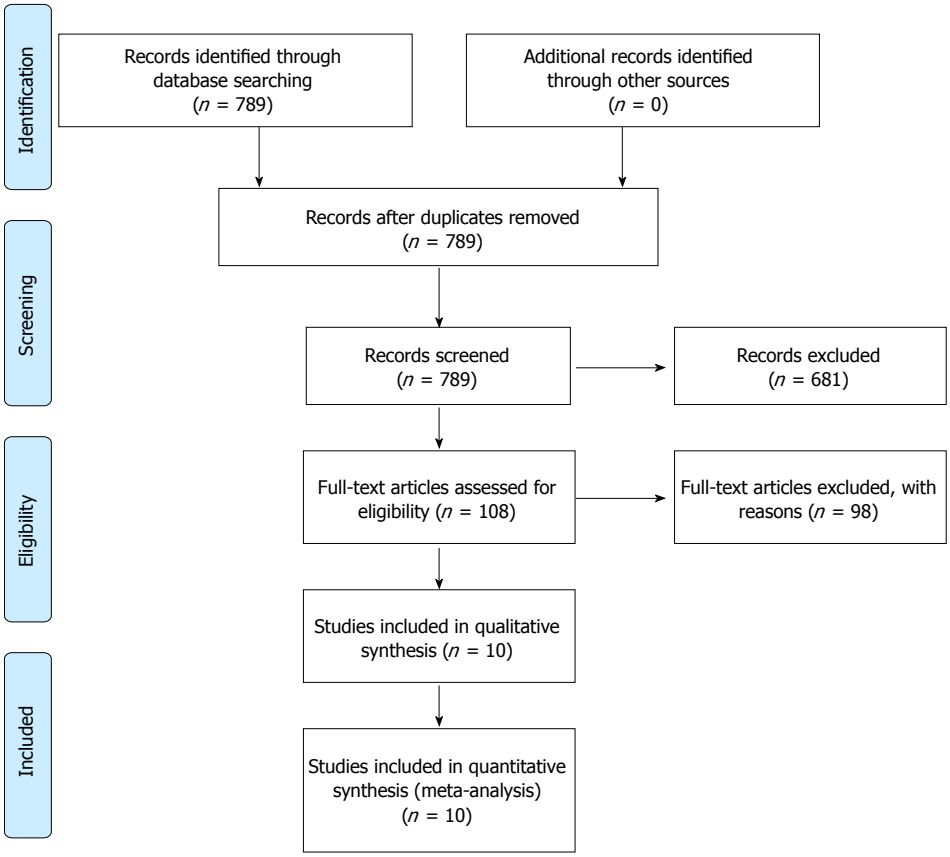

Figure 1 Flow chart: The number of articles in each step.

bevacizumab prolonged PFS[7-13,15], and one suggested that bevacizumab treatment shortened PFS[14]. Combined analysis showed high heterogeneity of nine studies (I^2 = 85.2%); thus only one study using adjuvant postoperative chemotherapy[14] resulting in potential heterogeneity was eliminated. The combined analysis of the remaining eight studies[7-13,15] revealed that heterogeneity fell to a moderate level (I^2 = 70.5%), and a combined HR of 0.617 (95%CI: 0.530-0.719, P < 0.001) was obtained using a random-effects model (Figure 3).

Effect of bevacizumab on overall response rate: overall response rate (ORR) data were provided by nine studies[6-13,15]. Among these, the response rates in the bevacizumab treatment and control groups were 33.5% and 28.3%, respectively. Combined analysis showed moderate heterogeneity for nine studies (I^2 = 73%), and a combined OR of 1.627 (95%CI: 1.199-2.207, P = 0.002) was obtained using a random-effects model (Figure 4).

Side effects

Effect of bevacizumab on hypertension (⩾ grade 3): Data for the incidence of hypertension were provided in nine studies[7-15]. The incidence of hypertension in the treatment and controls groups was 7.53%, and 1.32%, respectively. The heterogeneity of the combined results

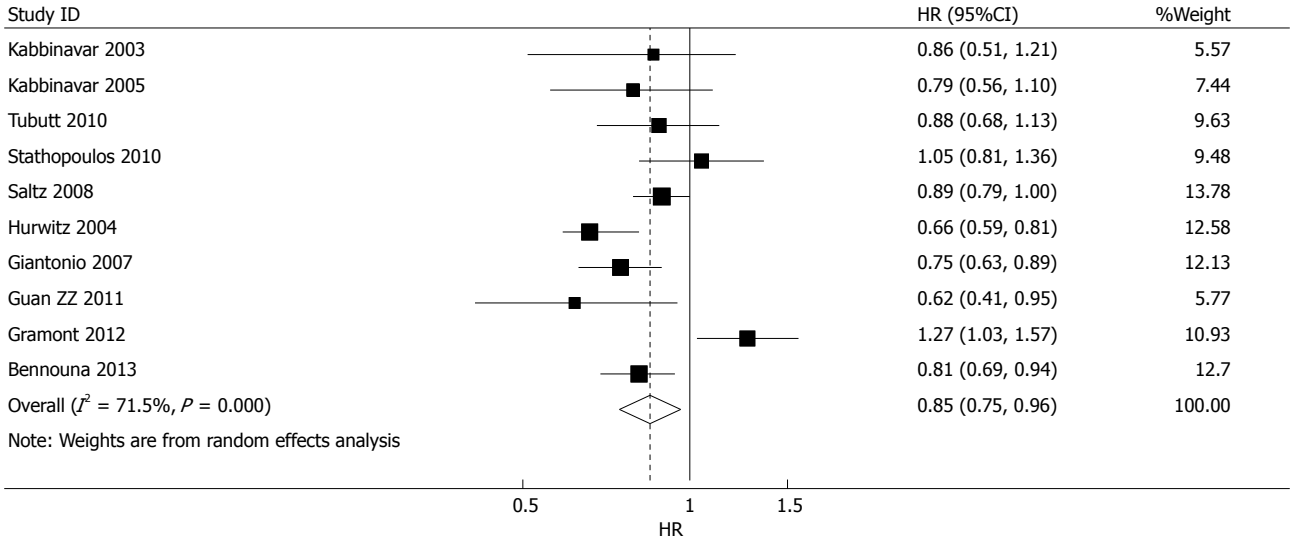

Figure 2 Forest plot of meta-analysis: Effect of bevacizumab on overall survival.

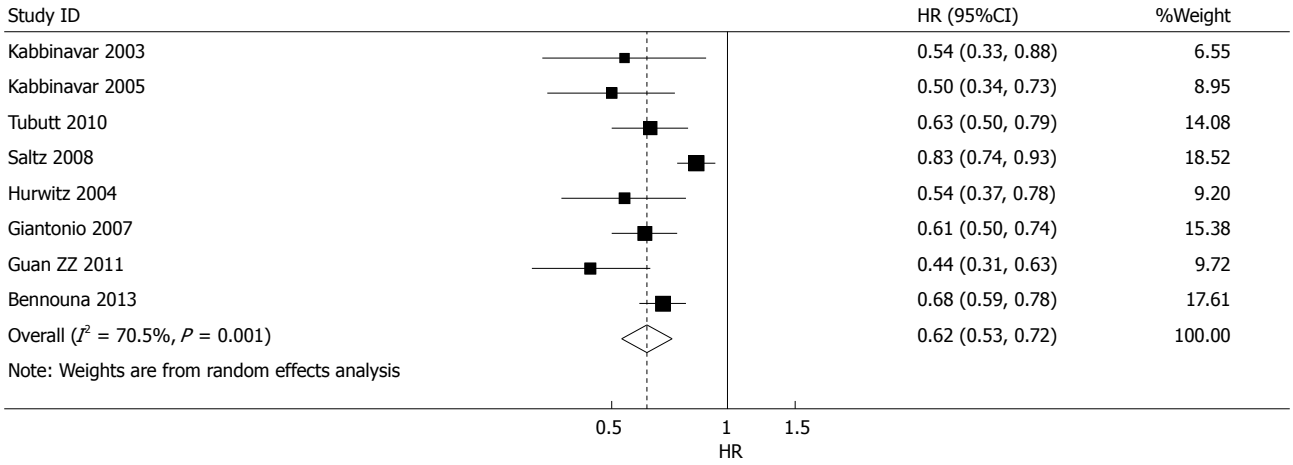

Figure 3 Forest plot of meta-analysis: Effect of bevacizumab on progression-free survival.

was moderate (I^2 = 40.9%), and a combined OR of 4.982 (95%CI: 3.069-8.087, P < 0.001) was obtained using a random-effects model (Figure 5).

Effect of bevacizumab on proteinuria (⩾ grade 3): Data for the incidence of proteinuria were provided in seven studies[7,8,10-14]. The incidence of proteinuria in the treatment and control groups was 0.89% and 0.18%, respectively. The combined results presented little heterogeneity (I^2 = 0.0%), and a combined OR of 3.856 (95%CI: 1.681-8.848, P = 0.001) was obtained using a fixed effects model (Figure 6).

Effect of bevacizumab on bleeding (⩾ grade 3): Data for the incidence of bleeding were provided in nine studies[7-15]. The incidence of bleeding in the treatment and control groups was 1.77% and 1.04%, respectively. The combined results presented low heterogeneity (I^2 = 15.3%), and a combined OR of 1.933 (95%CI: 1.279-2.922, P = 0.002) was obtained using a fixed effects model (Figure 7).

Effect of bevacizumab on thrombosis (⩾ grade 3): Data for the incidence of thrombosis were provided in seven studies[7-9,11,13-15]. The incidence of thrombosis in the treatment and control groups was 5.20% and 5.83%, respectively. The meta-analysis results indicated that the heterogeneity of the combined results was high (I^2 = 88.3%). The sensitivity analysis was performed using the exclusion method. After eliminating the study reported by de Gramont *et al*[14], the heterogeneity fell to I^2 = 0.0%. The high heterogeneity of these data was due to this study being conducted in postoperative patients; therefore, it was impossible to exclude the effects of the use of anticoagulants or hemostasis in the perioperative period on incidence of thrombus. By using a fixed effects model for the remaining six studies, the combined OR was 1.685 (95%CI: 1.262-2.249, P < 0.001) (Figure 8).

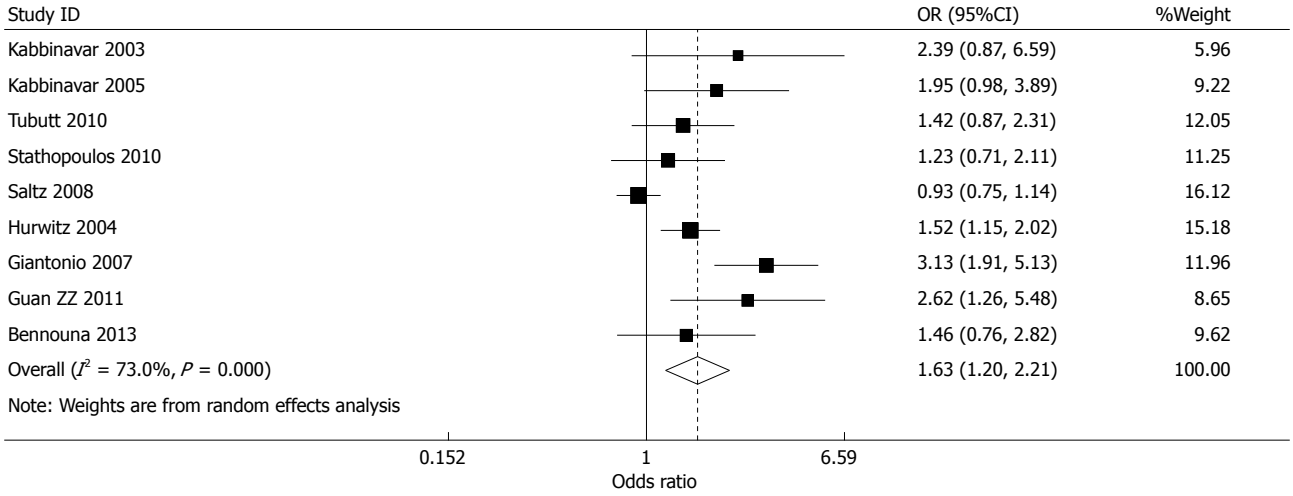

Figure 4 Forest plot of meta-analysis: Effect of bevacizumab on overall response rate.

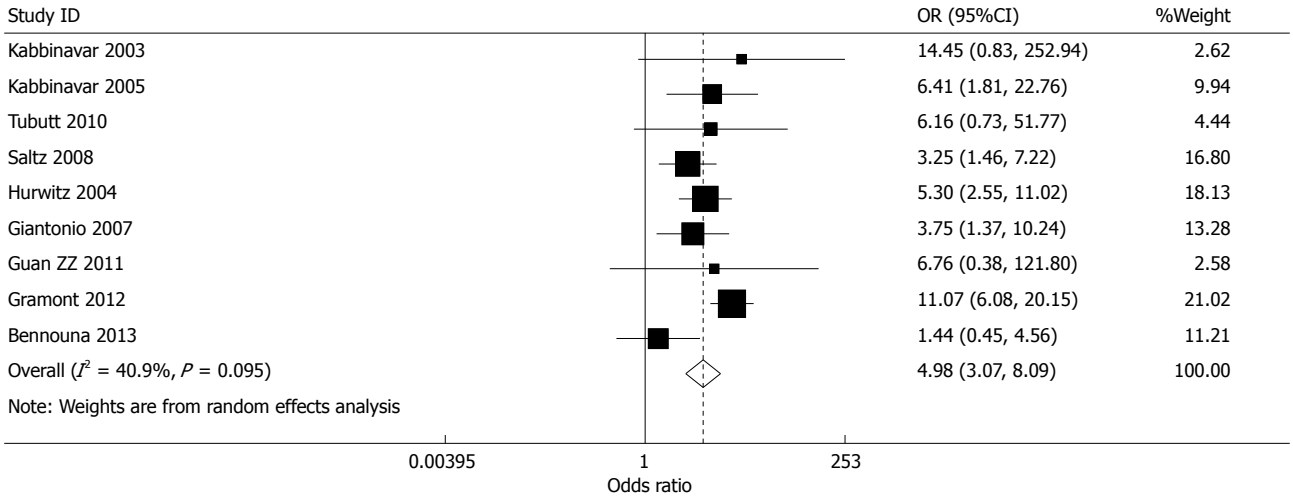

Figure 5 Forest plot of meta-analysis: Effect of bevacizumab on grade ⩾ 3 hypertension.

Effect of bevacizumab on gastrointestinal perforation: Data for the incidence of gastrointestinal perforation were provided in eight studies[6,7,10-15]. The incidence of gastrointestinal perforation in the treatment and control groups was 1.02% and 0.20%, respectively. The combined results presented little heterogeneity (I^2 = 0.0%), and a combined OR of 3.958 (95%CI: 1.866-8.397, P < 0.001) was obtained using a fixed effects model (Figure 9).

Publication bias: Publication bias was assessed in terms of HR for the OS at the primary endpoint. As seen from the funnel chart shown in Figure 10, all studies were evenly distributed on both sides, thus presenting basic symmetry. This indicated that there was no publication bias in the 10 reports included in this meta-analysis (Begg's test, P > 0.05).

DISCUSSION

Colorectal cancer is a common malignant neoplasm of the digestive system, with approximately 1.2 million new cases reported globally each year[16], ranking third in terms of global cancer incidence[17]. Approximately 25% of patients with colorectal cancer were found to have hepatic metastasis when initially diagnosed, and 25% of patients gradually developed liver or lung metastasis with disease progression[18]. Therefore, a safe and effective therapy for patients with colorectal cancer is urgently required. For patients in the early stages, surgery is still the most important method of treatment. However, for patients in advanced stage, most of whom are not suitable for surgery, the only remaining options are palliative therapies, such as chemotherapy, which is the most common choice, traditional Chinese medicine and biological target therapy. Common combined chemotherapy regimens include FOLFIRI[19], FOLFOX[20] and thymidylate synthase inhibitors (*e.g.*, capecitabine)[21]. The efficiency of these regimens is only 30%-40%, even in frontline application; therefore, in order to improve the therapeutic effect for patients with colorectal

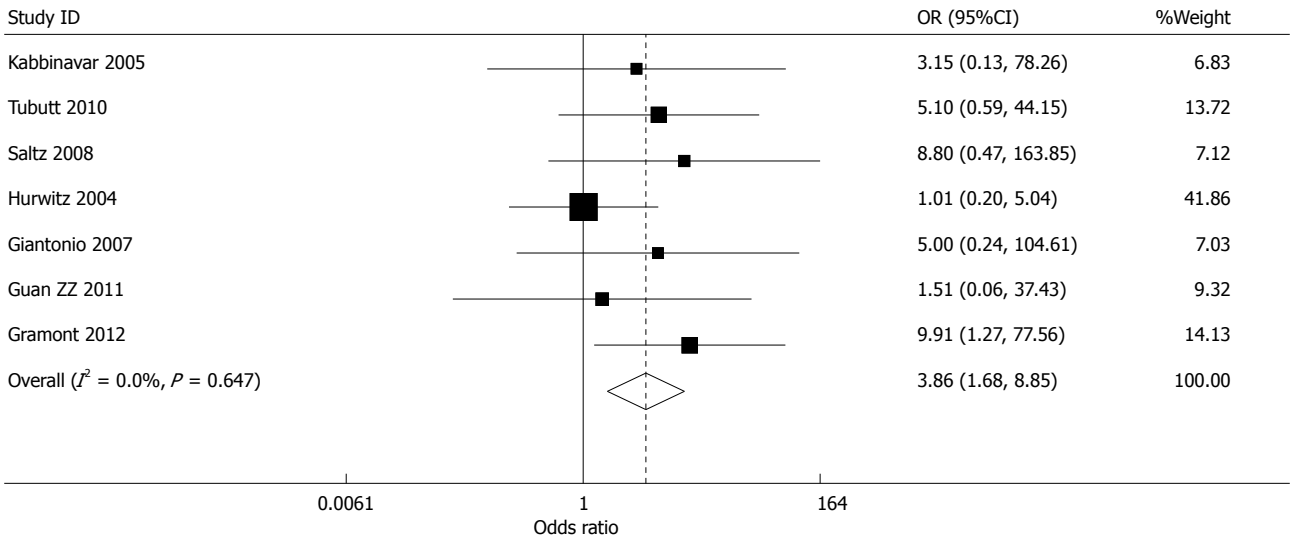

Figure 6 Forest plot of meta-analysis: Effect of bevacizumab on grade ⩾ 3 proteinuria.

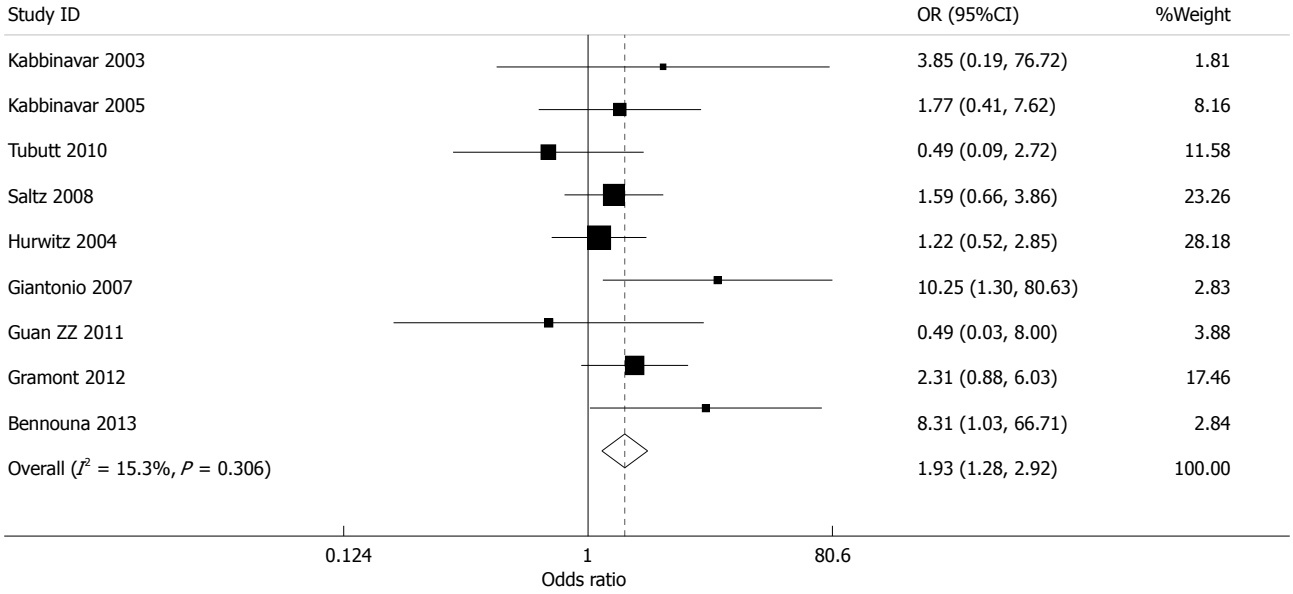

Figure 7 Forest plot of meta-analysis: Effect of bevacizumab on grade ⩾ 3 bleeding.

cancer, molecularly targeted therapies have been developed[22], among which, combined chemotherapy has become an important choice[1].

Molecular targeted drugs exploit specific changes in tumor cells to achieve relative target specificity. This not only enhances the anti-cancer effect, but also reduces the damage to normal cells. Anti-angiogenic drugs have become a key to targeted therapy[23,24]. Bevacizumab is a monoclonal antibody specific for recombinant humanized vascular endothelial growth factor (VEGF), which can block signal transduction *via* the VEGF receptor by binding to VEGF-A. This study assessed the efficacy and safety of bevacizumab in the treatment of colorectal cancer through meta-analysis of 10 randomized controlled trials including 6977 cases. Following grading using a modified Jadad scale, nine reports were deemed to be of high quality ones (4-7 points), and one of low quality (1-3 points). Funnel plots of the data revealed no significant publication bias.

OS data were provided for all 10 randomized controlled trials, and PFS and ORR data were provided for nine trials. The combined results suggested that, in the overall patient population, bevacizumab significantly increased OS (HR = 0.848, 95%CI: 0.747-0.963), prolonged PFS (HR = 0.617, 95%CI: 0.530-0.719), and improved the ORR (OR = 1.627, 95%CI: 1.199-2.207; P < 0.05 for all). These data indicated that bevacizumab treatment offers a significant survival advantage to patients with colorectal cancer.

Although studies have confirmed the survival benefit of bevacizumab, the toxic side-effects caused by bevacizumab should also be considered.

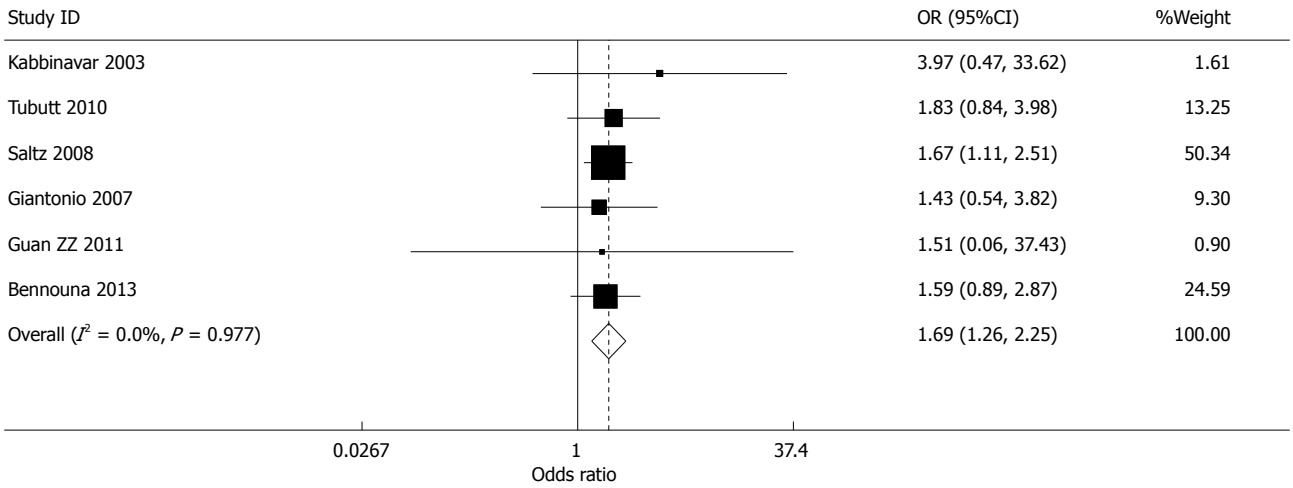

Figure 8 Forest plot of meta-analysis: Effect of bevacizumab on grade ⩾ 3 thrombosis.

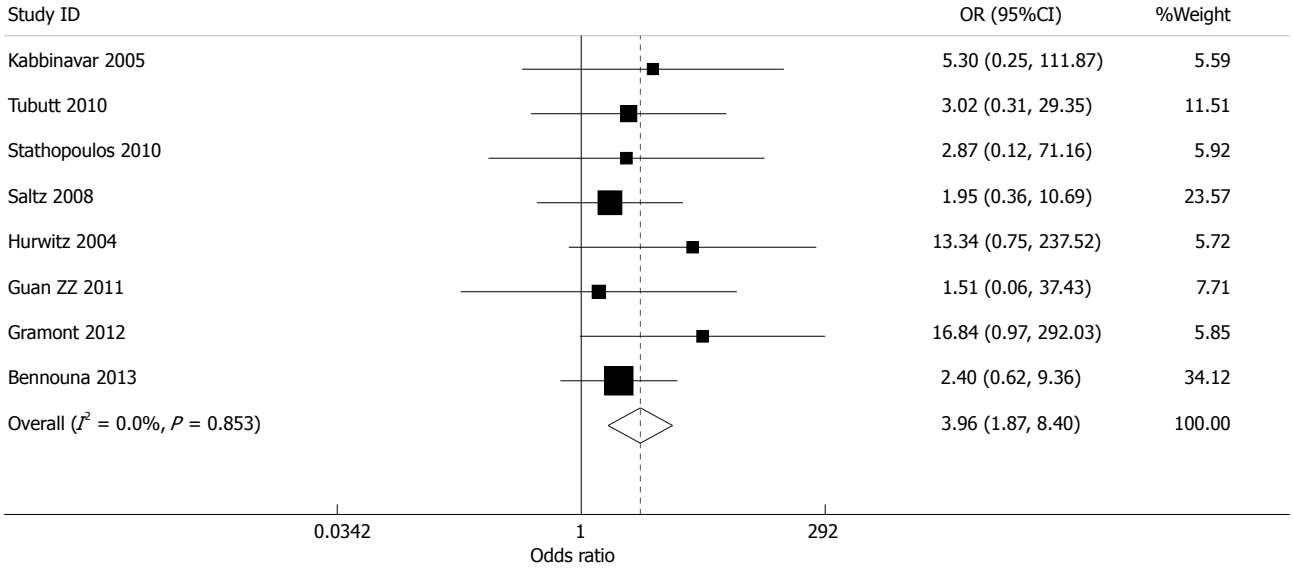

Figure 9 Forest plot of meta-analysis: Effect of bevacizumab on gastrointestinal perforation.

According to previous reports, the most frequent adverse reactions to bevacizumab are hypertension and proteinuria, with gastrointestinal perforation being the most serious. Other side-effects include thrombosis and bleeding[1-4]. The results of our combined analysis of these adverse reaction data are consistent with previous studies. Although the incidence of gastrointestinal perforation caused by bevacizumab was increased by approximately 3-fold in the treatment group compared with that of the control group, the incidence in the eight studies included in this meta-analysis is relatively low (approximately 1%), suggesting that bevacizumab is safe and non-toxic. Compared to the control group, the treatment group has significantly higher incidence of hypertension, proteinuria, bleeding, and thrombosis ($P < 0.05$). Although the results showed that incidence of hypertension (⩾ grade 3) in the treatment group was approximately 5 times higher than that of the control group, published studies have shown that this side-effect can be controlled by oral antihypertensive agents[10].

Although incidence of other side-effects increased, most adverse reactions were grade 1/2, with few adverse reactions graded 3/4. Most clinical adverse reactions can be controlled, and no dose-limiting toxicity has been reported[25]. The incidence of adverse reactions can be limited by effective monitoring and prevention during the course of treatment.

The result of this meta-analysis suggests that bevacizumab combined with chemotherapy can provide a significant survival advantage by prolonging OS and PFS in patients with colorectal cancer, thus improving the ORRs. Despite the increased risk of hypertension, proteinuria, bleeding, thrombosis, gastrointestinal perforation and other adverse events, bevacizumab combined with chemotherapy deserves further promotion in future clinical work due to its low

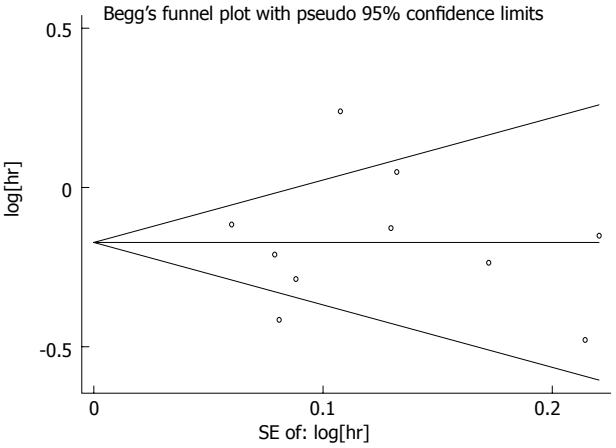

Figure 10 Assessment of publication bias. All studies are evenly distributed on both sides, thus presenting basic symmetry and indicating that there was no publication bias in the 10 reports.

incidence of serious adverse events, excellent overall tolerance and good safety.

COMMENTS

Background

Colorectal cancer ranked third in terms of global cancer incidence. Approximately 25% of patients with colorectal cancer were found to have hepatic metastasis when initially diagnosed. Therefore, a safe and effective therapy for patients with colorectal cancer is urgently required.

Research frontiers

Vascular endothelial growth factor (VEGF) has been proved to be closely related to the growth of tumors. Bevacizumab (BV) is a monoclonal antibody specific for recombinant humanized VEGF. It binds to VEGF and prevents its interaction with VEGF receptors (VEGFR-1 and VEGFR-2), mediating inhibition of growth in a variety of tumors.

Innovations and breakthroughs

There is not inherent innovation in this meta-analysis. However, it is informative to see the results of multiple studies compared next to each other.

Applications

Bevacizumab combined with chemotherapy provides a significant survival advantage in clinical use for patients with colorectal cancer.

Terminology

VEGF mediates growth in a variety of tumors. BV is a monoclonal antibody specific for recombinant humanized VEGF. It binds to VEGF and prevents its interaction with VEGF receptors (VEGFR-1 and VEGFR-2), mediating inhibition of growth in a variety of tumors.

Peer-review

The authors present a meta-analysis of the safety and efficacy of bevacizumab treatment for colorectal cancer. This paper is well-written and the analysis is thorough. The statistical methods used are appropriate, and the conclusions are solid. The quality of English in the manuscript is also excellent, and the content concise.

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META-ANALYSIS

Relationship between apurinic endonuclease 1 Asp148Glu polymorphism and gastrointestinal cancer risk: An updated meta-analysis

Zhi-Jun Dai, Yong-Ping Shao, Hua-Feng Kang, Wei Tang, Dan Xu, Yang Zhao, Di Liu, Meng Wang, Peng-Tao Yang, Xi-Jing Wang

Zhi-Jun Dai, Hua-Feng Kang, Yang Zhao, Di Liu, Meng Wang, Peng-Tao Yang, Xi-Jing Wang, Department of Oncology, the Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China
Zhi-Jun Dai, Yong-Ping Shao, Dan Xu, Center for Translational Medicine, Frontier Institute of Science and Technology, Xi'an Jiaotong University, Xi'an 710049, Shaanxi Province, China
Wei Tang, School of Chemistry and Chemical Engineering, Shaanxi Normal University, Xi'an 710061, Shaanxi Province, China
Author contributions: Dai ZJ, Shao YP and Kang HF contributed equally to this work; Dai ZJ, Kang HF and Wang XJ designed the research; Shao YP, Xu D, Zhao Y, Liu D and Yang PT performed the research; Tang W, Wang M and Wang XJ analyzed the data; Dai ZJ and Shao YP wrote the paper.
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Correspondence to: Zhi-Jun Dai, MD, PhD, Department of Oncology, the Second Affiliated Hospital of Xi'an Jiaotong University, No. 157, West 5th Road, Xi'an 710004, Shaanxi Province, China. dzj0911@126.com
Telephone: +86-29-87679226
Fax: +86-29-87679282
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Abstract

AIM: To evaluate the relationship between apurinic endonuclease 1 (APE1) Asp148Glu polymorphism and the susceptibility to gastrointestinal (GI) cancers.

METHODS: We searched PubMed, ISI Web of Knowledge, and Chinese National Knowledge Infrastructure (CNKI) databases updated on July 15, 2014 for relevant studies. Only case-control studies comparing APE1 Asp148Glu polymorphism and GI cancer risk were included. We excluded studies reporting only standardized incidence ratios without control groups and those without detailed genotyping data. Meta-analysis was performed on 17 studies involving 4856 cancer patients and 6136 cancer-free controls. Review Manager version 5.1 was used to perform the meta-analysis. The pooled odds ratios (ORs) and 95% confidence intervals (CIs) were estimated under the allele contrast, homozygous, heterozygous, dominant and recessive genetic models. We also conducted subgroup analyses stratified by ethnicity and cancer type. Publication bias was evaluated using Begg's test.

RESULTS: The meta-analysis showed a significant association between APE1 Asp148Glu polymorphism and GI cancer risk in three genetic models in the overall population (G *vs* T: OR = 1.18; 95%CI: 1.05-1.32; TG *vs* TT: OR = 1.28; 95%CI: 1.08-1.52; TG + GG *vs* TT: OR = 1.32; 95%CI: 1.10-1.57). Stratified analysis by ethnicity revealed a statistically increased GI cancer risk in Asians (G *vs* T: OR = 1.27; 95%CI: 1.07-1.51; GG *vs* TT: OR = 1.58; 95%CI: 1.05-2.38; TG *vs* TT: OR = 1.30; 95%CI, 1.01- 1.67; and TG + GG *vs* TT: OR = 1.38; 95%CI: 1.07-1.78), but not in Caucasians. Further

subgroup analysis by cancer type indicated that APE1 Asp148Glu polymorphism may contribute to gastric cancer risk. However, Asp148Glu has no significant association with colorectal or esophageal cancer risk in any genetic model.

CONCLUSION: This meta-analysis suggests that the APE1 Asp148Glu polymorphism G allele is associated with an increased GI cancer risk, especially in gastric cancer.

Key words: Apurinic endonuclease 1; Single nucleotide polymorphism; Gastrointestinal cancers; Cancer risk; Meta-analysis

Core tip: Apurinic endonuclease 1 (APE1) plays an important role in the DNA repair system and therefore has been implicated in human carcinogenesis. Many studies have suggested an association between the APE1 Asp148Glu polymorphism and gastrointestinal cancer susceptibility. However, the results remained inconclusive. We performed a meta-analysis on pooled data from previously published studies. The results showed that the APE1 Asp148Glu polymorphism G allele is associated with an increased gastrointestinal cancer risk, especially in gastric cancer.

Dai ZJ, Shao YP, Kang HF, Tang W, Xu D, Zhao Y, Liu D, Wang M, Yang PT, Wang XJ. Relationship between apurinic endonuclease 1 Asp148Glu polymorphism and gastrointestinal cancer risk: An updated meta-analysis. *World J Gastroenterol* 2015; 21(16): 5081-5089 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/5081.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.5081

INTRODUCTION

Gastrointestinal (GI) cancers, especially esophageal, gastric, and colorectal cancers, are the leading causes of cancer-related death worldwide[1]. GI cancers are multifactorial diseases caused by complex interactions between many genetic and environmental factors[1,2]. Allelic variations in oncogenes are candidate genetic risk factors that may alter the onset and outcome of GI cancers[3].

Apurinic/apyrimidinic endonuclease 1 (APE1) is an essential enzyme in the base excision repair pathway[4]. APE1 plays an important role in the DNA repair system and therefore has been implicated in human carcinogenesis[5]. The human APE1 is located on chromosome 14q11.2 and consists of five exons, spanning roughly 2.5 to 3 kb of DNA[6]. Many single nucleotide polymorphisms in the APE1 gene have been reported, including the commonly occurring Asp148Glu in the fifth exon and -141T/G in the promoter region[7]. These nonconservative amino acid alterations have been reported to reduce the DNA repair activity of APE1 and consequently increase cancer risk[8]. Our previous study has suggested that the APE1 -141T/G but not the Asp148Glu polymorphism may influence the susceptibility to and progression of breast cancer in the Chinese population[9]. However, a recent study reported that the APE1 148 GG genotype is associated with an increased risk of colorectal cancer (CRC)[10].

It is important to summarize inconclusive results from different studies to further validate the association of one polymorphism with cancer risk[11]. To clarify the role of the APE1 Asp148Glu polymorphism in GI cancer risk, we performed a meta-analysis on all eligible case-control studies to estimate the overall cancer risk associated with the APE1 Asp148Glu polymorphism. Furthermore, we conducted subgroup analyses stratified by ethnicity and cancer type.

MATERIALS AND METHODS

Methods

The procedures performed in this meta-analysis are in accordance with recent guidelines for the reporting of meta-analyses (PRISMA guidelines).

Publication search

We searched the electronic databases of PubMed, Web of Knowledge and Chinese National Knowledge Infrastructure databases to collect articles reporting case-control studies related to the association of APE1 Asp148Glu polymorphisms with GI cancer risk. The keywords used for search were as follows: apurinic/apyrimidinic endonuclease-1/APE1/APEX/HAP1/REF-1, gastrointestinal/esophageal/gastric/colorectal, cancer/carcinoma/tumor/neoplasm, polymorphism/genotype/SNP/variation. The latest search was updated on July 15, 2014. Furthermore, reference lists of main reports and review articles were also reviewed manually to identify additional relevant publications.

Selection criteria

The following criteria were used to select studies for further meta-analysis: (1) case-control studies; (2) studies that evaluated the associations between APE1 Asp148Glu polymorphism and GI cancer risk; (3) studies that contained at least two comparison groups (cancer *vs* control); and (4) studies that included detailed genotyping data.

Data extraction and synthesis

Articles were reviewed independently by two reviewers and data with discrepancies were discussed by all authors. For each included study, the following information was collected: first author, year of publication, country of origin, ethnicity, source of control, total numbers of cases and controls, genotyping methods as well as numbers of cases and controls with the different genotypes. Different ethnic groups were categorized as Caucasian,

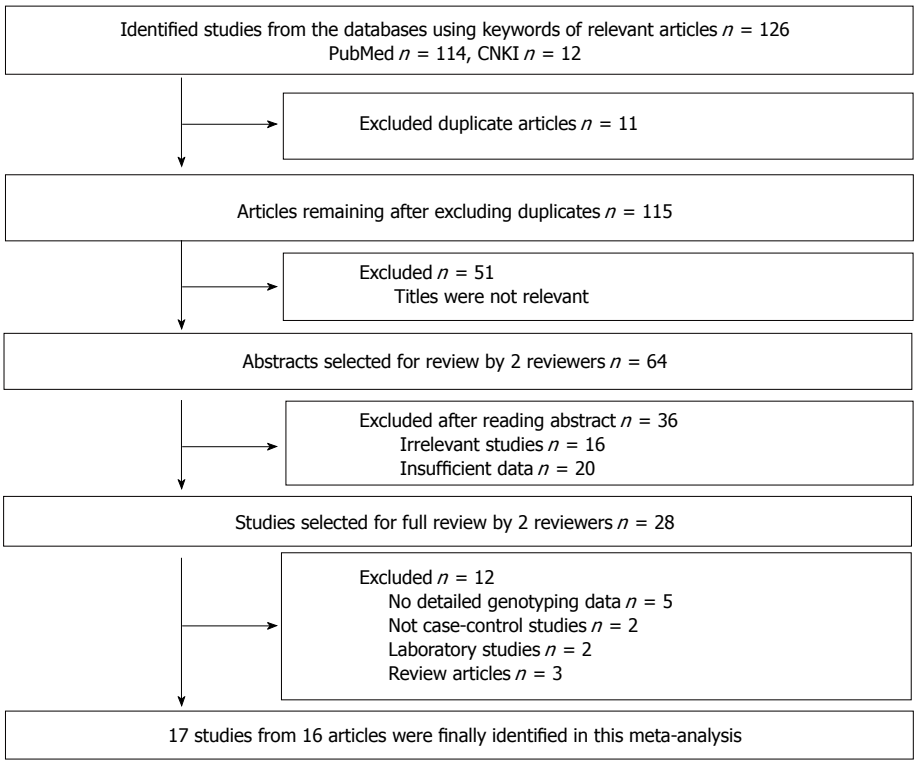

Figure 1 Flow diagram of study identification and selection.

Asian, African, and "mixed". All the case and control groups were well controlled. The non-cancer controls had no history of gynecologic disease, and there was no present evidence of any malignant disease.

Statistical analysis

The associations between APE1 Asp148Glu polymorphism and GI cancer risk were measured by odds ratio (OR) with 95% confidence interval (CI). The significance of the pooled OR was determined by the *Z*-test.

The meta-analysis assessed association by using five different genetic models: (1) allele contrast genetic model - A *vs* a (where "a" is the wild-type allele and "A" is the variant allele); (2) homozygous genetic model-comparison between the 2 homozygous genotypes (AA *vs* aa); (3) heterozygous genetic model-comparison between the heterozygous and homozygous wild-type genotype groups (Aa *vs* aa); (4) dominant genetic model-comparison between the wild-type homozygous genotype *vs* the variant allele-positive genotypes (AA + Aa *vs* aa); and (5) recessive genetic model-comparison between the variant homozygous genotype *vs* the rest (AA *vs* aa + Aa).

Statistical heterogeneity among studies was assessed with the *Q* and I^2 statistics. If the *P*-value of heterogeneity test was more than 0.1 ($P \geqslant 0.1$), the pooled OR estimate of the study was calculated using the fixed-effects model. Otherwise, the random-effects model was used[11]. The value of the I index was used to assess the degree of heterogeneity (I^2 < 25%: no heterogeneity; 25% < I^2 < 50%: moderate heterogeneity; 50% < I^2 < 75%: high heterogeneity; I^2 > 75%: extremely high heterogeneity). Publication bias was evaluated by the funnel plot and further assessed by the method of Egger's linear regression test. All statistical analyses were carried out with the Review Manager version 5.1 (Revman; The Cochrane Collaboration, Oxford, United Kingdom).

RESULTS

Characteristics of the included studies

As shown in Figure 1, a total of 126 potential publications were initially extracted. After reading the abstracts, we excluded 67 irrelevant studies, 20 studies with insufficient data, and 11 duplicated studies. After reading the full-texts, we excluded 5 articles with no detailed genotyping data, 2 non-case-control studies, 2 laboratory studies and 3 review articles. Finally, 17 studies from 16 articles were included in this meta-analysis.

Overall, 17 studies on APE1 Asp148Glu polymorphism and GI cancer risk were identified[10,12-26], including a total of 4856 cases and 6136 case-free controls. The characteristics of the included studies are listed in Table 1. Among the eligible 17 studies, nine were carried out in Caucasians from United States, Italy, Czech, Spain, Poland and Turkey, seven were based on Asian background and carried out in China and Japan, and one was based on mixed ethnic groups. All studies were case-controlled, including 11 CRC studies, 4 gastric cancer (GC) studies and 2 esophageal

Table 1 Characteristics of the studies included in the meta-analysis

Ref.	Year	Country	Ethnicity	Cancer type	Genotyping method	Source of controls	Total sample size (cases/controls)
Zhang *et al*[10]	2014	China	Asian	CRC	PCR-CTPP	PB	247/300
Li *et al*[12]	2013	China	Asian	CRC	PCR-RFLP	HB	451/631
Canbay *et al*[13]	2011	Turkey	Caucasian	CRC	PCR-RFLP	PB	79/247
Gu *et al*[14]	2011	China	Asian	GC	PCR-RFLP	PB	572/547
Li *et al*[15]	2011	China	Asian	GC	PCR-RFLP	PB	126/156
Canbay *et al*[16]	2010	Turkey	Caucasian	GC	PCR-RFLP	PB	40/247
Brevik *et al*[17]	2010	United States	Caucasian	CRC	TaqMan	HB	304/359
Jelonek *et al*[18]	2010	Poland	Caucasian	CRC	PCR-RFLP	PB	153/273
Palli *et al*[19]	2010	Italy	Caucasian	GC	TaqMan	PB	314/548
Ye *et al*[20]	2010	China	Asian	CRC	MassARRAY	HB	123/158
Kasahara *et al*[21]	2008	Japan	Asian	CRC	PCR-RFLP	HB	68/121
Pardini *et al*[22]	2008	Czech	Caucasian	CRC	TaqMan	HB	532/532
Tse *et al*[23]	2008	United States	Caucasian	EC	TaqMan	HB	312/454
Berndt *et al*[24]	2007	United States	Caucasian	CRC	TaqMan	PB	720/725
Berndt *et al*[24]	2007	United States	Mixed	CRC	TaqMan	PB	47/48
Moreno *et al*[25]	2006	Spain	Caucasian	CRC	Arrayed primer extension	PB	359/312
Hao *et al*[26]	2004	China	Asian	EC	PCR-RFLP	PB	409/478

CRC: Colorectal cancer; GC: Gastric cancer; EC: Esophageal cancer; PCR-CTPP: Polymerase chain reaction with confronting two-pair primers; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism; PB: Population based; HB: Hospital based.

Table 2 Apurinic endonuclease 1 Asp148Glu polymorphism genotype distribution and allele frequency in cases and controls

Ref.	Genotype (*n*)								Allele frequency (*n*)				MAF
	Cases				Controls				Cases		Controls		
	Total	TT	TG	GG	Total	TT	TG	GG	T	G	T	G	
Zhang *et al*[10]	247	87	90	70	300	121	137	41	264	230	381	219	0.47
Li *et al*[12]	451	123	247	81	631	186	335	110	493	409	707	555	0.45
Canbay *et al*[13]	79	28	43	8	247	151	63	33	99	59	365	129	0.37
Gu *et al*[14]	338	69	185	84	362	110	183	69	323	353	403	321	0.52
Li *et al*[15]	126	26	64	36	156	56	70	30	116	136	182	130	0.54
Canbay *et al*[16]	40	14	18	8	247	151	63	33	46	34	365	129	0.42
Brevik *et al*[17]	304	102	137	65	359	108	167	84	341	267	383	335	0.44
Jelonek *et al*[18]	113	49	59	5	273	70	141	62	157	69	163	143	0.31
Palli *et al*[19]	298	103	147	48	546	208	243	95	353	243	659	433	0.41
Ye *et al*[20]	123	37	86	0	158	52	106	0	160	86	210	106	0.35
Kasahara *et al*[21]	68	23	45	0	121	70	51	0	91	45	191	51	0.33
Pardini *et al*[22]	531	140	261	130	530	157	267	106	541	521	581	479	0.49
Tse *et al*[23]	311	75	162	74	454	123	228	103	312	310	474	434	0.50
Berndt *et al*[24]	692	175	364	153	710	204	335	171	714	670	743	677	0.48
Berndt *et al*[24]	47	11	23	13	48	18	22	7	45	49	58	36	0.48
Moreno *et al*[25]	359	95	177	87	312	99	147	66	367	351	345	279	0.49
Hao *et al*[26]	409	126	211	72	477	149	243	95	463	355	541	433	0.43

MAF: Minor allele frequency.

cancer (EC) studies. All GI cancers were confirmed by histology or pathology. The histological type of cancers in the included studies was adenocarcinoma except one EC study[26]. Moreover, controls were matched mainly by age. Eleven studies were population-based and six were hospital-based. Several genotyping methods were used in the studies, including polymerase chain reaction-restriction fragment length polymorphism, PCR-ligase detection reaction, TaqMan, MassARRAY, and Arrayed primer extension.

Meta-analysis results

As shown in Table 2, the frequency of the G allele varied widely across the 12 studies, ranging from 0.31 to 0.54. The average frequency of the G allele in the overall population, Caucasian population and Asian population was 0.47, 0.46, and 0.47, respectively. There was no significant difference between Asians and Caucasians ($P > 0.05$). The average frequency of the G allele in CRC, GC and EC was 0.46, 0.50, and 0.46, respectively.

Overall, there was evidence of an association between GI cancer risk and the variant genotypes when all the eligible studies were pooled into the meta-analysis. As show in Figure 2 and Table 3, there was a significant association between APE1 Asp148Glu polymorphism and GI cancer risk in three genetic models in the overall population (G *vs* T: OR = 1.18,

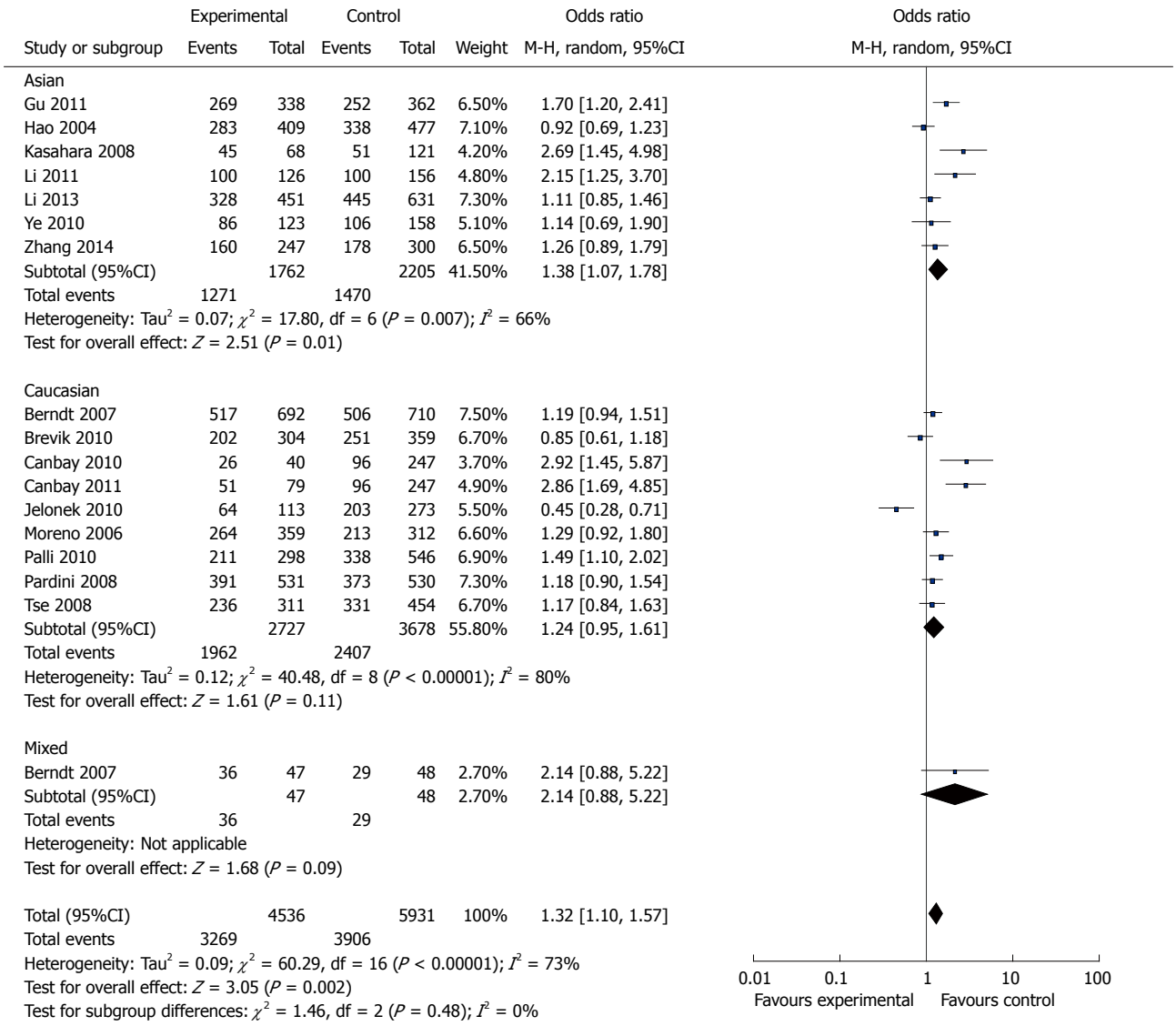

Figure 2 Forest plot of association of apurinic endonuclease 1 Asp148Glu polymorphism with gastrointestinal cancer risk stratified by ethnicity (TG + GG *vs* TT).

95%CI = 1.05-1.32, *P* = 0.004; TG *vs* TT: OR =1.28, 95%CI = 1.08-1.52, *P* = 0.004; TG + GG *vs* TT: OR = 1.32, 95%CI =1.10-1.57, *P* = 0.002). However, there was no significant association in the other two genetic models (GG *vs* TT: OR = 1.25, 95%CI = 0.98-1.59, *P* = 0.07; GG *vs* TT + TG: OR = 1.10, 95%CI = 0.90-1.34, *P* = 0.33).

In the stratified analysis by population, as shown in Figure 2 and Table 3, meta-analysis showed that Asp148Glu polymorphism had no association with GI cancer risk in Caucasians in all genetic models (allele contrast genetic model: OR = 1.09, 95%CI = 0.94-1.26, *P* = 0.24; homozygote comparison: OR = 1.25, 95%CI = 0.98-1.59, *P* = 0.81; heterozygote comparison: OR = 1.26, 95%CI = 0.98- 1.63, *P* = 0.07; the dominant model: OR = 1.24, 95%CI = 0.95-1.61, *P* = 0.11; and the recessive model: OR = 0.95, 95%CI = 0.75-1.20, *P* = 0.66).

There were 7 studies with 1587 cases and 1913 controls for assessing the relationship between Asp148Glu polymorphism and GI cancer susceptibility in Asians. As shown in Figure 2 and Table 3, Asp148Glu polymorphism was significantly associated with GI cancer risk in four genetic models (allele contrast genetic model: OR = 1.27, 95%CI = 1.07-1.51, *P* = 0.007; homozygote comparison: OR = 1.58, 95%CI = 1.05-2038, *P* = 0.03; heterozygote comparison: OR = 1.30, 95%CI = 1.01-1.67, *P* = 0.04; and the dominant model: OR = 1.38, 95%CI = 1.07-1.78, *P* = 0.01.

In the stratified analysis by cancer type, 11 studies including 3083 cases and 3706 controls were used to evaluate the relationship between APE1 Asp148Glu polymorphism and CRC risk. As shown in Table 3 and Figure 3, there was no significant association between APE1 Asp148Glu polymorphism and CRC risk under all genetic models (allele contrast genetic model: OR = 1.15, 95%CI = 0.99-1.33, *P* = 0.06; homozygote comparison: OR = 1.12, 95%CI = 0.79-1.59, *P* = 0.51; heterozygote comparison: OR = 1.23, 95%CI = 0.98-1.54, *P* = 0.08; the dominant model: OR =

Table 3 Meta-analysis results

Comparison	OR	95%CI	*P* value	Heterogeneity		Effects model
				I^2	*P* value	
G *vs* T	1.18	1.05-1.32	0.004[1]	71%	< 0.00001	Random
Caucasian	1.09	0.94-1.26	0.24	72%	0.0004	Random
Asian	1.27	1.07-1.51	0.007[1]	70%	0.003	Random
Colorectal cancer	1.15	0.99-1.33	0.06	73%	< 0.0001	Random
Gastric cancer	1.41	1.09-1.83	0.009[1]	70%	0.02	Random
Esophageal cancer	1.01	0.88-1.16	0.87	0%	0.40	Fixed
GG *vs* TT	1.25	0.98-1.59	0.07	73%	< 0.00001	Random
Caucasian	1.04	0.77-1.41	0.81	72%	0.0003	Random
Asian	1.58	1.05-2.38	0.03[1]	76%	0.002	Random
Colorectal cancer	1.12	0.79-1.59	0.51	79%	< 0.00001	Random
Gastric cancer	1.77	1.11-2.84	0.02[1]	63%	0.04	Random
Esophageal cancer	1.02	0.77-1.35	0.90	0%	0.34	Fixed
TG *vs* TT	1.28	1.08-1.52	0.004[1]	68%	< 0.0001	Random
Caucasian	1.26	0.98-1.63	0.07	76%	<0.0001	Random
Asian	1.30	1.01-1.67	0.04[1]	63%	0.01	Random
Colorectal cancer	1.23	0.98-1.54	0.08	73%	< 0.0001	Random
Gastric cancer	1.66	1.20-2.31	0.002[1]	51%	< 0.0001	Random
Esophageal cancer	1.04	0.83-1.31	0.72	0%	0.41	Fixed
GG+TG *vs* TT	1.32	1.10-1.57	0.002[1]	73%	< 0.00001	Random
Caucasian	1.24	0.95-1.61	0.11	80%	< 0.00001	Random
Asian	1.38	1.07-1.78	0.01[1]	66%	0.007	Random
Colorectal cancer	1.23	0.98-1.55	0.08	75%	< 0.0001	Random
Gastric cancer	1.77	1.40-2.24	< 0.0001[1]	19%	0.29	Fixed
Esophageal cancer	1.02	0.81-1.29	0.84	9%	0.29	Fixed
GG *vs* TT+TG	1.10	0.90-1.34	0.33	70%	< 0.0001	Random
Caucasian	0.95	0.75-1.20	0.66	64%	0.004	Random
Asian	1.36	0.95-1.95	0.10	77%	0.002	Random
Colorectal cancer	1.05	0.77-1.43	0.76	79%	< 0.00001	Random
Gastric cancer	1.25	0.99-1.56	0.06	33%	0.21	Fixed
Esophageal cancer	0.96	0.75-1.21	0.71	0%	0.38	Fixed

[1]Represent a significant association between APE1 Asp148Glu polymorphism and GI cancer risk in three genetic models in the overall population.

1.23, 95%CI = 0.98-1.55, *P* = 0.08; and the recessive model: OR = 1.05, 95%CI = 0.77-1.43, *P* = 0.76).

There were four studies including 1052 cases and 1498 controls used to evaluate the relationship between APE1 Asp148Glu polymorphism and GC risk. As shown in Table 3 and Figure 3, Asp148Glu polymorphism was significantly associated with an increased risk of GC under all genetic models (allele contrast genetic model: OR = 1.41, 95%CI = 1.09-1.83, *P* = 0.009; homozygote comparison: OR = 1.77, 95%CI = 1.11-2.84, *P* = 0.02; heterozygote comparison: OR = 1.66, 95%CI = 1.20-2.31, *P* = 0.002; and the dominant model: OR = 1.77, 95%CI = 1.40-2.24, *P* < 0.0001).

There were only two EC studies included in this meta-analysis. As shown in Table 3 and Figure 3, no significant association was detected between Asp148Glu polymorphism and EC risk.

Sensitivity analysis and publication bias

The influence of any single study on the overall estimate was analyzed by excluding one study at a time. No significant difference was observed when any of the studies was excluded. Therefore, our results were statistically reliable.

Funnel plot and Egger's test were performed to evaluate the publication bias. As shown in Figure 4, the funnel plots failed to detect any obvious asymmetry in all genotypes in the overall population, and the Egger's test revealed no publication bias (*P* > 0.05). Therefore, no significant publication bias was found in this meta-analysis.

DISCUSSION

The present meta-analysis, including 4856 cases and 6136 case-free controls from 17 case-control studies, was conducted to evaluate the association between APE1 Asp148Glu polymorphism and GI cancer risk. Our results indicated that the variant genotypes were associated with an increased risk of GI cancers, especially GC.

APE1 is a key multifunctional gene involved in the base excision repair pathway. It was reported that APE1 is associated with aggressive tumor biology and has an impact on survival of GC patients[27]. The Asp148Glu polymorphism is a common non-synonymous APE1 coding region variant. Previous studies on the association between the APE1 Asp148Glu polymorphism and GI cancer risk have shown controversial results[12-26].

In a previous meta-analysis, Gu *et al*[28] suggested that the APE1 Asp148Glu polymorphism may contribute

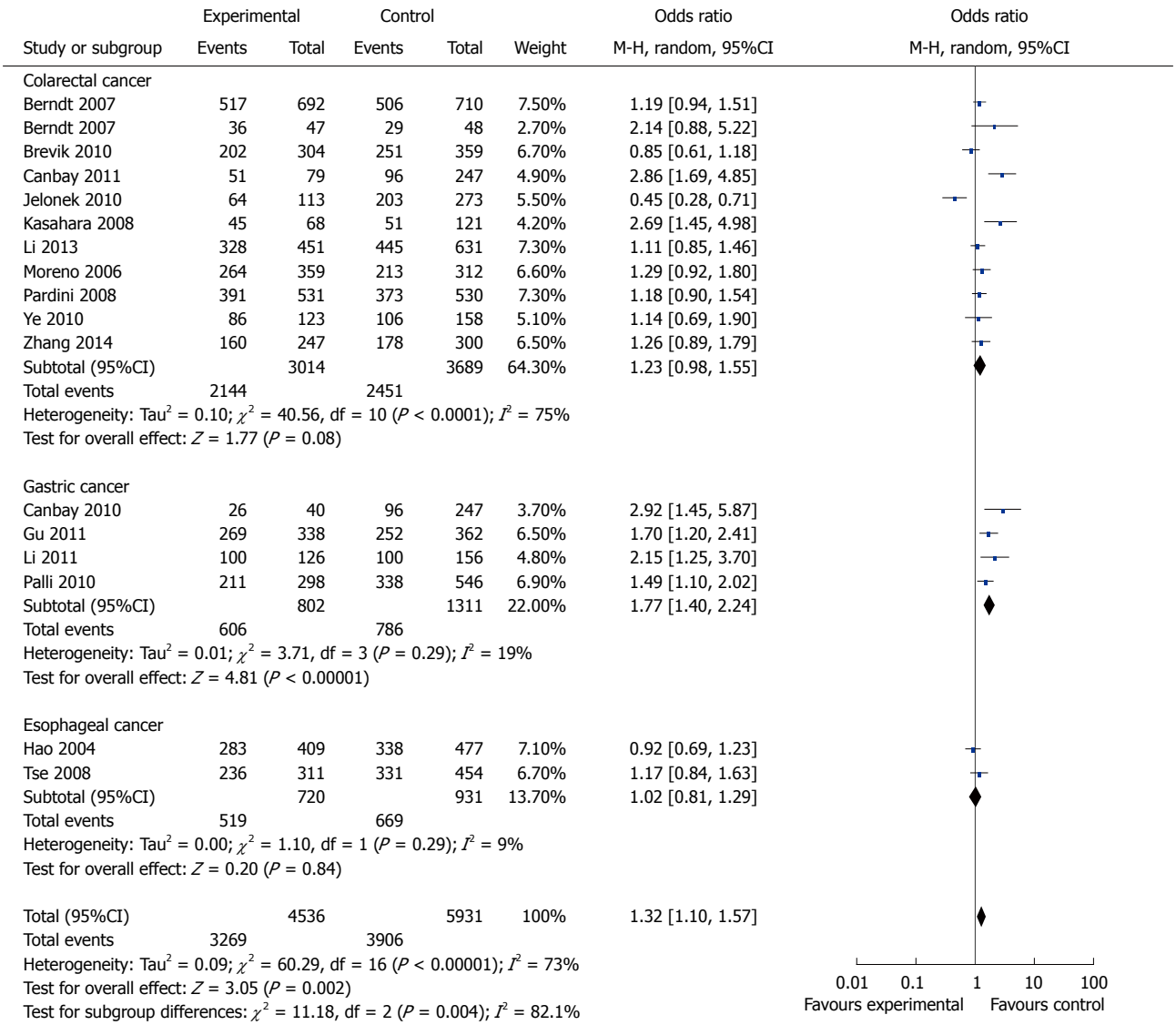

Figure 3 Forest plot of association of apurinic endonuclease 1 Asp148Glu polymorphism with GI cancer risk stratified by cancer type (TG + GG *vs* TT).

to genetic susceptibility to cancers, especially CRC. However, a recent meta-analysis by Shen *et al*[29] failed to detect an association between APE1 Asp148Glu polymorphism and CRC risk. We found several worthwhile queries in Shen's study. First, the ethnicity of Canbay's study was identified as Asian in Shen's meta-analysis[29]. While in the original article, the cases and controls were both based on Caucasians but not Asians[13]. Second, the study by Berndt *et al*[24] was carried out in Caucasians and a mixed non-Caucasian population, and therefore should probably be divided into two studies. Third, non-English literature database such as the CNKI should also be considered for the search of eligible case-control studies. We have found one eligible study published in Chinese and included it in this study[20].

In our study, 17 case-control studies were included. There was a significant association between APE1 Asp148Glu polymorphism and GI cancers risk in four genetic models in the overall population. Stratified analysis by ethnicity revealed that there was a statistically increased GI cancers risk in Asians. Further subgroup analysis by cancer type indicated that APE1 Asp148Glu polymorphism may contribute to GC risk.

There are some limitations of this meta-analysis that should be noted. First, this meta-analysis was based on pooled data while no individual data were available; thus, we could not assess the risk of cancer based on environmental factors, age, and other risk factors for GI cancers. Second, the small study effect, where the effects reported in small studies are larger, could not be avoided in some studies of a relative small size (< 200). Third, there was no significant association between Asp148Glu polymorphism and EC risk in this meta-analysis. Since only two EC studies with different pathological types[23,26] were included, this negative finding may result from a lack of statistical power. Larger scale multicenter studies are warranted to further validate the association between APE1 Asp148Glu polymorphism and GI cancer risk.

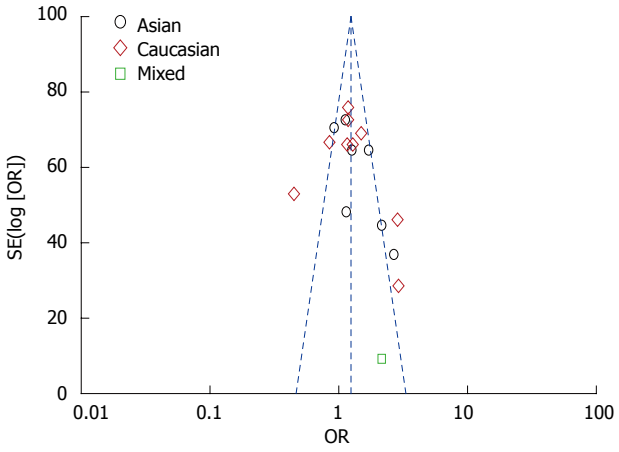

Figure 4 Funnel plot for publication bias.

In conclusion, our present meta-analysis provides evidence for the association between the APE1 Asp148Glu polymorphism and GI cancer risk. Results suggest that the APE1 Asp148Glu G allele was associated with an increased GI cancer risk among Asian subjects. Furthermore, the APE1 Asp148Glu polymorphism was associated with an increased risk GC. Further large-population based studies are needed to confirm the association between APE1 Asp148Glu polymorphism and EC risk.

COMMENTS

Background

Epidemiological studies have suggested that Asp148Glu polymorphism in the apurinic endonuclease 1 (APE1) gene is associated with gastrointestinal (GI) cancer risk. However, the results are still controversial.

Research frontiers

APE1 plays an important role in the DNA repair system and therefore has been implicated in human carcinogenesis. Epidemiologic studies suggested that single nucleotide polymorphisms (SNP) in APE1 may confer individuals' susceptibility to cancer. Recently, numerous studies have evaluated the association between APE Asp148Glu polymorphism and cancer risk. However, the results remain inconclusive.

Innovations and breakthroughs

The present meta-analysis was performed on all eligible case-control studies to estimate the association of the APE1 Asp148Glu polymorphism with GI cancer risk.

Applications

The present meta-analysis showed that the APE1 Asp148Glu G allele was associated with an increased GI cancer risk among Asian subjects. Furthermore, APE1 Asp148Glu polymorphism was associated with an increased risk of GC.

Terminology

SNP is DNA sequence variation occurring when a single nucleotide in the genome differs between members of a biological species or paired chromosomes. SNP in some genes may cause an increase or decrease in risk for some certain diseases.

Peer-review

This meta-analysis showed that the G allele of APE1 Asp148Glu polymorphism was associated with a higher gastrointestinal tract cancer risk. However, larger scale studies are warranted to further validate the association between this polymorphism and GI cancer risk.

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CASE REPORT

Case of arterial hemorrhage after endoscopic papillary large balloon dilation for choledocholithiases using a covered self-expandable metallic stent

Shuya Shimizu, Itaru Naitoh, Takahiro Nakazawa, Kazuki Hayashi, Katsuyuki Miyabe, Hiromu Kondo, Yuji Nishi, Shuichiro Umemura, Yasuki Hori, Akihisa Kato, Hirotaka Ohara, Takashi Joh

Shuya Shimizu, Itaru Naitoh, Takahiro Nakazawa, Kazuki Hayashi, Katsuyuki Miyabe, Hiromu Kondo, Yuji Nishi, Shuichiro Umemura, Yasuki Hori, Akihisa Kato, Takashi Joh, Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan

Hirotaka Ohara, Department of Community-based Medical Education, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan

Author contributions: Shimizu S designed the idea, collected clinical data and wrote the paper; Naitoh I designed the idea and wrote the paper; Nakazawa T designed the idea and wrote the paper; Hayashi K collected clinical data; Miyabe K collected clinical data; Kondo H collected clinical data; Nishi Y collected clinical data; Umemura S collected clinical data; Hori Y collected clinical data; Kato A collected clinical data; Ohara H collected clinical data; and Joh T designed the idea.

Ethics approval: The study was reviewed and approved by the Institutional Review Board of the Nagoya City University Graduate School of Medical Sciences (# 831).

Informed consent: The study was written informed consent was obtained from the patient prior to study enrollment.

Conflict-of-interest: The authors have no conflict of interest related to the manuscript.

Correspondence to: Itaru Naitoh, MD, PhD, Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan. inaito@med.nagoya-cu.ac.jp
Telephone: +81-52-8538211
Fax: +81-52-8520952

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Abstract

A 78-year-old male was admitted to our hospital because of choledocholithiasis. ERC demonstrated choledocholithiases with a maximum diameter of 13 mm, and we performed endoscopic papillary large balloon dilation (EPLBD) with a size of 15 mm. Immediately following the balloon deflation, spurting hemorrhage occurred from the orifice of the duodenal papilla. Although we performed endoscopic hemostasis by compressing the bleeding point with the large balloon catheter, we could not achieve hemostasis. Therefore, we placed a 10 mm fully covered self-expandable metallic stent (SEMS) across the duodenal papilla, and the hemorrhage stopped immediately. After 1 wk of SEMS placement, duodenal endoscopy revealed ulcerative lesions in both the orifice of the duodenal papilla and the lower bile duct. A direct peroral cholangioscopy using an ultra-slim upper endoscope revealed a visible vessel with a longitudinal mucosal tear in the ulceration of the lower bile duct. We believe that the mucosal tear and subsequent ruptured vessel were caused by the EPLBD procedure.

Key words: Endoscopic papillary large balloon dilation; Hemorrhage; Covered self-expandable metallic stent; Direct peroral cholangioscopy; Endoscopic hemostasis

Core tip: We present a case study of arterial hemorrhage after endoscopic papillary large balloon dilation (EPLBD) that was treated using a covered self-expandable metallic stent (SEMS). After 1 wk of SEMS placement, a direct peroral cholangioscopy revealed a visible vessel with a longitudinal mucosal tear in the ulceration of the lower bile duct. This image is important for understanding the mechanism of hemorrhage after EPLBD.

Shimizu S, Naitoh I, Nakazawa T, Hayashi K, Miyabe K, Kondo H, Nishi Y, Umemura S, Hori Y, Kato A, Ohara H, Joh T. Case of arterial hemorrhage after endoscopic papillary large balloon dilation for choledocholithiases using a covered self-expandable metallic stent. *World J Gastroenterol* 2015; 21(16): 5090-5095 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/5090.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.5090

INTRODUCTION

Arterial hemorrhagic complications following therapeutic endoscopic retrograde cholangiopancreatography (ERCP) are difficult to manage endoscopically because of the difficulty of observing the bleeding point and maintaining clear endoscopic vision despite the bleeding. Surgical operations or interventional radiology are usually performed to control hemorrhage after the failure of endoscopic hemostasis. However, these procedures are invasive, particularly in patients of advanced age and those with serious underlying diseases.

Endoscopic papillary large balloon dilation (EPLBD) is used for difficult extractions of large choledocholithiases because of the easy removal. The efficacy and safety of EPLBD has been reported[1,2]; however, the complications and long-term outcomes of EPLBD have not been clarified. Post EPLBD hemorrhage is one of the possible ERCP-related complications. Although there have been some reports of endoscopic hemostasis with covered self-expandable metallic stents (SEMS) against post endoscopic sphincterotomy (EST) bleeding[3-7], no post EPLBD hemorrhage cases treated with covered SEMS have been reported. Furthermore, there have been no direct cholangioscopic images to clarify the mucosal tear of the bile duct after EPLBD, which is useful for understanding the mechanism of post EPLBD hemorrhage.

In this paper, we describe a case of endoscopic hemostasis using a fully covered SEMS for a spurting hemorrhage from the lower bile duct following an EPLBD procedure with a notable cholangioscopic image of a mucosal tear in the bile duct.

CASE REPORT

A 78-year-old male was admitted to our hospital in July 2014 because of a fever elevation. He had undergone EST with a small incision in 2011 and had experienced endoscopic removal of choledocholithiasis several times. Laboratory findings on this admission revealed elevated serum levels of total bilirubin, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and γ-glutamyl transpeptidase to 5.6 mg/dL (normal range, 0.3-1.2 mg/dL), 368 U/L (normal range, 13-33 U/L), 210 U/L (normal range, 6-30 U/L), 1583 U/L (normal range, 100-340 U/L), and 336 U/L (normal range, 10-47 U/L), respectively. The serum level of C-reactive protein was 0.77 mg/dL (normal range, ≤ 0.3 mg/dL), and the white blood cell count was 9600/mm^3 (normal range, 3600-9600/mm^3). Computed tomography (CT) showed the presence of high-density lesions in the common bile duct and gallbladder with a slightly dilated biliary tract. Therefore, we diagnosed acute cholangitis because of the choledocholithiases and performed an emergency endoscopic biliary drainage. The patient did not take any anticoagulants or antiplatelet medicines. After choledocholithiases with a maximum diameter of 13 mm were identified on the cholangiogram (Figure 1), we decided to attempt EPLBD. Because the outer diameter of the therapeutic duodenoscope (TJF-260V; Olympus, Tokyo, Japan) was 13.5 mm, the diameter of the lower bile duct was estimated at 15 mm. Accordingly, we performed EPLBD using a Giga® wire-guided balloon dilator (Century Medical, Inc., Japan) with a maximum size of 15 mm that was placed across the papilla of Vater. The balloon was gradually inflated to an adequate size for stone removal with a contrast medium under endoscopic and fluoroscopic imaging until we confirmed the disappearance of the notch at the papilla of Vater. Immediately following the balloon deflation, spurting hemorrhage occurred from the orifice of the papilla of Vater. After extraction of the choledocholithiases, we performed endoscopic hemostasis by immediately compressing the bleeding point with a large balloon catheter several times;

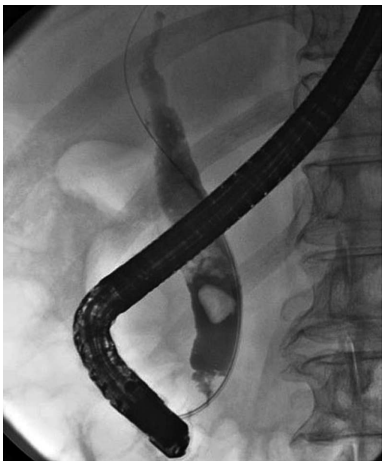

Figure 1 Choledocholithiases with a maximum diameter of 13 mm were identified on the cholangiogram.

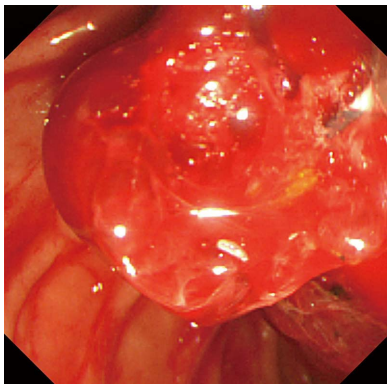

Figure 2 Endoscopic image of the papilla of Vater was gradually worsening because of the adhesion of a significant amount of coagulation.

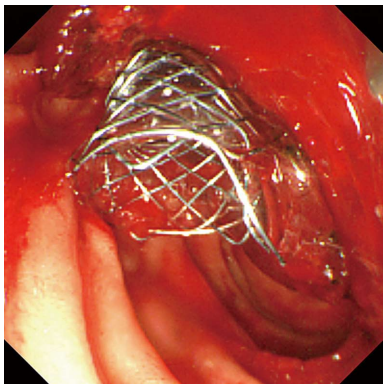

Figure 3 We placed a fully covered Wallflex® self-expandable metallic stent that was 10 mm with an 8-cm diameter across the papilla of Vater, and hemorrhage stopped immediately.

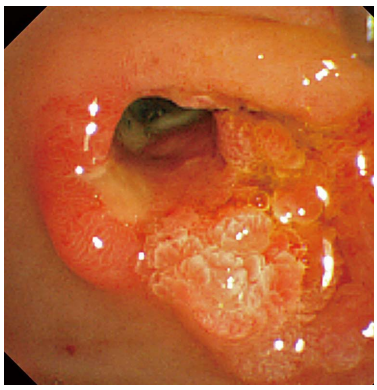

Figure 4 Re-bleeding was not observed after the self-expandable metallic stent removal, and an ulcerative lesion appeared from the orifice of the papilla of Vater to the lower bile duct.

however, we could not detect the bleeding point, and hemostasis could not be achieved. We were unable to control the hemorrhage, and the endoscopic image of the papilla of Vater was gradually worsening because of the adhesion and the significant amount of coagulation (Figure 2). We wanted to adopt a minimally invasive treatment to control hemorrhage because of serious dementia symptoms in this patient's background; therefore, we decided to place a covered SEMS for the purpose of astriction. We placed a fully covered Wallflex® SEMS (Microvasive Endoscopy, Boston Scientific Corporation, Natick, MA, United States) that measured 10 mm with an 8-cm diameter across the papilla of Vater, and hemorrhage stopped immediately (Figure 3). The hemoglobin level dropped from 11 g/dL to 7.5 g/dL; therefore, a blood transfusion of 2 units of packed red cell concentrate was performed. However, re-bleeding and early complications with respect to the SEMS placement, such as cholecystitis and pancreatitis, did not occur after endoscopic hemostasis with the SEMS placement. Thus, we could avoid surgical operation or interventional radiology (IVR) to manage the hemorrhage. In addition, acute cholangitis on admission improved after the therapeutic ERCP procedure.

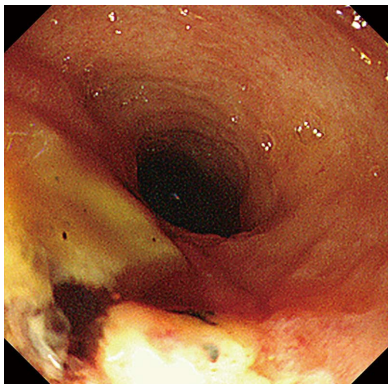

Figure 5 Direct peroral cholangioscopy revealed an exposed vessel with a laceration in the lower bile duct ulcerative area.

After 1 wk of covered SEMS placement, although the SEMS had migrated slightly to the duodenal site, hemorrhage was not observed in the endoscopic findings. Re-bleeding was not observed after the SEMS removal, and an ulcerative lesion appeared from the orifice of the papilla of Vater to the lower bile duct (Figure 4). Subsequently, we performed a direct peroral cholangioscopy using an ultra-slim upper endoscope (GIF-XP290N; Olympus, Tokyo, Japan). The cholangioscopy revealed an exposed vessel with a laceration in the lower bile duct ulcerative area (Figure 5), and we believe that this lacerative lesion was caused by mucosal damage of the lower bile duct during the EPLBD procedure.

Because re-bleeding was not observed after consuming a meal, the patient was safely discharged and is currently undergoing outpatient follow-up.

DISCUSSION

EPLBD has increasingly been performed as therapeutic ERCP against large common bile duct stones. The rates of hemorrhagic adverse events with EPLBD were reportedly 0%-6.7%[8-11]. There are no significant differences of hemorrhagic complication rates between

Table 1 Reported studies of endoscopic hemostasis using covered self-expandable metallic stents for severe post- sphincterotomy bleeding

Ref.	No. of Patients	Previous attempted treatments	SEMS type, diameter × length (mm)	Rate of complete hemostasis	The mean duration of SEMS placement
Canena *et al*[3] (2013)	4	Endoclips: 1 case Epinephrine injection: 4 cases Balloon tamponade: 2 cases	Fully covered type Hanarostent[1] or Niti-S[2] or Wallflex[3], 10 × 40: 1 case 10 × 60: 3 cases	100%	7.5 d
Itoi *et al*[5] (2011)	11	Endoclips: 7 cases Epinephrine injection: 9 cases Balloon tamponade: 11 cases	Covered type Wallstent[4]: 8 cases Wallflex: 2 cases Niti-S: 1 case 10 × 60	100%	8.2 d
Shah *et al*[6] (2010)	5	Endoclips: 1 case Epinephrine injection: 2 cases Thermal coagulation: 1 case IVR embolization: 1 case Balloon tamponade: 1case	Fully covered Wallflex, 10 × 40: 2 cases 10 × 60: 2 cases 8 × 60: 1 case	100%	4-5 wk
Di Pisa *et al*[7] (2010)	2	Endoclips: 1 case Epinephrine injection: 2 cases Balloon tamponade: 2 cases	Partially covered type Wallstent, 10 × 40	100%	3 wk

[1]Hanarostent (M.I. Tech, Seoul, South Korea); [2]Niti-S (Taewoong Medical, Seoul, South Korea); [3]Wallflex (Boston Scientific, Natick, MA, United States); [4]Wallstent (Boston Scientific, Natick, MA, United States). SEMS: Self-expandable metallic stent; IVR: Interventional radiology.

EPLBD and EST in some reports[11,12], and the rate of hemorrhage was lower in EPLBD than EST in other studies[13]. In general, the rate of hemorrhage after EPLBD is considered low. However, fatal hemorrhage with EPLBD has also been reported[14]. Accordingly, we should keep in mind the possibility of life-threatening massive bleeding with EPLBD. Furthermore, we should understand how to manage hemorrhagic complications.

Endoscopic interventions for bleeding complications include conventional injection therapy using hypertonic saline epinephrine, balloon tamponade, the application of coagulation current during a sphincterotomy, endoclip placement, *etc*. When complete endoscopic hemostasis cannot be achieved in spite of these procedures, invasive interventions such as surgical operations or IVR are required. There are several reports of endoscopic hemostasis using covered SEMS for post-EST bleeding in recent years[3-7] (Table 1). The authors placed a covered SEMS that was 10 mm in diameter across the papilla of Vater and removed it endoscopically 1 wk to 2 mo after successful hemostasis. According to these reported cases, complete hemostasis was achieved in all the patients. Therefore, covered SEMS placement is considered useful in selected patients with uncontrolled post-EST bleeding. However, there have been no reported cases of endoscopic hemostasis using covered SEMS after hemorrhage during an EPLBD procedure. In our case, we performed EPLBD with a maximum size of 15 mm, and the size of the SEMS was 10 mm in diameter. Therefore, theoretically, the bleeding point was not fully compressed by a 10 mm covered SEMS. Nevertheless, we could manage the bleeding, and the SEMS had not migrated at the time of placement. Presumably, even if we performed EPLBD with a maximum size of 15 mm, the dilated bile duct would become slightly smaller after deflation of the balloon dilation, and blood clots might occur between the bleeding point and the covered SEMS. Hence, we succeeded in endoscopic hemostasis with a covered SEMS 10 mm in size. The covered SEMS was removed 1 wk after the confirmation of controlled hemorrhage, as in the reported cases[3,5].

EPLBD is the procedure used to dilate the lower bile duct by randomly cutting off the sphincter of ampulla. We performed a direct peroral cholangioscopy after SEMS removal and confirmed a laceration with an exposed vessel in the ulcerative lesion of the lower bile duct. A direct cholangioscopic image after EPLBD has not been previously reported; however, there is literature on a linear mucosal tear after balloon dilation in the esophagus[15]. Adams *et al*[15] reported that 15% of treated achalasia cases were abnormal after balloon dilation, with complications such as complete rupture or incomplete tears of the esophageal wall and localized outpouch or diverticulum. Moreover, the patient had undergone EST in 2011, and we did not perform EST before EPLBD during this admission. Thus, we believe that the mucosal tear and subsequent ruptured vessel were caused by the EPLBD procedure. There is a possibility that a mucosal tear of the duodenal papilla or lower bile duct often occurs after EPLBD, similar to in the esophagus. This image is important for understanding the mechanism of hemorrhage after EPLBD. Arterial hemorrhage after EST usually occurs from the duodenal papilla. However, arterial hemorrhage after EPLBD might occur not only from the duodenal papilla but also from the lower or middle bile duct. We should consider the

possibility of hemorrhage from the bile duct when we encounter hemorrhage after EPLBD. It is difficult to manage hemorrhage from the lower bile duct by endoscopic hemostasis, such as through conventional injection therapy, application of coagulation current, or endoclip placement. In the present case, we finally obtained hemostasis using a covered SEMS. Endoscopic hemostasis using a covered SEMS is one of the optional procedures for post EPLBD hemorrhage after the failure of other endoscopic therapies. We believe the cholangioscopic image of laceration with an exposed vessel in an ulcerative lesion of the lower bile duct is beneficial and educational for endoscopists performing EPLBD because we can investigate the mechanism of hemorrhage after EPLBD.

In summary, we have described a case of endoscopic hemostasis using a fully covered SEMS for arterial hemorrhage from the lower bile duct following EPLBD. We must consider the possibility of hemorrhage from the bile duct when we encounter post EPLBD bleeding. Endoscopic hemostasis using a covered SEMS might be a useful option.

COMMENTS

Case characteristics

A 78-year-old male with choledocholithiasis.

Clinical diagnosis

Arterial hemorrhage from the orifice of the duodenal papilla after endoscopic papillary large balloon dilation (EPLBD).

Differential diagnosis

Hemorrhage occurred from the duodenal papilla or the lower bile duct.

Laboratory diagnosis

The hemoglobin level dropped from 11 g/dL to 7.5 g/dL; therefore, a blood transfusion of 2 units of packed red cell concentrate was performed.

Imaging diagnosis

The cholangioscopy revealed an exposed vessel with a laceration in the lower bile duct ulcerative area, which was caused by mucosal damage during the EPLBD procedure.

Pathological diagnosis

The authors did not perform a pathological examinations in this case study.

Treatment

The authors placed a 10 mm fully covered self-expandable metallic stent across The authors duodenal papilla after the failure of hemostasis by compressing the bleeding point with a large balloon catheter.

Related reports

There have been several reports of endoscopic hemostasis using a covered SEMS for post- endoscopic sphincterotomy bleeding in recent years. However, there have been no reported cases of endoscopic hemostasis using a covered SEMS after hemorrhage during an EPLBD procedure.

Term explanation

EPLBD is the procedure used to dilate the lower bile duct by randomly cutting off the sphincter of ampulla. EPLBD has increasingly been performed as therapeutic ERCP against large common bile duct stones.

Experiences and lessons

The authors should keep in mind the possibility of life-threatening massive bleeding with EPLBD. Arterial hemorrhage after EPLBD might occur from both the duodenal papilla and the lower or middle bile duct.

Peer-review

This is a straight forward case report whose main point is the cholangioscopic image demonstrating a bile duct tear following endoscopic balloon dilation for removal of a bile duct stone. The figures are adequate. The paper is likely of interest, not because of as the authors state "understanding the mechanism post EPLBD hemorrhage" but rather highlighting the use of fully covered stents in this situation (*i.e.*, for bleeding).

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CASE REPORT

Endoscopic removal of a tablespoon lodged within the duodenum

Takashi Watanabe, Kunihiko Aoyagi, Yoshitaka Tomioka, Hideki Ishibashi, Shotaro Sakisaka

Takashi Watanabe, Kunihiko Aoyagi, Yoshitaka Tomioka, Hideki Ishibashi, Shotaro Sakisaka, Department of Gastroenterology and Medicine, Fukuoka University School of Medicine, Fukuoka 814-0180, Japan
Author contributions: Watanabe T and Aoyagi K designed the report; Watanabe T, Tomioka Y and Ishibashi H collected the patient's clinical data; Watanabe T, Aoyagi K and Sakisaka S wrote the paper.
Supported by Department of Gastroenterology and Medicine, Fukuoka University School of Medicine, Fukuoka, Japan.

Correspondence to: Takashi Watanabe, MD, Department of Gastroenterology and Medicine, Fukuoka University School of Medicine, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan. takashii@minf.med.fukuoka-u.ac.jp
Telephone: +81-92-8011011-3355
Fax: +81-92-8742663
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Abstract

Here we report the case of a 34-year-old man who underwent endoscopic removal of a tablespoon from the stomach that was lodged within the duodenum. Removal required the use of a two-channel upper endoscope and polypectomy snares. Using the double-snare technique, the spoon was grasped at the proximal and distal parts of the handle. The double-snare was first pulled unsuccessfully and then pulled with simultaneous manual abdominal compression of the bulbus from the body surface. Compression was gently applied towards the stomach. As a result, the head of the spoon prolapsed from the bulbus, and was easily retracted from the stomach without any complications. In cases of foreign body lodging within the duodenum, the manual abdominal compression technique may help clinicians pull out the object and avoid surgery. The usefulness of manual compression is dependent on the foreign body's sharpness and the location.

Key words: Endoscopic removal; Tablespoon; Duodenum; Lodged

Core tip: Here we report the case of endoscopic removal of a tablespoon from the stomach that was lodged within the duodenum. Only the double-snare technique was first performed unsuccessfully and then pulled with simultaneous manual abdominal compression of the bulbus from the body surface. Compression was gently applied toward the stomach. As a result, the head of the spoon prolapsed from the bulbus and was easily retracted from the stomach without any complications. The usefulness of manual compression is dependent on the foreign body's sharpness and location.

Watanabe T, Aoyagi K, Tomioka Y, Ishibashi H, Sakisaka S. Endoscopic removal of a tablespoon lodged within the duodenum. *World J Gastroenterol* 2015; 21(16): 5096-5098 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/5096.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.5096

INTRODUCTION

Endoscopist has often encountered cases of foreign bodies in the gastrointestinal tract. Most foreign bodies pass spontaneously through the body, but 10%-20% require endoscopic removal and ⩽ 1% require surgery[1,2]. Surgical removal can be avoided with correct endoscopic removal.

Here we present a successful case of endoscopic removal of a tablespoon from the stomach that had lodged within the duodenum. This report is the first to detail the removal of a foreign body lodged within the duodenum using the manual abdominal compression technique.

CASE REPORT

A 34-year-old man was admitted to our hospital for the removal of a metal spoon from the stomach. Five days previously, he was hospitalized with schizophrenia in another hospital and deliberately swallowed the spoon. Plain radiography of the abdomen revealed a large spoon within the stomach (Figure 1). Surprisingly, only the handle of the spoon was actually in the stomach (Figure 2), while the head of the spoon was lodged within the bulbus of the duodenum. The entire spoon could not pass through the duodenum, so its handle extended to the wall of the lower part of the greater curvature of the stomach.

Using the double-snare technique, the spoon was grasped at the proximal and distal parts of the handle as described previously[3]. The double-snare was first pulled unsuccessfully, followed by simultaneous pulling with manual abdominal compression of the bulbus from the body surface (Figure 3). The compression technique was gently performed toward the stomach. As a result, the head of spoon prolapsed from the bulbus. Furthermore, this patient had severe hiatal hernia, which allowed the distal portion of the handle to be easily retracted through the esophagocardial junction. The spoon was 18 cm long and 4.0 cm at it's widest point (Figure 4). No complications such as hemorrhage or perforation were encountered.

DISCUSSION

In cases of foreign bodies in the gastrointestinal tract, treatments are chosen according to object type and location as well as the patient's general condition[4]. It is especially necessary to confirm the foreign body's size, shape, and material[5]. For example, long objects are generally difficult to extract from the stomach, while sharp foreign bodies present a high risk of mucosal injury including hemorrhage or perforation.

In the present case, the tablespoon was a long metallic object, prone to slipping and difficult to grasp[6]. The polypectomy snare is considered useful for retrieving sharp objects[7,8]. Spoon removal from the stomach utilizing the double-snare technique has been reported and its usefulness has been proven[3].

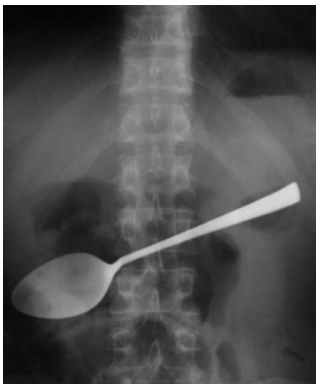

Figure 1 A plain radiograph of the abdomen revealing a tablespoon within the stomach.

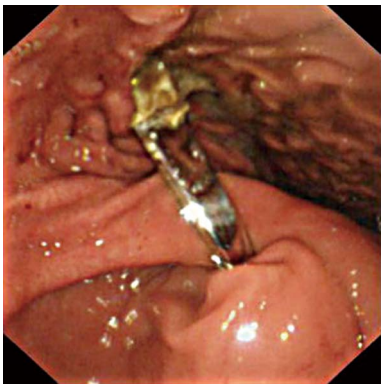

Figure 2 Endoscopic findings showing only the handle of the spoon in the stomach. The head of the spoon is incarcerated in the bulbus of the duodenum.

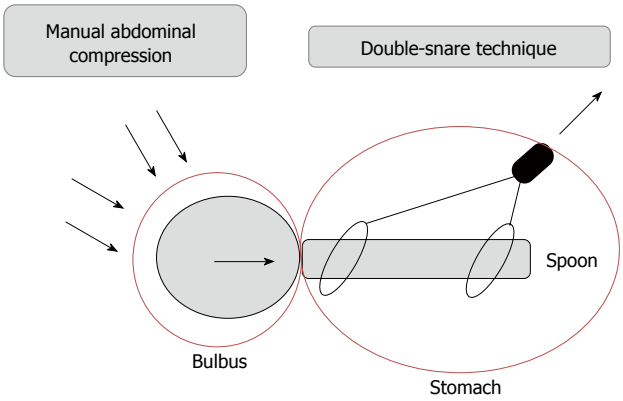

Figure 3 Image of the removal technique. The spoon was successfully removed by the combination of the double-snare technique as a pulling power and the manual abdominal compression as a pushing power.

However, since the head of the spoon was lodged in the bulbus of the duodenum, the extraction technique failed. Five days passed from the appearance of the condition, and the possibility of it spontaneously passing through the intestinal tract was low. If endoscopic extraction was not possible, the presence of the foreign body could cause pressure necrosis

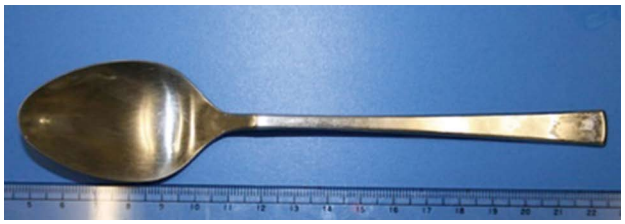

Figure 4 The retrieved spoon.

of the bowel mucosa as well as hemorrhage and perforation[9]. When a foreign body cannot be removed, surgery becomes the only option[10]. In this case, it was possible to extract the spoon from the duodenum using the manual abdominal compression technique on the body surface. However, manual compression may be dangerous if a sharp foreign body perforates the duodenal wall. Therefore, the usefulness of manual compression is dependent on the foreign body's sharpness and location.

COMMENTS

Case characteristics

A 34-year-old man with a tablespoon which lodged within the duodenum.

Clinical diagnosis

Foreign body as a tablespoon which lodged within at the duodenum.

Imaging diagnosis

The head of the spoon is incarcerated in the bulbus of the duodenum.

Treatment

The foreign body was removed by the double-snare technique and manual abdominal compression technique.

Related reports

There are a lot of cases of the large foreign bodies removed in the gastrointestinal by endoscopy.

Experiences and lessons

The authors report the case of endoscopic removal of a tablespoon from the stomach that was lodged within the duodenum. The usefulness of manual compression is dependent on the foreign body's sharpness and the location.

Peer-review

This is a interesting case which performed endoscopic removal of a tablespoon from the stomach that was lodged within the duodenum.

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CASE REPORT

Oxyntic gland adenoma endoscopically mimicking a gastric neuroendocrine tumor: A case report

Tae-In Lee, Jae-Young Jang, Seungmin Kim, Jung-Wook Kim, Young-Woon Chang, Youn-Wha Kim

Tae-In Lee, Jae-Young Jang, Seungmin Kim, Jung-Wook Kim, Young-Woon Chang, Division of Gastroenterology, Department of Internal Medicine, College of Medicine, Kyung Hee University, Seoul 136-705, South Korea
Youn-Wha Kim, Department of Pathology, College of Medicine, Kyung Hee University, Seoul 136-705, South Korea
Author contributions: Lee TI and Jang JY designed the report; Jang JY was the patient's attending doctor; Kim S, Kim JW and Chang YW organized the report; Kim YW performed the pathologic examinations; Lee TI wrote the paper.

Correspondence to: Jae-Young Jang, MD, PhD, Division of Gastroenterology, Department of Internal Medicine, College of Medicine, Kyung Hee University, 23 Kyungheedae-ro, Dongdaemun-gu, Seoul 130-872, South Korea. jyjang@khu.ac.kr
Telephone: +82-2-9588200
Fax: +82-2-9681848
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Abstract

Gastric adenocarcinoma is one of the most common malignancies worldwide. Histochemical and immunohistologic analyses classify the phenotypes of gastric adenocarcinoma into several groups based on the variable clinical and pathologic features. A new and rare variant of gastric adenocarcinoma with chief cell differentiation (GA-CCD) has recently been recognized. Studies reporting the distinct clinicopathologic characteristics proposed the term oxyntic gland polyp/adenoma because of the benign nature of the GA-CCD. Typically, GA-CCD is a solitary mucosal lesion that develops either in the gastric cardia or fundus. Histologically, this lesion is characterized by tightly clustered glands and anastomosing cords of chief cells. Immunohistochemically, GA-CCD is diffusely positive for mucin (MUC) 6 and negative for MUC2 and MUC5AC. However, other gastric tumors such as a gastric neuroendocrine tumor or fundic gland polyp have been difficult to exclude. Because GA-CCD tends to be endoscopically misdiagnosed as a neuroendocrine tumor or fundic gland polyp, comprehensive assessment and observation by an endoscopist are strongly recommended. Herein, we report a rare case of oxyntic gland adenoma endoscopically mimicking a gastric neuroendocrine tumor that was successfully removed by endoscopic mucosal resection.

Key words: Chief cell differentiation; Gastric carcinoma; Mucin 6; Neuroendocrine tumor; Oxyntic gland adenoma

Core tip: Gastric adenocarcinoma with chief cell differentiation is a new and rare variant of gastric adenocarcinoma. Due to difficulty in ruling out other gastric tumors, such as gastric neuroendocrine tumor, fundic gland polyp, or gastric adenoma, comprehensive examination and observation by an endoscopist are strongly recommended. We report a rare case of oxyntic gland adenoma endoscopically mimicking a gastric neuroendocrine tumor that was successfully removed by endoscopic mucosal resection.

Lee TI, Jang JY, Kim S, Kim JW, Chang YW, Kim YW. Oxyntic gland adenoma endoscopically mimicking a gastric neuroendocrine tumor: A case report. *World J Gastroenterol*

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INTRODUCTION

First reported in 2007, fundic gland type with chief cell differentiation represents a novel, rare variant of gastric adenocarcinoma[1]. Singhi *et al*[2] recently reported ten cases of gastric adenocarcinoma with chief cell differentiation (GA-CCD) in patients from multiple ethnic backgrounds. The term oxyntic gland polyp/adenoma was proposed instead of GA-CCD, as GA-CCD is regarded as benign based on the natural course of the disease described in the previous studies. GA-CCD is very rare, with unknown clinicopathologic features and natural course[1-3]. We herein report a rare case of oxyntic gland adenoma endoscopically mimicking a gastric neuroendocrine tumor that was successfully removed by endoscopic mucosal resection (EMR).

CASE REPORT

A 73-year-old man with a history of type 2 diabetes mellitus, dyslipidemia and benign prostate hypertrophy was admitted for evaluation of a suspected gastric neuroendocrine tumor following an esophagogastroduodenoscopy (EGD) screening of a gastric lesion. The patient had no history of alcohol use or smoking. Upon physical examination, the patient exhibited no specific symptoms or abnormalities. Blood test results were as follows: white blood cell count, 5.9 × 10^3/μL (normal range: 4.0-10.0 × 10^3/μL); hemoglobin, 15.9 g/dL (normal range: 12.0-16.0 g/dL); platelet count, 162 × 10^3/μL (normal range: 150-350 × 10^3/μL). All other laboratory findings were within normal limits. The serum gastrin level was 61.5 pg/mL (normal range: 0.0-180.0 pg/mL). EGD showed a 6 mm, slightly elevated, yellowish lesion with irregular surface vessels at the greater curvature of the upper body (Figure 1). The rapid urease test (CLO test; Delta West Pty Ltd., Bentley, Australia) was negative. Endoscopic ultrasonography revealed a 6.7 mm, homogeneous, hypoechoic mass invading the submucosal layer (Figure 2) with a thick mucosa. No specific gastric wall abnormalities or lymph node enlargements were noted on the abdominal CT scan.

EMR was performed under conscious sedation for the duration of the treatment in order to establish the definitive diagnosis of the lesion. The inject-lift-and-cut technique was employed through two separate channels of a double-channel endoscope; the grasping forceps were used to lift the lesion, and an electrocautery snare was used to remove the lesion (Figure 3). No complications, such as bleeding or perforation, were observed after the procedure. The patient started eating two days after EMR, and was discharged on the third hospital day.

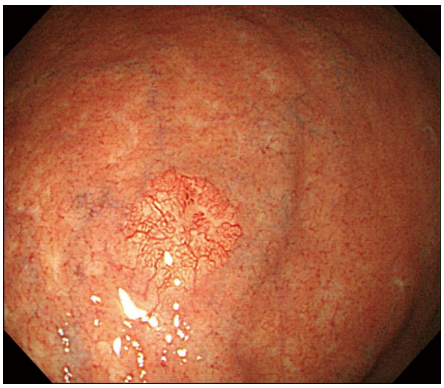

Figure 1 Esophagogastroduodenoscopy findings. Esophagogastroduodenoscopy revealed a 6 mm, slightly elevated, yellowish lesion with irregular surface vessels at the greater curvature of the upper body.

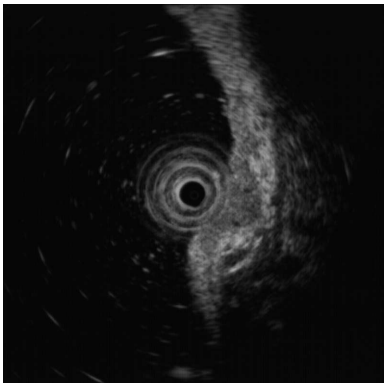

Figure 2 Endoscopic ultrasonography findings. Endoscopic ultrasonography revealed a 6.7 mm, homogeneous, hypoechoic mass that appeared to invade the submucosal layer.

Sections from the stomach showed proliferation of glands with complex architecture confined to the mucosa and no submucosal invasion (Figure 4A). A high-power view of the lesion showed a complex growth of glands lined with one to two cell layers of oxyntic epithelium containing abundant chief cells (Figure 4B). The lining cells showed moderate nuclear pleomorphism with no identifiable mitotic figures. Immunohistochemical analysis showed most of the glandular cells were positive for mucin (MUC) 6 (Figure 5) and negative for MUC2, MUC5AC and α-fetoprotein. Ki-67 index was < 1% (Figure 5E). Consequently, the patient was diagnosed with oxyntic gland adenoma with high-grade dysplasia that correlated with immunohistochemical staining. A follow-up EGD, performed two months after the EMR, showed a whitish scar with no recurrence.

DISCUSSION

Gastric adenocarcinoma is defined as a malignant epithelial tumor, originating from glandular epithelium

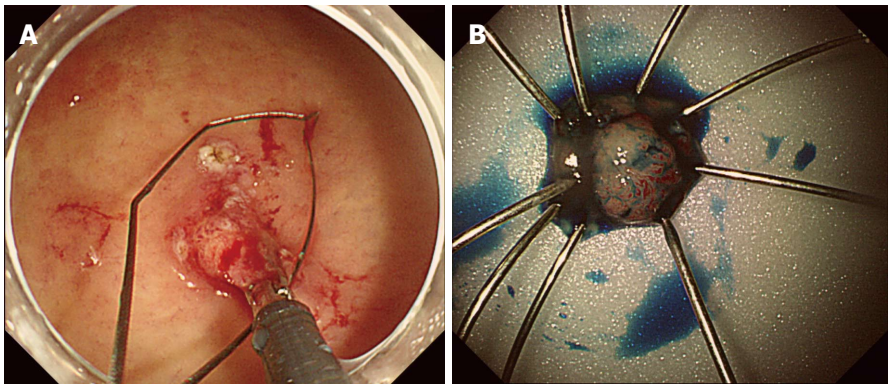

Figure 3 Endoscopic mucosal resection findings. A: The inject-lift-and-cut technique was performed; B: Pathologic specimen.

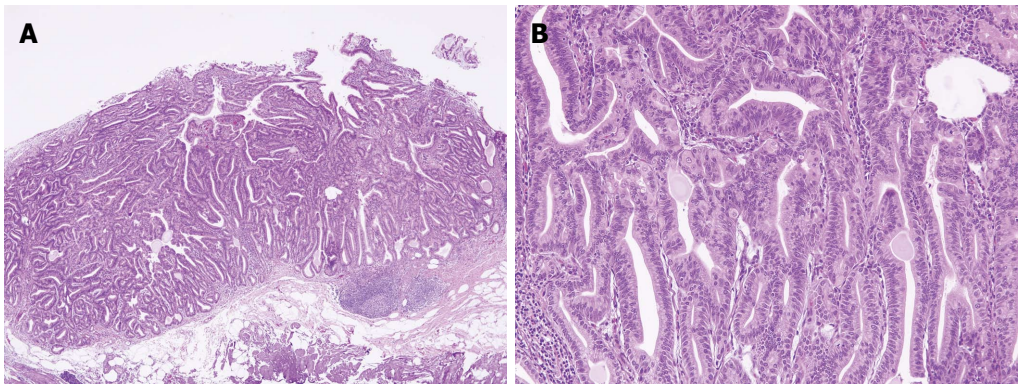

Figure 4 Pathologic examinations. A: Hematoxylin and eosin staining showed proliferation of glands with complex architecture, situated deep in the mucosal layer with surface involvement (× 40); B: A higher magnification showed complex growth of glands lined with one to two layers of oxyntic epithelium with abundant chief cells. The lining cells showed moderate nuclear pleomorphism, but no mitotic figures were identified (× 200).

Table 1 Recently published literature on oxyntic gland polyp/adenoma cases

Ref.	Journal	Year	Number of cases	Title
Tsukamoto *et al*[1]	*Pathol Int*	2007	1	Gastric adenocarcinoma with chief cell differentiation
Ueyama *et al*[3]	*Am J Surg Pathol*	2010	10	Gastric adenocarcinoma of fundic gland type (chief cell predominant type): proposal for a new entity of gastric adenocarcinoma
Park *et al*[4]	*Korean J Pathol*	2012	3	Gastric adenocarcinoma of fundic gland type: report of three cases
Singhi *et al*[2]	*Am J Surg Pathol*	2012	10	Gastric adenocarcinoma with chief cell differentiation: a proposal for reclassification as oxyntic gland polyp/adenoma
Ueyama *et al*[9]	*Endosc*	2014	10	Gastric adenocarcinoma of the fundic gland type (chief cell predominant type)

of the gastric mucosa[2]. Using previous histochemical and immunohistologic analyses for MUC, the phenotype of gastric adenocarcinoma can be classified into various subtypes[1]. The gastric phenotypes include the foveolar, pyloric gland, and fundic gland types[4]. Currently, little information is available concerning gastric adenocarcinoma featuring fundic gland cell types[1]. GA-CCD is a recently reported rare variant of gastric adenocarcinoma[2]. After the initial report on GA-CCD[1], the subsequent follow-up study of ten GA-CCD cases expanded on the clinicopathologic features of this disease[3]. Park *et al*[4] reported three cases of GA-CCD among Koreans (Table 1). Interestingly, Singhi *et al*[2] noted that the use of the term GA-CCD seemed to overemphasize the biologic potential of these lesions, which have not been reported to metastasize and display a lower proliferative index compared to fundic gland polyps. Considering the benign feature of GA-CCD, these authors proposed the term oxyntic gland polyp/adenoma to be used until further studies could clarify the pathogenesis of these lesions and their natural history[2]. In our case, a lesion endoscopically mimicking a gastric neuroendocrine tumor was diagnosed as oxyntic gland adenoma. Initial biopsy findings were chronic gastritis with intestinal metaplasia. However, EMR allowed for a definitive diagnosis and treatment of an oxyntic gland adenoma with high grade-dysplasia. The previously reported

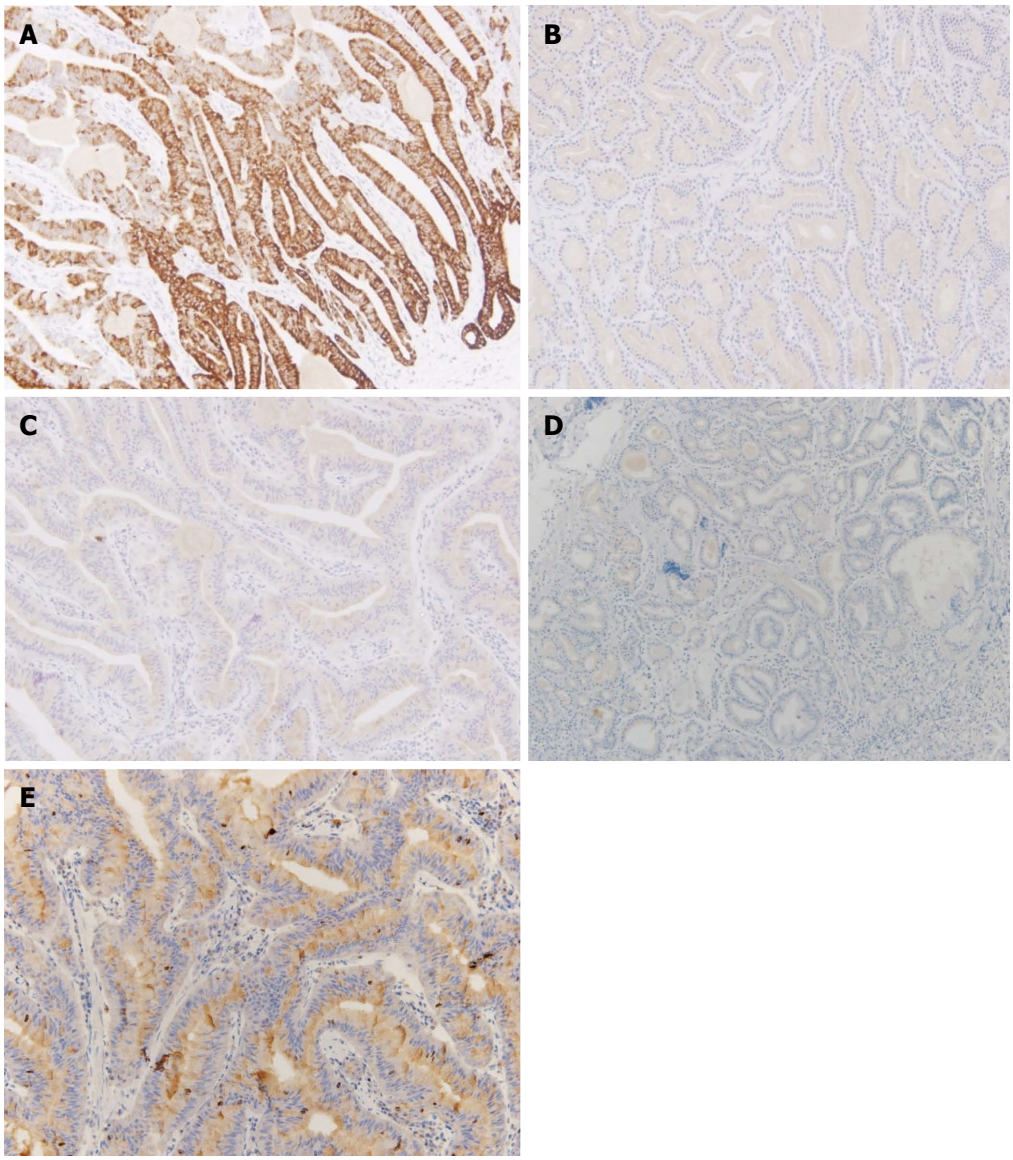

Figure 5 Immunohistochemical staining for mucin, α-fetoprotein, and Ki-67. A: Most of the glandular cells were positive for mucin (MUC) 6; B: Most of the glandular cells were negative for MUC2; C: Most of the glandular cells were negative for MUC5AC; D: Most of the glandular cells were negative for alpha fetal protein; E: Ki-67 immunolabeling demonstrates a low proliferation index (< 1%).

cases of oxyntic gland adenoma have had favorable prognoses[1-4]. Similarly, the present case was expected to have a good prognosis despite the presence of high-grade dysplasia. However, a more prolonged follow-up investigation of the patient is needed. Some studies have reported the presence of histologic variants of gastric adenocarcinoma featuring specific cell types based on MUC immunohistochemistry or electron microscopy, including choriocarcinoma, hepatoid carcinoma, carcinoma with lymphoid stroma, Paneth cell carcinoma, small cell carcinoma, and parietal cell carcinoma[5]. Depending on the variant, the prognosis was either better or worse than the typical gastric adenocarcinoma[5].

Histologically, GA-CCD is a well-differentiated adenocarcinoma composed primarily of cells resembling chief cells[1-4]. GA-CCD is typically centered in the deep mucosa and consists of clustered glands and irregular anastomosing cords of predominantly chief cells[1]. In our present case, no lymphovascular or submucosal invasion, geographic necrosis or mitotic figures were identified, which helped exclude malignancy despite the striking architectural disturbance. MUCs are high-molecular-weight glycoproteins synthesized by secretory epithelial cells as membrane-bound or secreted products[6]. Pinto-de-Sousa *et al*[7] showed the MUC phenotype associated with the tumor site. Normal gastric mucosa express MUC1, MUC4, MUC5AC, and MUC6[6]. According to the previous reports, GA-CCD is diffusely positive for MUC6, but negative for MUC2 and MUC5AC[1-4,8]. In our present case, immunohistochemical analysis revealed the lesion positive for MUC6 and negative for MUC2, MUC5AC, and α-fetoprotein with a low Ki-67 proliferative index,

excluding a neuroendocrine tumor and confirming the diagnosis.

In several studies, GA-CCD was located in the upper third of the stomach and arose from a deeper area of the gastric mucosa[1-3]. For this reason, endoscopic findings of GA-CCD may resemble those of fundic gland polyp or gastric neuroendocrine tumor[4]. Recently, Ueyama *et al*[9] analyzed the endoscopic findings of ten GA-CCD cases including the shape, color, vessels and background mucosa using the Sydney system to elucidate the endoscopic characteristics[10]. In terms of gross appearance, all of the lesions were classified into two subtypes: the submucosal tumor-shape type (60%) and a flat and depressed type (40%)[9]. Therefore, it was suggested that the submucosal tumor-shape type is needed to rule out other submucosal tumors. Consequently, the authors proposed that the discriminating characteristics for gastric neuroendocrine tumor were related to color tone, vessels, and hardness. Gastric neuroendocrine tumors have a yellow tone, few vessels on the surface, and a hard appearance, whereas GA-CCD are faded or whitish, have a soft appearance, and have more branched vessels on the surface[9]. Endoscopically, the four most frequent features of GA-CCD were submucosal tumor shape, whitish color, dilated vessels with branch architecture, and background mucosa without atrophic change[9]. In our present case, endoscopic findings showed that the lesion was hard, movable, and a submucosal tumor shape, with irregular and dilated vessels on the surface, consistent with two of the four most frequent features of GA-CCD. Further research is necessary to better our understanding of the endoscopic characteristics of GA-CCD.

Helicobacter pylori (*H. pylori*) plays an important role in gastric carcinogenesis, as the majority of non-cardia gastric cancers develop from *H. pylori*-infected mucosa[11]. Literature research did not yield any references describing a relationship between *H. pylori* infection and oxyntic gland adenoma. In the case of our patient, the rapid urease test was negative. Further research is necessary to elucidate the relationship between *H. pylori* infection and oxyntic gland adenoma.

In conclusion, the lesion was diagnosed as GA-CCD based on the fact that the lesion endoscopically mimicked a gastric neuroendocrine tumor with shared characteristics of chief cells, was positive for unique histopathologic features, and expressed MUC6. Because GA-CCD tends to be endoscopically misdiagnosed as a neuroendocrine tumor or fundic gland polyp, a tailored examination and observation by an endoscopist are strongly recommended.

COMMENTS

Case characteristics

A 73-year-old man with a history of type 2 diabetes mellitus, dyslipidemia and benign prostate hypertrophy was admitted for evaluation of a suspected gastric neuroendocrine tumor following esophagogastroduodenoscopy screening of a gastric lesion.

Clinical diagnosis

On physical examination, the patient exhibited no specific symptoms or abnormalities.

Differential diagnosis

Gastric adenocarcinoma; gastric neuroendocrine tumor; gastric polyp.

Laboratory diagnosis

White blood cell, 5.85 × $10^3/\mu L$; hemoglobin, 15.90 gm/dL; platelet, 162 × $10^3/\mu L$; serum gastrin level, 61.5 pg/mL; metabolic panel and liver function test were within normal limits.

Imaging diagnosis

Endoscopic gastroduodenoscopy revealed a 6 mm, slightly elevated, yellowish lesion with irregular surface vessels at the greater curvature of the gastric upper body.

Pathological diagnosis

Biopsy revealed oxyntic gland adenoma with high-grade dysplasia, positive for mucin (MUC) 6, and negative for MUC2, MUC5AC, and α-fetoprotein.

Treatment

The patient was treated with endoscopic mucosal resection without complications.

Related reports

There was a clear margin of endoscopically resected lesion with no lymphovascular invasion.

Experiences and lessons

Because gastric adenocarcinoma with chief cell differentiation tends to be endoscopically misdiagnosed as a neuroendocrine tumor or fundic gland polyp, a comprehensive examination and observation by an endoscopist are strongly recommended.

Peer-review

This case report is of great interest as the authors describe a rare case of oxyntic gland adenoma endoscopically mimicking gastric neuroendocrine tumor that was successfully removed by endoscopic mucosal resection.

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CASE REPORT

Nonconvulsive status epilepticus disguising as hepatic encephalopathy

Yong Min Jo, Sung Wook Lee, Sang Young Han, Yang Hyun Baek, Ji Hye Ahn, Won Jong Choi, Ji Young Lee, Sang Ho Kim, Byeol A Yoon

Yong Min Jo, Sung Wook Lee, Sang Young Han, Yang Hyun Baek, Ji Hye Ahn, Won Jong Choi, Ji Young Lee, Department of Internal Medicine, Dong-A University School of Medicine, Busan 602-715, South Korea
Sang Ho Kim, Byeol A Yoon, Department of Neurology, Dong-A University School of Medicine, Busan 602-715, South Korea
Author contributions: Jo YM and Lee SW performed the majority of clinical practice and experiments; Han SY and Baek YH provided the collection of all the patient medical records; Ahn JH and Choi WJ involved in editing the manuscript; Kim SH and Yoon BA interpreted electroencephalographic results of the patient; Jo YM and Lee JY wrote the manuscript.

Correspondence to: Sung Wook Lee, MD, PhD, Department of Internal Medicine, Dong-A University School of Medicine, 3 Ga 1, Dongdaesin-dong, Seo-gu, Busan 602-715, South Korea. sunglee@dau.ac.kr
Telephone: +82-51-2405627
Fax: +82-51-2402087
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Abstract

Nonconvulsive status epilepticus has become an important issue in modern neurology and epileptology. This is based on difficulty in definitively elucidating the condition and its various clinical phenomena and on our inadequate insight into the intrinsic pathophysiological processes. Despite nonconvulsive status epilepticus being a situation that requires immediate treatment, this disorder may not be appreciated as the cause of mental status impairment. Although the pathophysiology of nonconvulsive status epilepticus remains unknown, this disorder is thought to lead to neuronal damage, so its identification and treatment are important. Nonconvulsive status epilepticus should be considered in the differential diagnosis of patients with liver cirrhosis presenting an altered mental status. We report a case of a 52-year-old male with liver cirrhosis presenting an altered mental status. He was initially diagnosed with hepatic encephalopathy but ultimately diagnosed with nonconvulsive status epilepticus by electroencephalogram.

Key words: Liver cirrhosis; Hepatic encephalopathy; Nonconvulsive status epilepticus; Electroencephalogram

Core tip: This case highlights the probability of nonconvulsive status epilepticus in patient with liver cirrhosis and the utility of electroencephalogram to evaluate for patient with liver cirrhosis who presents an altered mental status. It is important to consider the possibility of nonconvulsive status epilepticus, evaluating for patient with liver cirrhosis who presents with an altered mental status, especially who do not respond to empirical treatment.

Jo YM, Lee SW, Han SY, Baek YH, Ahn JH, Choi WJ, Lee JY, Kim SH, Yoon BA. Nonconvulsive status epilepticus disguising as hepatic encephalopathy. *World J Gastroenterol* 2015; 21(16): 5105-5109 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/5105.htm DOI: http://dx.doi.

org/10.3748/wjg.v21.i16.5105

INTRODUCTION

Hepatic encephalopathy is defined as a reversible disturbance in central nervous system function that is observed in patients with chronic or acute liver failure. This complex neuropsychiatric disorder is mainly caused by biochemical disturbance and is a commonly diagnosed and well-recognized disorder that has been studied for many years. However, this disorder still continues to be a primary clinical problem and should be considered in any patient with liver cirrhosis who has neuropsychiatric symptoms but no brain abnormalities. Treatment depends on immediate awareness of the precipitating factors and initiation of empirical treatment.

Status epilepticus is a life-threatening condition involving constant seizures. This disorder is a well-recognized medical emergency requiring immediate treatment to prevent morbidity and mortality. However, nonconvulsive status epilepticus may not be recognized as the cause of a mental status change without obvious convulsions, so treatment can be delayed. This underdiagnosis may lead to injurious results. Nonconvulsive status epilepticus, such as the easily recognized convulsive status epilepticus, is a fatal condition requiring immediate treatment. It is often difficult to make a diagnosis, as many other conditions can cause an altered mental status in patients with liver cirrhosis.

It is important to understand the possibility of nonconvulsive status epilepticus being disguised as hepatic encephalopathy, especially when evaluating a patient with liver cirrhosis who presents an altered mental status.

Herein, we report the case of a patient with a high clinical suspicion of hepatic encephalopathy who was ultimately diagnosed as having nonconvulsive status epilepticus. To the best of our knowledge, this is the first reported case of such a presentation in Korea.

CASE REPORT

A 52-year-old man with liver cirrhosis secondary to hepatitis B and a 2 cm-sized hepatocellular carcinoma in the S4 segment of the liver was brought into the emergency department by ambulance for a confused and lethargic mental status.

On arrival, the patient had a blood pressure of 130/80 mmHg, a pulse of 65 beats per minute, a temperature of 36.5 ℃, a respiratory rate of 20 breaths per minute, and oxygen saturation of 97% in room air. The patient did not have a history of hepatic encephalopathy. Additionally, the patient did not have a history of seizures, brain trauma or other brain diseases.

Physical examination revealed hepatosplenomegaly with ascites. The patient's Glasgow Coma Scale score was 11 (eyes, 3; verbal, 4; motor, 4). There were no signs of traumatic injury anywhere on his body. His pupils were equally round and reactive to light, and his neck was soft, without the Brudzinski sign. Cardiopulmonary and abdominal examinations were unremarkable. The patient could move all extremities equally but showed shaking in both hands. The Babinski sign was normal in both feet.

Chest radiography did not reveal any pathologic lesion. Laboratory tests revealed a white blood cell count of 4830/mm^3, with 64% neutrophils, hemoglobin of 14.4 g/dL, hematocrit of 39.8%, and platelets at 77000/mm^3. The electrolyte and liver function test results are as follows: sodium, 137 mmol/L; potassium, 3.9 mmol/L; chloride, 105 mmol/L; bicarbonate, 21 mEq/L; calcium, 8.3 mg/dL; blood urea nitrogen, 11 mg/dL; creatinine, 1.0 mg/dL; total bilirubin, 3.7 mg/dL; aspartate aminotransferase, 86 IU/L; alanine aminotransferase 22 IU/L; alkaline phosphatase, 374 IU/L; albumin, 3.5 g/dL; lipase, 56 IU/L; prothrombin time (INR), 1.33; and ammonia, 88 μmol/L (reference range, 10-47 μmol/L). The random blood glucose level was 92 mg/dL. The arterial blood gas was pH 7.45.

According to the history and the laboratory data, hepatic encephalopathy grade Ⅲ was suspected. Rifaximin 400 mg was administered to the patient *via* a nasogastric tube every eight hours, and lactulose 30 mL was administered *via* a nasogastric tube every six hours to induce three to four bowel movements daily. Despite the patient immediately improving neurologically during the emergency department stay, with a Glasgow Coma Scale score of 15 (eyes, 4; verbal, 5; motor, 6), physical examination still showed shaking in both hands, even when the ammonia levels had decreased to 63 μmol/L.

The patient was admitted to the general ward. Administration of empirical antibiotics was initiated in the emergency department but was interrupted because an infectious etiology was not demonstrated and because the culture results were negative.

On the second day of his hospitalization, the patient suddenly entered a stupor, could not respond to simple commands and showed a decerebrate response to painful stimuli. At that time, his Glasgow Coma Scale score was 4 (eyes, 1; verbal, 1; motor, 2), and his ammonia levels had increased to 112 μmol/L.

Despite continuation of empirical treatment for hepatic encephalopathy, the mental status of the patient remained comatose. A neurologist was consulted. The results of a magnetic resonance imaging brain scan exhibited symmetrical high-signal abnormalities in the basal ganglia on T1-weighted images (Figure 1).

An electroencephalogram revealed generalized sequential rhythmic delta to theta activity, with

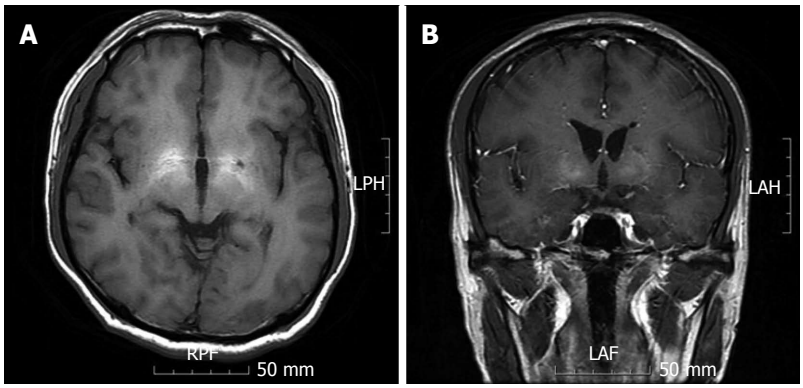

Figure 1 Magnetic resonance imaging. Results of a magnetic resonance imaging brain scan exhibited symmetrical high-signal abnormalities in the basal ganglia on T1-weighted images, consistent with liver cirrhosis (A and B).

decreasing termination and increasing onset (Figure 2A). After administration of intravenous lorazepam 4 mg, the electroencephalogram showed that the epileptiform discharge had resolved (Figure 2B and C). Because the patient had no obvious convulsions, he was diagnosed with nonconvulsive status epilepticus according to Young's criteria for electrographic status epilepticus. Levetiracetam was administered, ultimately leading to resolution of status epilepticus, as revealed by an electroencephalogram. Despite the patient being treated with levetiracetam for 4 d, he did not improve neurologically. He was transferred to another facility for constant treatment at the family's request. Unfortunately, the patient expired five days after the transfer because of multiple organ dysfunction, including hepatorenal syndrome.

DISCUSSION

Hepatic encephalopathy is often diagnosed when a patient with liver cirrhosis presents an altered mental status. The symptoms of hepatic encephalopathy generally include personality changes, poor concentration, poor judgment, disorientation and confusion[1]. Although not clearly defined, the common pathophysiological hallmark of the disorder is a synergic interaction between high ammonemia and an inflammatory response, causing astrocyte swelling and ultimately cerebral edema. Additionally, diverse metabolic components may be involved, including short-chain fatty acids, mercaptans, phenols, and false neurotransmitters[2]. An electroencephalogram can be a useful diagnostic tool for hepatic encephalopathy. Electroencephalographic findings in hepatic encephalopathy show high-amplitude, low-frequency waves and triphasic waves. Hyperammonemia has been well investigated as a contributing factor to triphasic waves on electroencephalogram in hepatic encephalopathy[3-5].

Nonconvulsive status epilepticus is a clinical disorder defined as prolonged seizure activity without major motor signs for at least 30 min. Nonconvulsive status epilepticus can be difficult to diagnose because of the absence of overt convulsive activity[6].

Despite the lack of convulsive activity, this disorder is thought to result in neuronal damage, so awareness about it and treatment are critical[7]. This disorder should be treated rapidly to prevent serious neuronal damage. In intensive care units, nonconvulsive status epilepticus may be provoked by diverse conditions, such as infections, sleep deprivation, severe electrolyte imbalance, toxic or metabolic disturbances, antiepileptic drug or alcohol withdrawal and acute or remote structural brain injury[5,8]. Because nonconvulsive status epilepticus cannot be easily distinguished from other, similar conditions, diagnosis and awareness of the precipitating factors is important[9].

The present case involved a patient with a high clinical suspicion of hepatic encephalopathy who was ultimately diagnosed with nonconvulsive status epilepticus. The patient was initially diagnosed with hepatic encephalopathy grade Ⅲ. Empirical treatment of hepatic encephalopathy was initiated, but the condition did not ultimately improve. The clinical features, the results of a magnetic resonance imaging brain scan and an electroencephalogram were suggestive of nonconvulsive status epilepticus on the basis of Young's criteria for electrographic status epilepticus.

Reports of nonconvulsive status epilepticus in patients with liver cirrhosis are very rare. Additionally, the pathophysiology of nonconvulsive status epilepticus in patients with liver cirrhosis remains unknown. Various metabolic factors may be suggested as the cause. Plasma ammonium levels are consistently elevated, and other factors, such as short-chain free fatty acids, phenols, mercaptans and false neurotransmitters, have also been implicated.

This case emphasizes the reality of nonconvulsive status epilepticus in patients with liver cirrhosis and the utility of an electroencephalogram to evaluate the patient with liver cirrhosis who presents an altered level of consciousness. It is important to consider the possibility of nonconvulsive status epilepticus, evaluating the patient with liver cirrhosis who presents

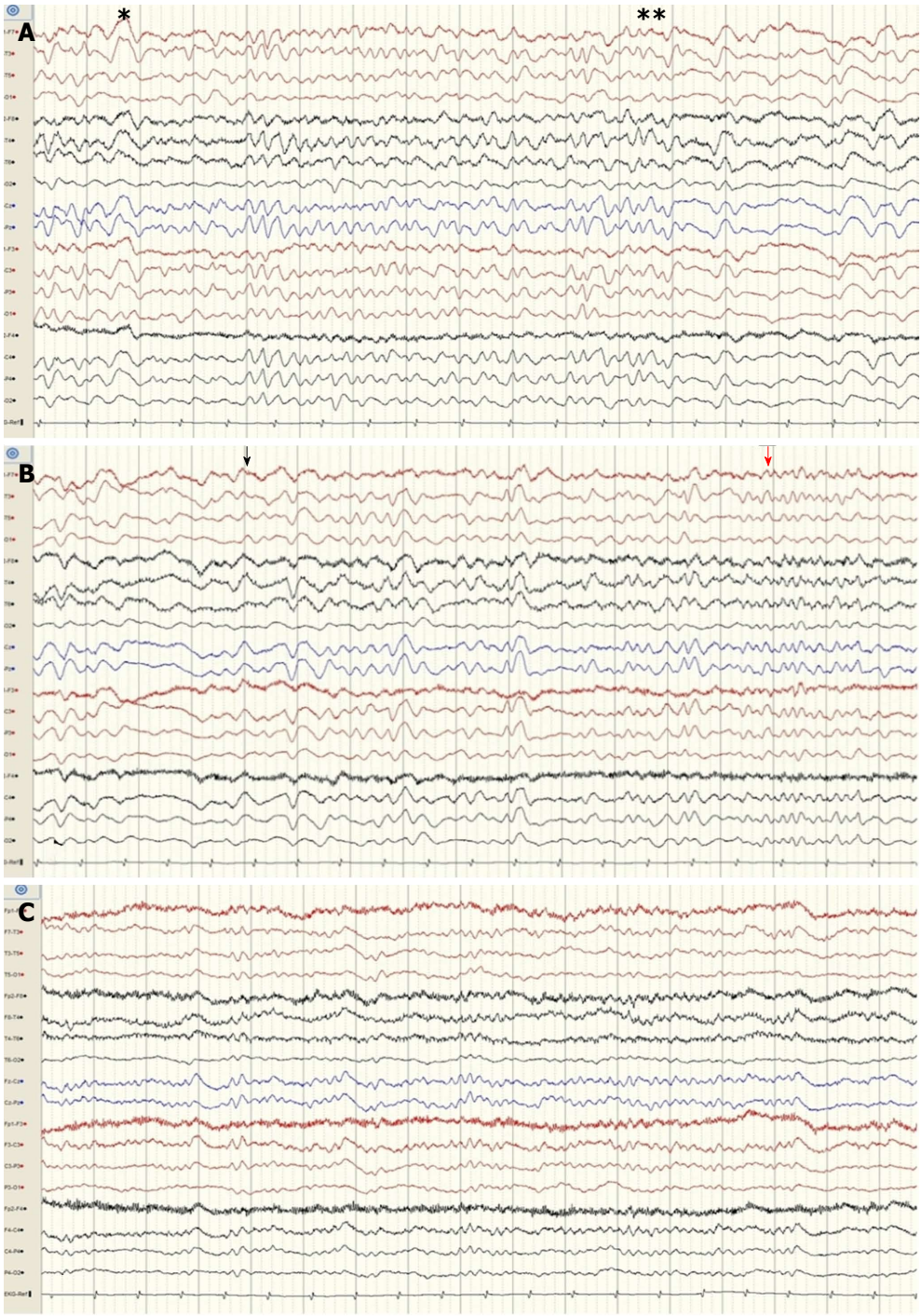

Figure 2 Electroencephalogram. An electroencephalogram revealed generalized rhythmic delta to theta activity with decreasing termination (single asterisk) and increasing onset (double asterisk) (Figure 2A). After administration of intravenous lorazepam (black arrow), the electroencephalogram showed that the epileptiform discharge had resolved (red arrow from Figure 2B to C).

with an altered mental status, and especially the patient who does not respond to empirical treatment. An emergent electroencephalogram can be useful in the evaluation of patients with hepatic encephalopathy who have alterations in their level of consciousness.

COMMENTS

Case characteristics

A 52-year-old man with liver cirrhosis secondary to hepatitis B and a 2 cm-sized hepatocellular carcinoma in the S4 segment of the liver was brought into the emergency department by ambulance for a confused and lethargic mental status.

Clinical diagnosis

He was diagnosed with nonconvulsive status epilepticus on the basis of Young's criteria for electrographic status epilepticus.

Differential diagnosis

Hepatic encepathopathy, Infectious meningoencephalitis, Central nervous system hemorrhage, Central venous system infarction.

Laboratory diagnosis

Ammonia, 88 μmol/L.

Imaging diagnosis

The results of a magnetic resonance imaging brain scan exhibited symmetrical high-signal abnormalities in the basal ganglia on T1-weighted images, consistent with liver cirrhosis.

Treatment

Despite the patient being treated with levetiracetam for 4 d, he did not improve neurologically.

Related reports

To the best of the authors' knowledge, this is the first reported case of such a presentation in Korea.

Term explanation

Nonconvulsive status epilepticus is an approximately clinical disorder defined as prolonged seizure activity without major motor signs for at least 30 min.

Experiences and lessons

It is important to consider the possibility of nonconvulsive status epilepticus, evaluating the patient with liver cirrhosis who presents with an altered mental status, and especially the patient who does not respond to empirical treatment.

Peer-review

It is important to understand the possibility of nonconvulsive status epilepticus disguising as hepatic encephalopathy in evaluating for especially patient with liver cirrhosis who presents an altered mental status.

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CASE REPORT

Management of post-gastrectomy anastomosis site obstruction with a self-expandable metallic stent

Ra Ri Cha, Sang Soo Lee, Hyunjin Kim, Hong Jun Kim, Tae-Hyo Kim, Woon Tae Jung, Ok Jae Lee, Kyung Soo Bae, Sang-Ho Jeong, Chang Yoon Ha

Ra Ri Cha, Sang Soo Lee, Hyunjin Kim, Hong Jun Kim, Tae-Hyo Kim, Woon Tae Jung, Ok Jae Lee, Chang Yoon Ha, Department of Internal Medicine, Gyeongsang National University School of Medicine, Jinju, Gyeongnam 660-702, South Korea
Kyung Soo Bae, Department of Radiology, Gyeongsang National University School of Medicine, Jinju, Gyeongnam 660-702, South Korea
Sang-Ho Jeong, Department of Surgery, Gyeongsang National University School of Medicine, Jinju, Gyeongnam 660-702, South Korea
Author contributions: Cha RR and Ha CY contributed equally to this work; Ha CY designed the study; Ha CY, Lee SS, Kim H, Kim TH, Kim HJ, Jung WT, Lee OJ, Bae KS and Jeong SH performed data collection and analysis; Cha RR, Ha CY and Jeong SH wrote the paper.
Ethics approval: The study was reviewed and approved by the Gyeongsang National University Hospital Institutional Review Board (GNUH 2014-12-007).
Informed consent: All study participants or their legal guardian provided informed written consent prior to study enrollment.
Conflict-of-interest: No potential conflicts of interest relevant to this article were reported.

Correspondence to: Chang Yoon Ha, MD, Department of Internal Medicine, Gyeongsang National University School of Medicine, 79 Gangnam-ro, Jinju-si, Gyeongnam 660-702, South Korea. cyha@gnu.ac.kr
Telephone: +82-55-7508057
Fax: +82-55-7589122
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Abstract

Post-gastrectomy anastomosis site obstruction is a relatively rare complication after a subtotal gastrectomy. We present a case of a 75-year-old man who underwent a truncal vagotomy, omental patch, gastrojejunostomy, and Braun anastomosis for duodenal ulcer perforation and a gastric outlet obstruction. Following the 10th postoperative day, the patient complained of abdominal discomfort and vomiting. We diagnosed post-gastrectomy anastomosis site obstruction by an upper gastrointestinal series and an upper endoscopic examination. We inserted a self-expandable metallic stent (SEMS) at the anastomosis site. The stent was fully expanded after deployment. On the day following the stent insertion, the patient began to eat, and his abdominal discomfort was resolved. This paper describes the successful management of post-gastrectomy anastomosis site obstruction with temporary placement of a SEMS.

Key words: Efferent loop syndrome; Postgastrectomy syndrome; Self-expandable metallic stent

Core tip: Post-gastrectomy anastomosis site obstruction is a relatively rare complication in patients following gastric surgery; however, it could cause serious discomfort and carries a risk of deterioration of the condition of the patient if a re-operation is required. We report successful management for post-gastrectomy anastomosis site obstruction with temporary placement of a self-expandable metallic stent.

Cha RR, Lee SS, Kim H, Kim HJ, Kim TH, Jung WT, Lee OJ, Bae KS, Jeong SH, Ha CY. Management of post-gastrectomy anastomosis site obstruction with a self-expandable metallic stent. *World J Gastroenterol* 2015; 21(16): 5110-5114 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i16/5110.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i16.5110

INTRODUCTION

Post-gastrectomy anastomosis site obstruction is a relatively rare complication following gastric surgery; it can cause serious discomfort and carries a risk of deterioration of the condition of the patient resulting from a re-operation. We present a case of post-gastrectomy anastomosis site obstruction after a truncal vagotomy, omental patch, gastrojejunostomy, and Braun anastomosis, and successful management with temporary placement of a self-expandable metallic stent (SEMS).

CASE REPORT

A 75-year-old man was referred to our hospital with hematemesis with epigastric pain the previous day. He had been admitted to our hospital several times for a gastric ulcer, duodenal ulcer, and gastric outlet obstruction. He had a history of smoking and daily alcohol use. On this admission, the physical examination revealed whole abdominal tenderness and rebound tenderness with rigidity. The patient presented with a fever (38.3 ℃), tachycardia (100 beats/min), and an elevated white blood cell count (12100/μL). The serum glucose was 222 mg/dL (normal range: 70-110 mg/dL), and the levels of total protein, albumin, and C-reactive protein were 5.5 g/dL (normal range: 6.4-8.3 g/dL), 3.0 g/dL (normal range: 3.4-4.8 g/dL), and 242 mg/L (normal range: 0-5 mg/L), respectively; there were no remarkable abnormalities in the other biochemical tests including the renal and hepatic function tests. The abdominal CT scans demonstrated fluid collection and pneumoperitoneum around the pylorus.

The patient underwent an emergency operation (truncal vagotomy, omental patch, gastrojejunostomy, and small-size Braun anastomosis) for the duodenal ulcer perforation and gastric outlet obstruction (Figure 1). After the 5th postoperative day, he began to eat. However, after the 10th postoperative day, he complained of abdominal discomfort and vomiting without bilious. An upper gastrointestinal series (UGIS) showed decreased peristalsis and severe distension of the stomach with no visible contrast passage to the gastrojejunostomy, and an endoscopy was performed (Figure 2). The endoscopy revealed extensive food material and anastomosis site obstruction. We diagnosed anastomosis site stricture of the gastrojejunostomy from postoperative stricture or torsion of the efferent

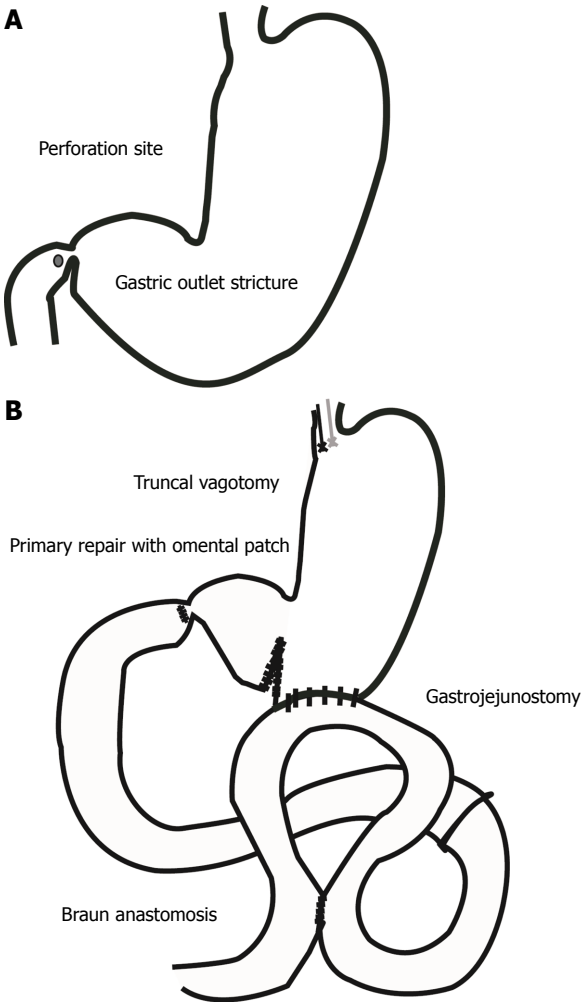

Figure 1 Schematic model of the operation. A: Before the operation; B: The primary repair and omental patch, truncal vagotomy, gastrojejunostomy, and Braun anastomosis for the duodenal ulcer perforation and gastric outlet stricture.

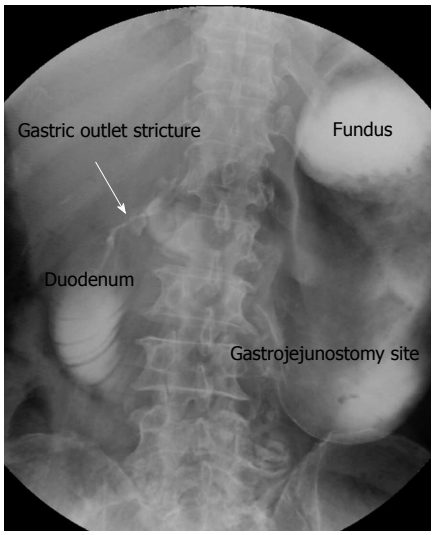

Figure 2 Upper gastrointestinal series finding on the 10th postoperative day. Decreased peristalsis and severe distension of the stomach were observed as well as stricture on the duodenal perforation site. There was no visible contrast passage to the gastrojejunostomy.

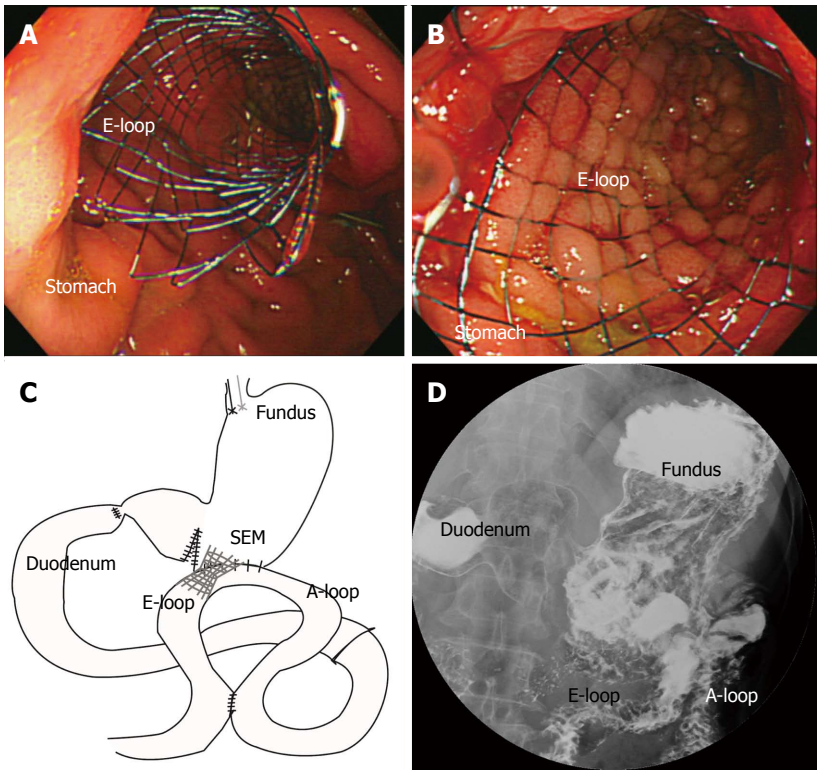

Figure 3 Endoscopic findings. A, B: A Self-expandable metallic stent was inserted at the gastrojejunostomy anastomosis site over a guide wire; C: Schematic model of the post-insertion state; D: The upper gastrointestinal series finding after the stent insertion showing good passage of the contrast material from the esophagus to the jejunum, with no evidence of leakage at the anastomosis site. A-loop: Afferent loop; E-loop: Efferent loop.

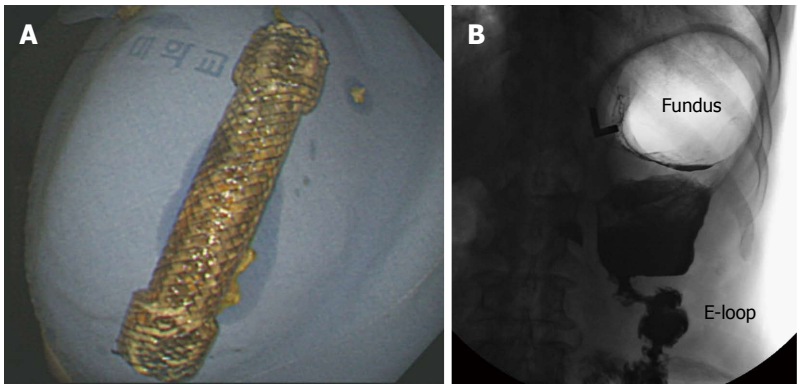

Figure 4 Follow-up upper gastrointestinal series. A: Removal of the self-expandable metallic stent; B: The upper gastrointestinal series finding showed good passage of the contrast material to efferent loop (E-loop).

loop of the gastrojejunostomy. We inserted a SEMS (a duodenal/pyloric non-covered stent; Sewoon Medical Co., Ltd., Chungcheongnam-Do, South Korea) at the anastomosis site (Figure 3A and B). Because the patient was older and did not have good general health, a reoperation was not advisable. The stent was introduced and inserted over a guide wire. The length of the stent body was 60 mm, with a diameter of 20 mm. The stent was fully expanded after deployment (Figure 3C). The UGIS showed good passage of the contrast material from the esophagus to the jejunum and no evidence of leakage at the anastomosis site (Figure 3D). On the day following the stent insertion, the patient began to eat, and his abdominal discomfort was resolved. The patient subsequently recovered, and there were no abdominal pain episodes. At the one-year follow-up, the stent had migrated into the stomach and was removed using alligator forceps with endoscopy. The UGIS revealed that the contrast dye passed the anastomosis site well. The patient survived, without other complications (Figure 4).

DISCUSSION

Post-gastrectomy anastomosis site obstruction is a rare complication (approximately 0.3%-1.0%)[1], and a re-

operation is typically performed to resolve a gastric outlet obstruction; however, re-operations cause deterioration of the patient's condition, and can be limited in elderly patients.

Two mechanical problems in post-gastric surgery complications are leakage and obstruction. First, anastomotic leakage is a life-threatening complication that requires prompt recognition and treatment[2]. Recent reports of successful endoscopic closure of anastomotic leakage suggest that endoscopic techniques might be a feasible alternative to surgical approaches[3]. Second, two loop syndromes of obstruction might occur after certain types of gastrectomies. Afferent or efferent loop syndrome is a purely mechanical problem caused by the obstruction of gastric emptying at or near the site of a gastrectomy[4]. Less invasive non-surgical procedures, such as insertion of gastrointestinal stents under endoscopy, percutaneous transhepatic biliary drainage, and percutaneous enterostomies have been used as a palliative option for post-gastrectomy obstruction[5,6].

Efferent loop syndrome is a rare post-gastrectomy complication. The major cause of the syndrome is an intestinal hernia, and minor causes include an adhesive band and kinking[7,8]. The clinical symptoms of efferent loop syndrome are characterized by abdominal cramps and bilious vomiting[7]. The treatment of efferent loop syndrome varies depending on the cause of the syndrome. Complete loop obstruction with a mechanical cause requires surgical intervention. Because of recent advances in endoscopic intervention, various treatment methods have been attempted. However, treatment has predominantly been reported in afferent loop syndrome, and very little documentation of the treatment of efferent loop syndrome exists. In several cases, endoscopic stent insertion was reported for the treatment of an obstruction caused by tumor recurrence or peritoneal seeding[8]. In this case, we used SEMS insertion to treat a patient with anastomosis site stricture in efferent loop syndrome caused by a benign stricture.

Recently, SEMS insertion has been used for palliative treatment of malignant gastric and duodenal obstructions because it requires a shorter procedure time and provides more prompt restoration of oral intake than surgical treatment. Some cases of SEMS insertion have been reported for the treatment of postoperative gastric outlet obstruction[9]. Most of these cases were for recurrent malignant gastric outlet obstruction; however, there are a few cases for benign gastric outlet obstruction. In our case, the patient underwent surgery for benign diseases including a gastric ulcer, duodenal ulcer, and gastric outlet obstruction. Temporary placement of a SEMS might be effective for symptomatic improvement in benign pyloric stenosis as well as for malignant stricture, although an inserted stent could migrate. A successful insertion of a SEMS in patients with post-gastrectomy anastomosis site obstruction is reported herein for the first time. For patients with post-gastrectomy anastomosis site obstruction who are poor candidates for surgery, the placement of SEMS might be an effective treatment method. The risk of anastomosis leakage could increase at the anastomosis site as a result of SEMS insertion. This procedure should be performed in selected cases in which the obstruction results from torsion instead of in cases of pure anastomosis site stricture.

In conclusion, placement of SEMS in selected patients with post-gastrectomy anastomosis site obstruction is a technically feasible and clinically effective procedure.

COMMENTS

Case characteristics

A 75-year-old man who underwent a truncal vagotomy, omental patch, gastrojejunostomy, and Braun anastomosis for duodenal ulcer perforation and a gastric outlet obstruction.

Clinical diagnosis

Anastomosis site torsion of the efferent loop of the gastrojejunostomy.

Differential diagnosis

Anastomosis site stricture of the gastrojejunostomy from postoperative stricture.

Imaging diagnosis

An upper gastrointestinal series showed decreased peristalsis and severe distension of the stomach, with no visible contrast passage to the gastrojejunostomy, and an endoscopy revealed extensive food material and anastomosis site obstruction.

Treatment

The authors inserted a self-expandable metallic stent (SEMS) at the anastomosis site.

Related reports

Insertion of SEMSs has been used for palliative treatment of malignant gastric and duodenal obstructions.

Experiences and lessons

Placement of SEMSs in selected patients with post-gastrectomy anastomosis site obstruction is a technically feasible and clinically effective procedure.

Peer-review

The authors report a rare case of a successful management for post-gastrectomy anastomosis site obstruction with temporary placement of a SEMS. The topic is interesting and this procedure could be an effective treatment for older patients who suffer anastomosis site obstruction after gastrointestinal anastomosis who are not suitable for reoperation.

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