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EDITORIAL

Typhoid intestinal perforation in developing countries: Still unavoidable deaths?

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Abstract

Typhoid fever is a public health challenge mostly concentrated in impoverished, overcrowded areas of the developing world, with lack of safe drinking and sanitation. The most serious complication is typhoid intestinal perforation (TIP), observed in 0.8% to 39%, with a striking rate difference between high-income and low-middle-income countries. Although the mortality rate consequent to TIP in resource-poor countries is improved in the last decades, it is still fluctuating from 5% to 80%, due to surgical- and not surgical-related constraints. Huge economic costs and long timelines are required to provide a short- to middle-term solution to the lack of safe water and sanitation. Inherent limitations of the currently available diagnostic tools may lead to under-evaluation as well as over-evaluation of the disease, with consequent delayed treatment or inappropriate, excessive antibiotic use, hence increasing the likelihood of bacterial resistance. There is a need for immunization programs in populations at greatest risk, especially in sub-Saharan Africa. Uniform surgical strategies and guidelines, on the basis of sound or prospective surgical studies and adapted to the local realities, are still lacking. Major drawbacks of the surgical treatment are the frequent delays to surgery, either for late diagnosis or for difficult transports, and the unavailable appropriate intensive care units in most peripheral facilities. As a consequence, poor patient's conditions at presentation, severe peritoneal contamination and unsuitable postoperative care are the foremost determinant of surgical morbidity and mortality.

Key words: Typhoid bacterial resistance; Typhoid fever; Typhoid intestinal perforation; Developing countries; Low- Middle-Income Countries; Postoperative care; Typhoid vaccination

Core tip: Typhoid perforation in low-middle-income countries has still a disappointing outcome, related to surgical and not surgical constraints: (1) safe water and sanitation are lacking in high risk settings like slums or overcrowded areas; (2) currently available diagnostic facilities have inherent limitations; (3) multiple drugs resistant bacteria are an increasingly

threatening problem; (4) vaccination programs in some high risk regions, like sub-Saharan Africa, have not yet been carried out; (5) surgery is often delayed; (6) in peripheral facilities postoperative intensive care is problematic and often unsuitable; and (7) surgical standards and guidelines are not available due to the lack of sound prospective studies.

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INTRODUCTION

Typhoid fever is a public health challenge, mostly occurring in impoverished, overcrowded areas of the developing world, with lack of safe drinking and sanitation[1]. Although there is some evidence that typhoid fever incidence rates have declined over the past several decades, still the global estimation of typhoid fever episodes in 2010 was of 13.5 million[2]. The majority of disease burden has been observed in South and South-East Asia[3] and in sub-Saharan Africa, primarily in the low income neighborhoods of the capital cities but also in rural areas[3,4]. Data collection is substantially underestimating the morbidity and mortality of typhoid[5,6], for the inherent limits of evaluations based on extrapolation of data across regions and age groups. Moreover, reliable data are particularly scanty where the burden of the disease is mostly concentrated.

Typhoid fever and typhoid intestinal perforation

Globally, typhoid fever has a case-fatality rate of 10%-30% without effective treatment, reduced to 1%-4% with appropriate management[7,8]. The true incidence of complications is unknown[9], but alarming problems may arise in 10% to 15 % of patients, especially when the disease is lasting for two or more weeks[10]. The commonest GI complication is intestinal bleeding, usually not severe and managed conservatively[11], while typhoid intestinal perforation (TIP) is the most serious one[11,12]. It has been reported in 0.8% to 39%of patients[13-15], with a striking difference between high-income and poor resources countries[16,17] A higher propensity to perforation has been observed in sub-Saharan Africa than in Asiatic countries, suggested to be consequent to more virulent agents[18], though likely more related to data coming from referral hospitals, where the very ill patients are seen, than to a true local disease virulence[19].

Clinical features and mortality of TIP

Clinical features may be misleading: Peritoneal irritation can be almost absent before perforation, and peritoneal response delayed afterward. Unlike other perforations, the omentum does not migrate to the perforation site[14]. The number and size of perforated ulcers do not affect the severity of the symptoms. Although the mortality consequent to TIP is certainly improved[20] when compared to the 58% of death rate almost 50 years ago in Nigeria[21], still the reported mortality rate is fluctuating from 5% in the best settings to 80% in peripheral facilities[10,22-26], with a not negligible death rate reported in tertiary hospitals[27-30]. Conversely, developed countries observed a decline in mortality to less than 5%, due mainly to timely surgery and appropriate pre- and post-operative intensive care[13,31,32].

Several constraints contribute to this disappointing death rate, either related to the surgical management or linked to local settings and primary health care strategies.

NON SURGICAL RELATED CONSTRAINTS

Lack of water and sanitation, overcrowding

A more diffuse access to water safety and sanitation is fundamental for the control of typhoid fever, but the related huge economic costs and long timelines will not allow a short- to middle-term solution. Healthcare systems of poor resources countries, especially when affected by internal or external conflicts, may not afford the cost of these socioeconomic improvements. Conversely, targeted interventions on densely populated urban communities like slums, where typhoid fever is a serious problem, could be a possible way out[33,34]. In the meantime fewer resources could be directed towards rural areas with lower population density where enteric fever is less common[35,36].

Inadequacy of immunization programs

Almost all public health typhoid vaccination programs in the groups of populations at greatest risk have been performed in Asia (Table 1), with the strongest impact in endemic settings and in the short- to medium-term[37]. The oral vaccine was found to be highly cost-effective when targeting ages 1-14 years in high-burden/high-risk districts, as well urban slums and rural areas without improved water[38]. Remarkably, no vaccination experience has been reported from sub-Saharan Africa, where emerging threats, including multidrug resistance and increasing urbanization, would warrant concentration on immunization programs[4,39,40].

The recently proposed Typhoid Risk Factor (TRF) index[41], which takes into account the drinking water sources, toilet facility types, and population density, seems a reliable tool to evaluate variations in the disease burden, helping decision makers to identify high risk areas and prioritize the right populations for vaccination.

Increasing antibiotics resistance

Resistance to commonly used antibiotics in typhoid fever is becoming an emergent problem in endemic

Table 1 Reported experiences with typhoid vaccination strategies[37]

Vaccination strategies	Countries
Preemptive community-based routine vaccination	China, India
Preemptive community-based routine vaccination campaign	China, India, Pakistan, Vietnam
Preemptive disaster-response community-based vaccination campaign	Fiji, India Pakistan
Preemptive school-based vaccination	Chile, China, Indonesia, Nepal, Pakistan, Vietnam
Reactive (outbreak response) community-based vaccination campaign	Fiji, Tajikstan
Reactive (outbreak response) school-based vaccination	China

areas[42]. In resource-limited countries the few remaining effective antimicrobials are either unavailable or too expensive and at the moment the development of new effective low-cost drugs has a little short-term perspective. More than one third of patients in many endemic areas are affected by Multi Drug Resistant (MDR) bacteria[43]: nearly 75% of *S. Typhi* isolated from a population-based surveillance in Kenya were multi-drug resistant[36].

Delay in diagnosis

Since clinical features are not always reliable, typhoid may be differentiated with difficulty from other co-endemic acute febrile illnesses. Validated prediction rules from clinical features and laboratory results are not available[44,45]. Blood culture remains the gold standard for diagnosis, especially in the first week of illness[46], but with a large range of sensitivity (40%-80%) and is less reliable during antibiotic treatment. Stool cultures have lower sensitivity (< 40%). Widal test can be performed with minimal laboratory infra-structure and might be a good diagnostic support, especially in the second week of disease, but misuse and misinterpretation can be critical[47]. Poorly reliable tests may lead either to under-evaluation or over-evaluation of the disease, delaying a correct treatment or leading to inappropriate and excessive antibiotic use.

Delay in surgical treatment

A timely surgical treatment can prevent the severe peritoneal contamination observed in up to 70% of patients[18,23-25,48,49], associated with a high mortality rate[50,51]. Moreover, early surgery might reduce the need for extensive surgical procedures, with their contribution to a high morbidity and mortality[52,53]. From 30% to 100% of perforated patients may wait a long period before surgery, especially in rural areas and peripheral facilities. Indeed the diagnosis can be challenging in very young patients, in those who perforate while on medical treatment[18] or in presence of a generalized septic state, but if symptoms are evocative, diagnostic confirmation by either abdominal x-ray or ultrasound, should not delay surgery[54]. Similarly, adjustment of electrolytes and fluids imbalance or anemia correction should postpone surgery only for a short time as prolonged resuscitation can adversely affect the outcome[55,56].

Frequent causes of surgical delay are protracted or late referral from inadequate health facilities, difficult transport systems (both ambulances and roads), difficulties sourcing funds for treatment and diversion of patients to alternative medical therapies[18] before consulting the hospital.

SURGICAL RELATED CONSTRAINTS

Non-operative treatment has been proposed in the past in moribund patients or for long-standing perforations[22], but there is now uniform agreement that the ultimate treatment for TIP should be a surgical one, although the best surgical management remains controversial. Actually, the type of surgical technique might have limited influence on the outcome, that is likely more related to the preoperative clinical conditions of the patients, to the degree of abdominal contamination and to the quality of pre- and post-operative care[57].

Scarcity of prospective studies and guidelines

Several surgical solutions have been proposed for the treatment of TIP, with a consequent variability of morbidity and mortality. Indeed, explicit surgical guidelines, particularly aimed to resource-poor countries, are lacking. Most reports are retrospective, often including a small number of patients with not rarely incomplete data and poor statistical analysis. Surgical morbidity and mortality are often reported without any risk adjustment based on the severity of the disease, delay of treatment *etc*. The few available prospective[50,55,57-60] studies highlighting that patient's conditions have a more significant impact on patient's outcome than the type of surgical procedure, are shown in Table 2, which is including all prospective studies found in the literature about TIP.

Unavailable appropriate postoperative care

Postoperative care may be quite complex in these very fragile patients, frequently presenting with a septic state, coexistent diseases and an impaired immunological status. Moreover, intensive care units supplied for possible renal or respiratory failures, with available appropriate antibiotics for overwhelming infections and with accessible tools for nutritional support, are found infrequently in resource-poor countries, especially in peripheral or rural settings.

Surgical technique

The type of the surgical procedure does not appear to influence the mortality of TIP[13,19,56]; conversely, sound surgical judgment and experience are required to select the appropriate surgery according to the surgical

Table 2 Prospective studies reported in literature about surgical management of typhoid intestinal perforation

Ref.	Conclusions
Haider *et al*[60], 2002	Late presentation, delay in operation, multiple perforations, and drainage of copious quantities of pus and fecal material from the peritoneal cavity adversely affected the incidence of fecal fistula and the mortality rate.
Adesunkanmi *et al*[50], 2003	Peritonitis assessment by APACHE II score (50% perforations). A modified APACHE II score greater than 15 was associated with a significantly greater mortality.
Bashir *et al*[67], 2003	Primary ileostomy *vs* simple repair *vs* resection anastomosis: ileostomy is a good life saving procedure (statistical evaluation not reported).
Shukla *et al*[68], 2004	Single layer *vs* double layer repair: good closure of the perforation rather than single- or double-layer repair that determines the outcome in patients with enteric perforation.
Edino *et al*[55], 2007	Mortality is significantly affected by multiple perforations, severe peritoneal contamination and burst abdomen.
Gedik *et al*[58], 2008	Mannheim Peritonitis Index and perforation-operation interval were found independent risk factors affecting morbidity.
Mohil *et al*[57], 2008	Disease severity assessed by POSSUM score. Severity of disease rather than surgical procedure has a significant impact on the outcome.
Pandey *et al*[69], 2008	T-tube inserted into the bowel lumen after closing all distal perforations *vs* primary closure *vs* resection. In children with multiple perforations and poor general condition, the use of T-tube may be an effective management option (statistical evaluation not reported).
Tade *et al*[59], 2011	ASA class is a significant predictor of mortality in patients treated for typhoid intestinal perforation.
Ibrahim *et al*[70], 2014	Single layer *vs* double layer repair: single layer repair of the perforated ileum due to typhoid enteric perforation with peritonitis in children was effective by reducing complication rates.
Chaudhary *et al*[75], 2015	Temporary loop ileostomy for perforation peritonitis due to benign systemic diseases like typhoid fever and tuberculosis confers a very high morbidity.

findings and especially in advanced diseases[25,60].

Primary repair is usually performed for single or isolated perforations–by single or two suture layers. Segmental resection and anastomosis is preferred in presence of multiple adjacent perforations, while wedge resection is reported infrequently[21,61-63]. Simple repair has generally a lower mortality rate than resection, although death rate remains high when abdominal contamination is severe[64-66]. Few studies[67-70] evaluated prospectively single *vs* double layer repair without achieving definite conclusions, as shown in Table 2. The correlation between a high number of perforations, perhaps due to a highly virulent causative organism[17,18], and a poor surgical outcome is questionable[12,56,59]. Enterocutaneous fistula is the most alarming complication, with a mortality up to 67%[71,72], that is likely underestimated because death can occur months after surgery[73].

Ileostomy is usually reserved to patients with severe disease, delayed presentation and very contaminated abdomen, with a high risk of suture leakage[11,13]. Ileostomy has been also described as a routine primary procedure[74] although it is associated with high morbidity rate and complications like prolapse, stricture, retraction, parastomal hernia, mainly when performed in patients with critical conditions[75]. Moreover, loss of intestinal fluids from ileostomy can be managed with difficulty in austere environment and shortage of suitable ileostomy bags, with consequent skin damage around ileostomies, not rarely induces the patient to a self-limitation of food intake.

Delayed primary closure of the abdominal wall has been recommended for heavily contaminated wounds since a long time[76], but to date the optimal method of closure in such situations remains controversial[77]. Vacuum assisted closure appears promising but may not be feasible in peripheral facilities. Scheduled re-laparotomies, allowing early recognition of complications and a more appropriate cleaning the abdominal cavity, have been performed with a positive impact on survival[64]. However, this policy has the disadvantage to submit the patients to multiple surgical trauma and increases the workload of the operative theater.

A laparoscopic approach to TIP has been occasionally carried out with acceptable results[78,79]. There is no evidence that laparoscopy is more advantageous than open surgery, although it could be considered as an advantageous diagnostic tool in doubtful abdomens. A concern is the need of a highly technological equipment and of an appropriate maintenance, often lacking in poor resources countries.

CONCLUSION

Treatment and outcome of the TIP are still unsatisfactory in LMICs, with barriers related to local settings, local health strategies and specific surgical issues. An estimate of the burden of enteric fever and enteric fever drug resistance, especially in sub-Saharan Africa, is still inadequate. Local public health planning in high risk settings is essential to improve safe water availability and sanitation, but this compulsory achievement will be forcedly slow. Selected immunization programs, should be considered in areas at high risk, like slums or overcrowded places, especially in sub-Saharan Africa, where vaccination programs were never carried out despite the high burden of the disease. The recently proposed TRF index may help decision makers to identify high risk areas. Currently available diagnostic tools for typhoid fever have limitations in terms of speed, sensitivity, infra-structure requirements, and suitability. New approaches are needed to address many of these limitations for resource-poor countries. The emergence of multiple drugs resistant bacteria is

a threatening problem for the already overstretched health care systems of LMICs, taking into account the scarce resources to pay for effective antibiotics. A long delay before surgery, either due to a late diagnosis or to a protracted referral time, may strongly condition the surgical outcome. Prospective studies about surgical treatment of TIP in LMICs are lacking and should be encouraged in order to provide clear-cut surgical standard and guidelines. No one single surgical procedure can be recommended as a standard treatment on the basis of sound surgical studies. Any timely surgery carried out in a short time and allowing a swift clearing of peritoneal contamination, is the most likely to give the best outcome. A problematic pre- and post-operative care, due to lack of intensive care units, especially in peripheral hospitals, is a further shortcoming affecting the surgical outcome, independently on the type of surgery.

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REVIEW

Insights on the use of biosimilars in the treatment of inflammatory bowel disease

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Abstract

Biologic therapy, such as those that target tumor necrosis factor (TNF) signaling, has proven to be an efficacious method of treatment for patients with inflammatory bowel disease (IBD) with regards to symptom management and mucosal healing. However, the rising prevalence of IBD worldwide and the ever-increasing burden of biologic pharmaceuticals in the health care industry is alarming for insurance companies, clinicians, and patients. The impending patent expiry and the relatively high costs of biologics, particularly anti-TNF agents, have paved the way for biosimilar development for IBD. The United States Food and Drug Administration defines a biosimilar as a biological product that is highly similar to its reference medicinal product, with no clinically meaningful differences in terms of safety, purity, and potency. The hope with biosimilars is that their entry into the market will be able to drive competition between pharmaceutical companies to reduce prices like that of the generic market, and that access to appropriate biologic treatments for IBD patients is increased in the long-term. Yet, there are challenging issues such as indication extrapolation and interchangeability that are still being debated in the field of IBD and must be addressed in future issued guidance. This review will discuss the issues and implications concerning the use of biosimilar therapy for IBD.

Key words: Biosimilar; Biologic; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Indication extrapolation; Interchangeability

Core tip: The expiration of patent protection for various biologics and increasing health care expenses has paved the way for biosimilars to enter the market. The introduction of biosimilars is expected to produce cost savings in the health care industry as well as provide patients with inflammatory bowel disease with wider access to treatment.

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INTRODUCTION

There are two conditions that mainly characterize inflammatory bowel disease (IBD): ulcerative colitis (UC) and Crohn's disease (CD). These are chronic, relapsing, immune-mediated inflammatory diseases of the gastrointestinal tract. Whereas UC is an inflammatory condition that only affect the colon, CD is a chronic inflammatory condition with pathological features such as patchy transmural inflammation and fibrostenosis[1]. Urbanization, industrialization, and lifestyle are all factors that contribute to the rising incidence of IBD worldwide[2]. It has been estimated that approximately 1.4 million Americans are affected by IBD and afflicted with recurrent symptoms of bloody diarrhea, abdominal pain, bowel obstruction, and other co-morbid conditions[3,4].

The introduction of biologic therapy for IBD proved to be a breakthrough for patients with the disease[5]. Biologic products are highly complex molecules that are manufactured using living organisms[6]. In the pharmaceutical industry, biologics that are classified as monoclonal antibodies (mABs), particularly those that serve to antagonize tumor necrosis factor (TNF) signaling, have provided specialists and IBD patients with a proven and efficacious method of symptom management, mucosal healing, and prevention of long-term complications[7,8]. TNFα is a cytokine responsible for causing an inflammatory response towards tissue damage, and it was discovered to play an important role in the pathophysiology of chronic immunological diseases, including IBD and rheumatoid arthritis (RA)[9]. In addition, mABs that antagonize the α4β7 integrin have been developed to treat IBD. The α4β7 integrin was found to be involved in interactions that facilitate T-cell extravasation into the GI tract[10]. Patients who fail to respond or demonstrate hypersensitivity to anti-TNF therapy may also be treated with biologics that target the interleukin (IL)-12 and IL-23 pathways. IL-12 and IL-23 are proinflammatory cytokines that play a role in the differentiation of T-helper cells into type 1 T-helper cells as well as T-helper cell proliferation[11]. Currently, four anti-TNF biologics (infliximab, adalimumab, golimumab, and certolizumab) and two anti-integrin biologics (natalizumab and vedolizumab) have been approved for use in IBD treatment, while one anti-IL biologic that targets IL-12 and IL-23 (ustekinumab) has been approved for CD treatment[12,13].

Despite the effectiveness of biologics in treating IBD, the approaching patent expiry of certain anti-TNF agents has triggered the development of highly similar versions of these drugs known as "biosimilars" (Table 1). The approval of these biosimilar therapies is expected to generate competition in the pharmaceutical market that will reduce the financial burden of patient care and allow more patients to access treatment. However, the effectiveness of biosimilars is being debated due to several factors including an expedited regulatory approval process for biosimilar therapy and the notion that once approved, a biosimilar may be approved for all other indications for which the reference medicinal product (RMP) has been approved, without the need for clinical trials for the latter indications[14,15]. The purpose of this review is to discuss the emergence and implications of biosimilar market entry and to evaluate the progress of biosimilar therapy for IBD.

THE RISE OF BIOSIMILARS

What are biologics?

Biologic medicines are considerably more complex than small-molecule chemical generics. Compared with small-molecule medicines, which can be synthesized relatively easily and replicated chemically, biologics are large and complex three-dimensional structures produced using living cell lines and are difficult to replicate[16].

Whereas chemical generics only require about 50 critical tests during the manufacturing process, biologics demand a highly regulated manufacturing process consisting of 250 or more tests and a sophisticated quality control protocol[17]. In order to produce biologic agents, the gene for the protein of interest is inserted into a cell that produces and secretes the biologic agent in culture. After harvesting, the biologic undergoes protein purification before product formulation and packaging for clinical use[16]. Biologics are typically made in living cells that are highly sensitive to environmental changes and external conditions (such as temperature, light, and shear forces). As a result, different batches of the same biologic will vary in structural properties such as size, post-translational modifications, and folding pattern[17,18].

For patients with IBD, biologic treatment is an effective therapy. Infliximab (IFX) is a human-murine chimeric mAB that blocks the action of TNFα (anti-TNF) and is used to treat various immune-mediated inflammatory diseases[19]. Remicade, an IFX biologic used in the treatment of various auto-immune and inflammatory diseases, has been approved as therapy for induction and maintenance of moderate-to-severe CD and UC in both adult and pediatric IBD patients[20]. Subsequent to the approval of IFX, three other anti-TNF drugs (adalimumab, certolizumab pegol, and golimumab) and two anti-integrin biologics (natalizumab and vedolizumab) are approved therapies to treat IBD, while one anti-IL biologic that targets IL-12 and IL-23 (ustekinumab) has been approved for CD treatment[12,13].

Table 1 Comparison of biologics and biosimilars

	Biologics	Biosimilars
Development costs[26,103]	Approximately $2 billion	Approximately $100-250 million
Characterization	Exhibits heterogeneity	Exhibits heterogeneity
Patent duration	20 yr; up to 12-yr exclusivity period	No patent licensing
Approval process	Submission of a BLA	Submission of an aBLA
Immunogenicity	Possible risk	Possible risk
Indication extrapolation	Not permitted	Case-by-case basis

aBLA: Abbreviated biologics license application; BLA: Biologics license application.

What are biosimilars?

The regulatory pathway of a biologic drug is a time-consuming process that requires successful clinical trials that demonstrate clinical efficacy as well as approval from regulatory agencies such as the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA)[14,21,22]. However, in the context of biosimilars, regulatory agencies only need to ensure that high similarity or comparability is demonstrated between the biosimilar and its RMP before a biosimilar candidate can be approved and marketed, resulting in a simpler approval pathway[15].

According to the FDA, a biosimilar is a biological product that is highly similar to a RMP, with no clinically meaningful differences in terms of safety, purity, and potency[22]. Biosimilars and generic drugs both represent competition towards brand-name drugs. Although a manufactured generic is an exact copy of the original small-molecule medicine, it is not possible to generate identical copies of a biologic[23]. Since biologics are difficult to replicate, biosimilars are manufactured using alternate methods such that the final product is almost identical to the RMP with respect to the primary amino acid sequence[24]. Due to the inherent variability of the living bacteria-based systems used to make biosimilar drugs, there is micro-heterogeneity between biosimilar and RMP[25].

The emergence of biosimilar therapies is an inevitable outcome of patent expiration. From the date of filing, a drug's patent lasts up to 20 years, with exclusivity lasting up to 12 years, according to the Biologics Price and Competition Innovation Act of 2009[26]. Pharmaceutical companies rely on patent exclusivity and protection to benefit from investment return. Once a patent expires, companies are immediately able to market generics, which usually have lower prices driven by competition[27]. The anticipation with biosimilars is that their entry into the market will be able to drive competition between pharmaceutical companies, to reduce prices comparably to how the generic market has, and to increase overall patient access to appropriate biologic treatments in the long-term. Currently, only two biosimilars have been approved for use in IBD in the United States: infliximab-dyyb and adalimumab-atto[28,29]. However, multiple anti-TNF biosimilars have either been proposed, are being tested in late stage clinical trials, or are awaiting approval from regulatory agencies (Table 2).

COMPARING BIOLOGICS AND BIOSIMILARS

Mechanism of action

IBD is characterized by immune dysregulation in a genetically predisposed individual, resulting in overproduction of TNFα by macrophages, monocytes, and T cells[30,31]. Anti-TNF therapy is an efficacious method that can treat IBD by blocking proinflammatory mediator TNF. Anti-TNF mAbs can also induce the formation of regulatory immunosuppressive macrophages and anti-inflammatory cytokines to further treat IBD[31]. Certain mAbs such as IFX and adalimumab (ADA), but not certolizumab, have the ability to mediate antibody-dependent cell-mediated cytotoxicity (ADCC), an immune response characterized by the lysis of target cells by activated effector cells, including natural killer cells, monocytes, macrophages, neutrophils, and eosinophils[32]. The crystallizable fragment of the IgG1 antibodies of these mAbs is necessary to exhibit ADCC[33].

Molecules in the same class, such as TNF inhibitors, may be extrapolated across all indications because they share the same mechanism of action. Extrapolation across indications is a process that may be considered when there are changes in manufacturing from an originator biologic or route of administration[18]. Typically, clinical data that corresponds to one indication may be extrapolated to additional indications based on information on comparability. Because clinical efficacy of the RMP is already established, the number of preclinical and clinical studies required for approval may be less for biosimilars, and studies may only be required for a subset of indications[34]. Clinical studies and analytical tests that observed comparability in physiochemical features and mechanism of action between RMP and biosimilar supported the approval of infliximab-dyyb across all indications of IFX by the FDA and EMA[18,28,35].

Pharmacokinetic profile

Pharmacokinetics (PK) refers to various factors (absorption, bioavailability, distribution, metabolism, and excretion) involved with the movement of a drug into,

Table 2 Proposed anti-tumor necrosis factor biosimilars[1]

Reference medicinal product	Biosimilar name
Infliximab	Infliximab-dyyb (Celltrion)[2]
	SB2 (Samsung Bioepis)
	PF-06438179 (Sandoz)
	BOW015 (Epirus)
Adalimumab	Adalimumab-atto (Amgen)[2]
	SB5 (Samsung Bioepis)
	ZRC-3197 (Zydus Cadila)
	MSB11022 (Merck KGaA)
Certolizumab pegol	PF688 (PFEnex)
Golimumab	BOW100 (Epirus)

[1]Information for each biosimilar was derived from the website of its respective drug company; [2]Approved by the United States Food and Drug Administration.

through, and out of the body. In addition to patient-related factors (*e.g.*, genetic makeup, sex, body mass index, age, and disease severity), chemical properties can also influence PK parameters[36]. Compared with small-molecule medicines, biologics and biosimilars will have a slower rate of absorption, smaller volume of distribution, different mechanisms of paracellular and transcellular movement, and different routes of clearance[37]. A biosimilar must display a comparable PK profile to the RMP. Results from a randomized study indicated that three formulations of IFX [infliximab-dyyb, United States RMP, and European Union (EU) RMP] had highly similar PK and safety profiles[38]. In addition, an understanding of PK is necessary to optimize therapeutic development and dosing in patients[37].

Immunogenicity

Biologics have been shown to elicit an immunogenic response in some patients, characterized by a release of antibodies by antibody-secreting B cells[39]. When present, anti-drug antibodies can neutralize the clinical efficacy of a biologic as well as cause unpredictable side effects and loss of response[40]. Immunogenicity is a major health concern with all biologics as well as biosimilars. Manufacturing, post-translational modifications, route of administration, and patient characteristics are several factors known to influence immunogenicity[41].

Concomitant use of immunomodulators such as azathioprine (AZA) and methotrexate (MTX) can prevent immunogenicity by decreasing the formation of anti-drug antibodies and reducing systemic inflammation[42]. The SONIC study evaluated the safety of efficacy of treating CD patients with IFX or AZA alone or in combination therapy. At week 26, there was a greater occurrence of corticosteroid-free remission and mucosal healing in those treated with combination therapy than with monotherapy. Additionally, there were fewer patients that developed serious infections in the combination therapy group, compared with both the IFX and AZA groups[43]. A similar study, UC SUCCESS, was performed to evaluate combination therapy in UC patients. At week 16, greater occurrence of mucosal healing and corticosteroid-free remission was observed those treated with combination therapy than with IFX or AZA alone[44]. The COMMIT study evaluated the safety and efficacy of IFX alone or in combination with MTX in CD patients. Although combination therapy was well tolerated, the number of patients who achieved corticosteroid-free remission at week 14 and maintained remission at week 50 was similar in both groups. However, only 4% of patients who received MTX developed antibodies to IFX, compared with 20% who received IFX alone. Furthermore, trough serum concentrations of IFX was also higher, albeit not statistically significant, in patients who received MTX[45].

The use of immunomodulatory agents on biosimilar treatment has shown to be feasible. In an extension of the PLANETRA study, all enrolled patients received intravenous infliximab-dyyb and concomitant methotrexate. Both the maintenance and switch groups displayed a similar proportion of patients with anti-drug antibodies[46]. Notably, the EMA also mentions the concomitant use of methotrexate in the European public assessment report for infliximab-dyyb[35].

THE BENEFITS OF BIOSIMILARS

Potential cost savings with biosimilars

The high prices of biologic pharmaceuticals have placed a burden on the healthcare industry, accounting for a continually increasing share of drug spending in the United States and limiting patient access to appropriate treatment. The Office of the Assistant Secretary for Planning and Evaluation estimates that United States drug spending totaled about $457 billion in 2015, making up 16.7% of overall health care spending. Notably, prescription drug expenditures are rising at a faster rate than overall spending, due to factors such as population growth, inflation, and a higher number of prescriptions per patient[47].

The estimated total costs of IBD in the United States range from $14.6B to $31.6B[48]. The growing prevalence of the disease worldwide, in conjunction with the high costs, is concerning for the economy and may lead to unsustainable healthcare costs in the future. Compared to patients without the disease, direct medical expenditures have been found to be around $13663 to $17434 higher for patients with CD and $10039 to $12615 higher for patients with UC[49].

Biosimilars are expected to produce savings across the board in the health care industry as a result of various factors, such as reduced research and development costs, competition driven by patent expiry, and a simpler approval pathway. An Excel-based model of Remsima for the treatment of various inflammatory autoimmune diseases was created to estimate the budget impact of Remsima. The model, which covers

five countries (Germany, the United Kingdom, Italy, the Netherlands, and Belgium) projects the biosimilar to induce cost savings over one year of $63 million (pounds converted to dollars) and the treatment of 3900 additional patients[50]. Furthermore, another budget impact model of Remsima in six different countries (Bulgaria, Czech Republic, Hungary, Poland, Romania, Slovakia) was developed while taking into two scenarios: BSc1 (interchangeability disallowed) and BSc2 (interchangeability allowed, 80% of patients taking IFX are interchanged to biosimilar). In this model, which estimates budget impact of Remsima in the treatment of RA only, savings of $21M (BSc1) and $29M (BSc2) are projected over 3 years, as well as the treatment of an additional 1200 to 1800 patients[51].

The EU has provided the healthcare industry with a preliminary impression of biosimilar market entry. Biosimilars have been available in the EU since 2006, and the observed average list prices are 30% lower than the RMP, compared to the 70% to 80% savings that generics induce[26,52]. Because biosimilars are more difficult to manufacture, the cost reduction is not expected to be as drastic as seen with generics.

Currently, filgrastim-sndz (Zarxio), an anti-cancer drug, infliximab-dyyb, and adalimumab-atto are the only biosimilars approved in the United States[28,29,53]. The entry of biosimilars into the United States market is important for the overall development and financial success of the pharmaceutical industry, bearing in mind that a majority of world biologics sales come from the United States[54]. From 2014-2024, it is anticipated that the entry of the 11 most likely biosimilars into the market will lead to $250 billion in savings for the American healthcare industry, with the possibility of greater disease control and reduced inpatient stays and outpatient visits[55].

Wider accessibility for patients

The entry of biosimilars to market is expected to give patients more choices and greater access to treatment. Prior to the development of biosimilars, those who required biologic therapy were either restricted to a limited number of costly treatment options or placed on a waiting list. A cross-sectional study, performed in 49 European countries, revealed that RA patients in lower income countries struggle with affordability and have less access to biologic and synthetic disease-modifying drugs[56]. Fortunately, due to projected cost reductions associated with biosimilars, a large number of patients are expected to have a larger complement of options available to them earlier in the course of the disease.

Furthermore, if switching between a particular RMP and its biosimilar are observed to be clinically noninferior to continued treatment of the RMP, then concerns about biologic shortages and waiting lists would potentially be alleviated. In 2014, there were 1000 additional patients in the Czech Republic who were able to initiate treatment than in the previous year, due to the cost savings of biosimilars[57].

CHALLENGES WITH BIOSIMILARS

Indication extrapolation

There is uncertainty as to the level of efficacy of certain biologic molecules in different indications. While IFX and etanercept (ETN) are effective in treating RA, ETN was determined to be futile in treating CD[58]. Studies show that in patients with CD, both IFX and ETN are successful in TNF blocking, but only IFX is capable of inducing apoptosis in order to reduce the number of inflammatory cells[59]. Notably, IFX provides clinical improvement in RA, but not by the induction of apoptosis[60].

The extrapolation of indications for infliximab-dyyb for the indications of IFX has also prompted questioning. IFX is effective in multiple tissues and organ systems (joints, axial skeleton, GI tract, and skin). However, even though the approval of infliximab-dyyb in the EU was mainly supported by studies in ankylosing spondylitis (AS), a chronic inflammatory disease that affects the spinal vertebrae and sacroiliac joints, and RA, the specific distribution and effectiveness of IFX and infliximab-dyyb to affected tissues is not known, presenting a potential problem in indication extrapolation[34,61-63]. In 2014, Health Canada approved infliximab-dyyb for all indications except for UC and CD due to a lack of clinical data demonstrating proper mechanism of action in all indications of IFX, and residual uncertainty regarding the role and impact of small differences in ADCC[34,64,65]. Because anti-TNF agents may also depend on ADCC in addition to TNFα neutralization, changes in ADCC tests pose a challenge for extrapolation[33].

Immunogenicity

As with biologics, it is important to take into consideration the unpredictable risk of immunogenicity when introducing a biosimilar to the market. While effective for treating inflammatory diseases such as IBD, some patients either fail to respond or develop a loss of response. Because indication extrapolation for a biosimilar requires less clinical data than the initial approval of a biologic would, information regarding immunogenicity of the biosimilar in patients for indications without substantial data becomes difficult to support without performing extensive clinical trials[34]. The FDA and World Health Organization have advised that immunogenicity be investigated in populations that are at the highest risk of an immune response and immune-related adverse events[15,66]. Furthermore, performing *in vivo* and *in vitro* assays (*e.g.*, size exclusion, western blots, and enzyme-linked immunosorbent assays) throughout development can lessen the probability of an immunogenic response[39].

Recent data suggests that it may not be appropriate to extrapolate immunogenicity data from the RMP to the biosimilar. Following results of a study which revealed cross-reactivity between anti-IFX antibodies and infliximab-dyyb, the European League Against Rheumatism stated that switching from IFX to infliximab-dybb may not be appropriate for all patients[67]. Given the unpredictability of anti-drug antibody formation, diagnostic tests have been developed in order to better estimate the efficacy of biologics and biosimilars in patients with IBD. The Anser IFX and Anser ADA, developed by Prometheus Laboratories, were designed to measure the serum levels and antibodies of patients being treated with IFX or ADA, respectively. In a cohort study of patients with acute UC (n = 115), detectable trough serum concentrations of IFX were shown to predict improved outcomes[68]. Recently, the Anser IFX was validated for use in patients who are treated with infliximab-dyyb[69].

Interchangeability

One of the major obstacles for the entry of biosimilars into the market is interchangeability. The Abbreviated New Drug Application (ANDA) is an application that uses bioequivalence as a basis to demonstrate that a new generic is similar enough to the original branded drug. Most generics are considered interchangeable once the ANDA is approved, and pharmacists are allowed to switch branded drugs for generics at the point of purchase, subject to state law[70,71]. Conversely, interchangeability of biosimilars is not immediately granted upon ANDA approval, which poses a challenge for clinical use[72].

Manufacturers face concerns with both clinician and patient acceptance, as well as the reluctance to use the biosimilar in treatment, especially if such a change is to alter a long established prescribing practice[73]. The FDA states that an interchangeable product is "expected to produce the same clinical result as the RMP in any given patient and, if the biological product is administered more than once to an individual, the risk in terms of safety or diminished efficacy of alternating or switching between the use of the biological product and the RMP is not greater than the risk of using the RMP without such alternation or switch[74]". As a result, a biosimilar product may not necessarily be interchangeable. Because an application for interchangeability requires the fulfillment of additional criteria, manufacturers may decide not to pursue the "interchangeable" designation. Without investing in extra clinical trials, patient and clinician confidence in non-interchangeable biosimilars could potentially decrease, despite a more rapid market entry[70].

In the EU, a majority of biosimilars had relatively little market share because of a lack of interchangeability[75]. A majority of United States states and Puerto Rico have either considered, passed legislation, or enacted law regarding the automatic substitution of biologics for biosimilars at the pharmacy level[76]. Automatic substitution allows pharmacists to replace biologics with biosimilars without informing or obtaining approval from the prescribing physician[77]. There are currently no studies that demonstrate the implications of cross-switching (switching between two biosimilars), reverse-switching (switching from a biosimilar to its RMP), or switching between multiple biosimilars. However, it is possible that switching between multiple biosimilars may lead to an immunogenic reaction and reduced efficacy of the drug. Because antibodies can develop within 2 to 3 treatments, an updated statement from the European Crohn's and Colitis Organization advises against switching within six months of initiating treatment for non-medical reasons[78]. Ultimately, the FDA is expected to issue their official guidance on interchangeability by the end of 2017[79].

THE STATE OF BIOSIMILAR DEVELOPMENT

Infliximab-dyyb

The results of two randomized and double-blind clinical studies, PLANETRA and PLANETAS, contributed to the approval of infliximab-dyyb in the EU for all the indications of the RMP (infliximab): RA, CD, UC, AS, psoriatic arthritis (PsA), and psoriasis (PsO)[80]. Six hundred and six RA patients enrolled in PLANETRA were randomized to receive either IFX (n = 304) or infliximab-dyyb (n = 302) in combination with MTX and folic acid. In PLANETAS, 250 AS patients were randomized to receive either IFX (n = 125) or infliximab-dyyb (n = 125) alone. At weeks 14 and 30 of both studies, the biosimilar was shown to have demonstrated to have highly similar PK, efficacy, safety, and immunogenicity[61,62]. In 2016, results from a secondary analysis of PLANETAS were made available. Through week 54, the observed PK parameters and immunogenicity remained similar in the two treatment groups. Withdrawal rates were similar in both the biosimilar (n = 19) and RMP (n = 21) treatment groups, with the most common cause being the case of a treatment-emergent adverse event (TEAE). The most common TEAEs (occurring in over 10% of patients in each treatment group) were abnormal liver function test and infusion-related reaction[63].

Several studies have been performed to address infliximab-dyyb induction in IBD (Table 3). Mixed results suggest that infliximab-dyyb and IFX may not have similar clinical efficacy and safety in patients with the disease. In Ireland, a study was performed to compare 14 IBD patients taking an IFX biosimilar (Inflectra) from January to July 2014 to 22 IBD patients commenced on IFX from December 2011 to December 2013. Results indicated that Inflectra demonstrated a significant decrease in clinical efficacy, with a 29%

Table 3 Clinical studies on infliximab-dyyb induction in inflammatory bowel disease

Ref.	Study	Population	Results	Safety
Jahnsen *et al*[84]	Prospective observational	CD = 46; UC = 32	Clinical remission rate at week 14: 79% (CD), 56% (UC) Significant decrease in CRP, calprotectin	No adverse events reported
Jung *et al*[82]	Retrospective multicenter	CD = 32; UC = 42	Clinical response at week 54: 87.5% (CD), 100% (UC) Clinical remission rate at week 54: 75% (CD), 50% (UC)	Adverse events in 11% of UC patients
Gecse *et al*[85]	Prospective, multicenter, nationwide cohort	CD = 126; UC = 84	Clinical response at week 14: 81.4% (CD), 77.6% (UC) Clinical remission rate at week 14: 53.6% (CD), 58.6% (UC)	Adverse events in 17.1% of all patients
Murphy *et al*[81]	Descriptive	IBD = 36 (Remicade = 22; Inflectra = 14)	CRP levels: increase in 93% of Inflectra patients, decrease in 100% of Remicade patients	29% increase in hospital readmission and 75% increase in surgery rates with Inflectra patients
Sieczkowska *et al*[86,104]	Switch from RMP to Infliximab-dyyb	Pediatric CD = 32; Pediatric UC = 7	Clinical remission rate: 88% (CD), 57% (UC) Decrease in PCDAI, CRP, ESR	No adverse events reported
Smits *et al*[87]	Prospective, observational, cohort switch	CD = 57; UC = 26	No significant change in DAI, CRP, calprotectin at week 16	No adverse events reported

CD: Crohn's disease; CRP: C-reactive protein; DAI: Disease Activity Index; ESR: Erythrocyte sedimentation rate; HBI: Harvey-Bradshaw Index; IBD: Inflammatory bowel disease; PCDAI: Pediatric Crohn's Disease Activity Index; UC: Ulcerative colitis.

increase in surgery rate and 75% increase in hospital readmission[81]. Another retrospective multi-center study evaluated the efficacy and safety of infliximab-dyyb in anti-TNF naïve UC (n = 42) and CD (n = 32) patients. After switching, therapeutic efficacy was maintained in 93% (25/27) of CD patients and 67% (6/9) UC patients at 54 wk. There were adverse events reported in 11% of UC patients, but the results indicated comparable efficacy, safety, and interchangeability between RMP and biosimilar[82]. Clinical data from 46 CD and 32 UC patients in a infliximab-dyyb induction study demonstrated comparable efficacy and safety to the RMP. At week 14, 76% (32/42) of CD patients and 56% (18/32) of UC patients were in clinical remission, and decreases in the Harvey-Bradshaw Index (HBI), calprotectin levels, and C-reactive protein (CRP) levels were observed in both indications. In addition, no adverse events were reported[83,84]. A prospective, multi-center, nationwide cohort that examined infliximab-dyyb induction in CD (n = 126) and UC (n = 84) revealed clinical response in 81.4% (CD) and 77.6% (UC) of patients as well as remission rates of 53.6% (CD) and 58.6% (UC). Adverse events were reported in 17.1% of all patients[85].

Currently, there is limited data that addresses the switching to a biosimilar from its RMP in IBD. However, studies have suggested that switching between biosimilar and RMP in IBD patients is feasible[86,87]. NOR-SWITCH was a randomized, double-blind, parallel-group study in Norway that evaluated the safety and efficacy of a single switch from IFX to infliximab-dyyb in patients with various inflammatory diseases (RA, spondyloarthritis, PsA, UC, CD, and chronic PsO). The study began in October 2014 and is expected to be completed in January 2017[88]. Data presented at the United European Gastroenterology Week 2016 revealed that switching to infliximab-dyyb was not inferior to continued treatment with RMP IFX[89].

SB2

Samsung Bioepis's SB2 (Flixabi), an IFX biosimilar, was approved in the EU for all the indications of infliximab, as listed above. The approval of Flixabi was facilitated by a randomized, double-blind Phase 3 study which demonstrated comparable PK and immunogenicity to IFX, and equivalent values for ACR20 in both the SB2 and IFX treatment groups at week 30 and 54[90,91].

PF-06438179

Sandoz acquired the rights to Pfizer's IFX biosimilar, PF-06438179, in February 2016[92]. In September 2013, a Phase 1 study, REFLECTIONS (B537-02), comparing PF-06438179 to IFX in healthy volunteers (n = 146) indicated comparability in the PK and immunogenicity profiles of both treatment groups[93]. REFLECTIONS (B537-02) is an ongoing randomized, double-blind Phase 3 clinical study comparing PF-06438179 to IFX in combination with methotrexate in patients with acute RA. The study began in August 2014 and is expected to be completed in May 2017[94].

BOW015

In September 2014, Epirus Biopharmaceutical's BOW015 became the first IFX biosimilar to be approved in India, facilitated by Phase 3 clinical data of BOW015 in RA patients[95]. Currently, Epirus has launched another Phase 3 study in Europe, the UNIFORM study, comparing BOW015 and IFX in patients with active RA. Data is expected after the study's primary completion

date in July 2017[96].

Adalimumab-atto

Amgen submitted an abbreviated Biologics License Application (aBLA) to the FDA in November 2015 for adalimumab-atto (Amjevita), a biosimilar candidate to its ADA biologic, Humira, following the completion of two phase 3 studies[97]. The FDA approved Amjevita across all eligible indications of Humira in September 2016. Amgen's first study was a randomized, double-blind, active-controlled phase 3 comparative study performed to demonstrate comparable safety, efficacy, and immunogenicity of ABP 501 and ADA with patients with moderate-to-severe RA. Amgen believes that the study met the primary endpoint of ACR20. Secondary endpoints, ACR50 and ACR70, as well as the incidence of TEAEs were also comparable between ADA and ABP 501[98]. Another randomized, double-blind phase 3 study of Amjevita was performed in patients with moderate-to-severe plaque PsO. Results achieved the primary endpoint for efficacy of the study with a percent improvement in Psoriasis Area and Severity Index from baseline to week 16 of treatment, and safety and immunogenicity were observed to be comparable between ADA and Amjevita[99].

ZRC-3197

In December 2014, the first ADA biosimilar, Zydus Cadila's ZRC-3197 (Exemptia) launched in India[100]. Approval of ZRC-3197 was facilitated by a randomized, double-blind study comparing Exemptia and ADA in patients with RA, yielding comparability data demonstrating high similarity between the biosimilar and the RMP in terms of efficacy, tolerability, and safety. The 12-wk study saw only 3 of 120 subjects drop out, all due to adverse events, and no deaths were reported[101].

MSB11022

In March 2016, Merck KGaA announced the initiation of AURIEL-PsO, a randomized double-blind study to evaluate the safety and efficacy of its ADA biosimilar candidate, MSB11022, compared with ADA in patients with moderate-to-severe plaque PsO. Data is expected in December 2016, with the study to be completed around September 2017[102].

CONCLUSION

Biologic therapy has greatly facilitated treatment for IBD, and the introduction of biosimilars has the potential to be a breakthrough development for IBD patients. Increasing prescription drug expenditures have limited patient access to the appropriate biologic treatment, contributing to a heightened interest in biosimilars, which are expected to trigger cost savings upon biologic patent expiry. Reflecting upon the biosimilar experience in the EU, savings of around 30% from the RMP were observed. Moreover, current studies and experience provide optimism with regards to future cost savings and interchangeability with their RMPs. The FDA's recent approval of Inflectra marks significant progress in the emergence of biosimilar therapy in the United States. Ultimately, as more biosimilars enter the market, competition is expected to drive prices down.

Perhaps the greatest hurdle that pharmaceutical companies face is clinician and patient acceptance. Issues such as immunogenicity and interchangeability cannot be avoided. It has been suggested that the development of anti-drug antibodies may have an inhibitive effect on clinical response and patient outcomes. Although diagnostic tests such as the Anser IFX are able to provide some clarification to patients, additional studies are necessary in order to clear up any uncertainty with regards to the influence of anti-drug antibodies. In addition, taking steps to improve manufacturing processes and production may contribute to avoid changes that influence an immunogenic response in patients. As more data becomes available, biosimilars have the opportunity to increase patient access to a more affordable form of appropriate treatment. Given the large number of studies in progress, it is conceivable that more promising results will expedite the transition towards biologic and biosimilar interchangeability as well as higher confidence in interchangeability and active switching between biosimilar and RMP.

Hopefully, when the appropriate guidance is finalized, the FDA will be able to answer many of the questions that manufacturers and companies have pertaining to biosimilar labeling and interchangeability. With the necessary data and guidance at their disposal, it will be feasible for clinicians to develop a treatment plan that is more personalized and tailored towards specific patients than before.

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REVIEW

Role of autophagy in the pathogenesis of inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) results from a complex series of interactions between susceptibility genes, the environment, and the immune system. Recently, some studies provided strong evidence that the process of autophagy affects several aspects of mucosal immune responses. Autophagy is a cellular stress response that plays key roles in physiological processes, such as innate and adaptive immunity, adaptation to starvation, degradation of aberrant proteins or organelles, antimicrobial defense, and protein secretion. Dysfunctional autophagy is recognized as a contributing factor in many chronic inflammatory diseases, including IBD. Autophagy plays multiple roles in IBD pathogenesis by altering processes that include intracellular bacterial killing, antimicrobial peptide secretion by Paneth cells, goblet cell function, proinflammatory cytokine production by macrophages, antigen presentation by dendritic cells, and the endoplasmic reticulum stress response in enterocytes. Recent studies have identified susceptibility genes involved in autophagy, such as *NOD2*, *ATG16L1*, and *IRGM*, and active research is ongoing all over the world. The aim of this review is a systematic appraisal of the current literature to provide a better understanding of the role of autophagy in the pathogenesis of IBD. Understanding these mechanisms will bring about new strategies for the treatment and prevention of IBD.

Key words: Autophagy; Inflammatory bowel disease; Genome-wide association study; Ulcerative colitis; Crohn's disease

Core tip: Recent studies provide strong evidence that the process of autophagy affects several aspects of mucosal immune responses. Autophagy is a cellular stress response that plays key roles in physiological processes. Dysfunctional autophagy is recognized as a contributing factor in many chronic inflammatory diseases, including inflammatory bowel disease (IBD). Autophagy plays multiple roles in IBD pathogenesis. Recent studies have identified susceptibility genes involved in autophagy, such as *NOD2*, *ATG16L1*, and

IRGM, and active research is ongoing around the world. The aim of this review is a systematic appraisal of current literature to provide a better understanding of the role of autophagy in IBD pathogenesis.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disease involving idiopathic inflammation, mainly in the gastrointestinal tract; defined more specifically, it comprises ulcerative colitis (UC) and Crohn's disease (CD). Both are characterized by onset at a young age, and the number of affected patients has risen sharply in recent years in Europe and the United States, as well as in Japan[1]. Thus, there is a pressing need to understand their pathologies and create effective treatments. Researchers, mainly in Europe and the United States, have been trying to identify disease-susceptibility genes for IBD. Nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) was the first susceptibility gene identified for CD[2,3], and in recent years genome-wide association studies (GWAS) have made it possible to perform comprehensive searches for susceptibility genes. In 2007, autophagy-related 16-like 1 (*ATG16L1*) was identified as an autophagy-related gene[4]. This was the first study to show a relationship between autophagy and a specific disease. Since then, the role of autophagy in the pathogenesis of IBD has been investigated all over the world.

This review will evaluate the current literature to provide a better understanding of the role of autophagy in the pathophysiology of IBD.

PATHOLOGY AND PATHOGENESIS OF IBD

The gastrointestinal tract not only absorbs fluid and nutrients, but is constantly involved in regulating and maintaining the gut flora, immune responses to food antigens and other substances, and homeostasis. IBD occurs when this homeostasis is impaired. Recent research has shown that IBD is caused by chronic intestinal inflammation, which occurs because of gene variations that can lead to disease susceptibility, changes in the structure of the intestinal flora needed to maintain intestinal homeostasis, and abnormal intestinal mucosal immune responses[5-7].

The role of genetic factors in IBD has been previously reported[8], and several researchers are seeking disease-susceptibility genes and trying to find customized treatments for individual patients[9]. To date, approximately 200 loci have been identified as being associated with both forms of IBD. Within these 200 loci, based upon single nucleotide polymorphism frequencies in IBD subjects versus controls, are approximately 1500 potential associated genes[10,11]. Representative autophagy-related genes are *NOD2*, *ATG16L1*, and immunity related guanosine triphosphatase M (*IRGM*)[1-3,12]. Autophagy has been linked to a variety of diseases, but its link to IBD is currently the subject of much debate.

AUTOPHAGY

Autophagy (from the Greek "auto" oneself and "phagy" to eat) refers to any cellular degradative pathway that involves the delivery of cytoplasmic cargo to the lysosome. During this process, the endoplasmic reticulum or other membranous cellular structures respond to stimuli by generating a double-membrane structure called a phagophore. On this phagophore, *ATG16L1* forms a complex with an *ATG5-ATG12* conjugate, which multimerizes and then lipidates *LC3 (LC3-II)*. Simultaneously, the phagophore elongates to envelop the cytoplasm or organelle to be degraded, forming an autophagosome, a unique double-membrane organelle. The outer membrane of the autophagosome then fuses with a lysosome to form an autolysosome, and the inner membrane degrades and absorbs its contents (Figure 1)[13-15]. This process, along with the ubiquitin proteasome pathway (UPP) system, triggers the intracellular protein degradation mechanism. The process is also responsible for mechanisms such as adaptation to starvation, defense against infections, carcinogenesis, antigen presentation, and quality control of intracellular proteins. It maintains appropriate cellular homeostasis and provides the structural processes necessary for organ renewal[16]. Yet, unlike the UPP system, autophagy is also able to degrade mitochondria and other organelles.

A remarkable analysis of autophagy-related factor groups showed that, in addition to its role in metabolism, autophagy plays an important role in the innate immune response[13]. Innate immunity is a mechanism by which nearly all multicellular organisms protect themselves from pathogens. Innate immunity signaling pathways are activated when the structural patterns of a pathogen's components are recognized (*i.e.*, the cell wall components of a bacterium or the genome of a virus). As noted above, autophagy was initially considered to be a nonspecific mechanism for degrading substances by incorporating them into a membrane structure, but recent research has shown that autophagosomes selectively isolate a variety of substrates[17]. However, besides autophagy of pathogens (xenophagy)[18,19] and autophagy of damaged mitochondria (mitophagy)[20,21], very little

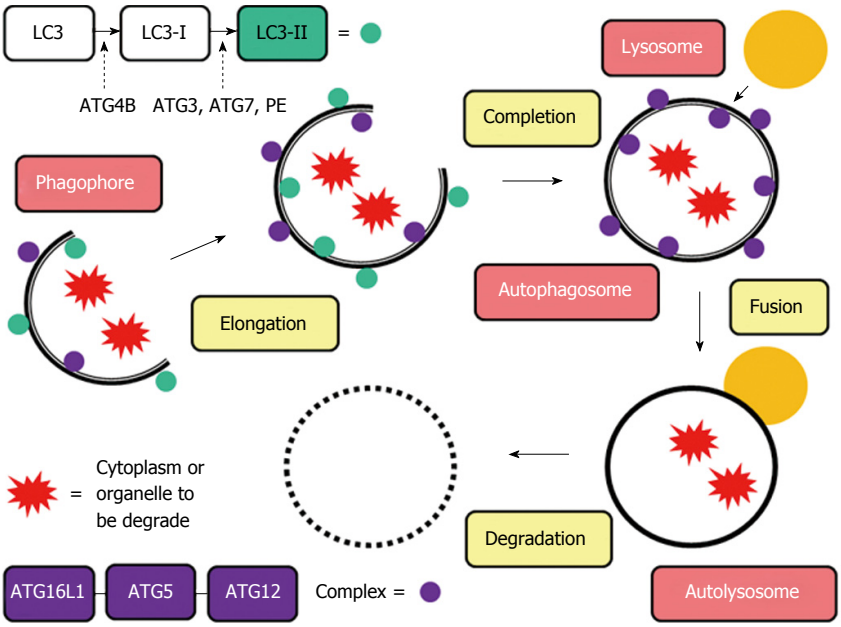

Figure 1 Autophagy mechanism. The autophagy pathway. During this process, the endoplasmic reticulum or other membranous cellular structures respond to stimuli by generating a double-membrane structure called a phagophore. On this phagophore, *ATG16L1* forms a complex with an *ATG5-ATG12* conjugate, which multimerizes and then lipidates LC3 (LC3-II). Simultaneously, the phagophore elongates to envelop the cytoplasm or organelle to be degraded, forming an autophagosome, a unique double-membrane organelle. The outer membrane of the autophagosome then fuses with a lysosome to form an autolysosome, and the inner membrane degrades and absorbs its contents.

Table 1 Genetic variants related to inflammatory bowel disease and autophagy

Gene	Chromosomal site	Relation to autophagy
NOD2	16q12.1	Intracellular bacterial sensing Autophagosome formation
ATG16L1	2p37.1	Autophagosome formation Suppressing Paneth cells
IRGM	5q33.1	Phagosome maturation Virus-induced autophagy
IL-23R	1p31.3	Through effects on IL-1 secretion
XIAP	Xq25	Physiological inhibitor of autophagy
LRRK2	12q12	Autophagosomal-lysosomal degradation
ULK1	12q24.33	Regulated by TORC1 and AMPK
VDR	12q13.11	Regulate the expression of *NOD2*
MTMR3	22q12.2	Autophagosome formation

is understood about which substrates autophagy degrades when it functions as part of innate immunity.

IBD- AND AUTOPHAGY-RELATED GENETIC VARIANTS

Autophagic dysfunction causes several diseases[22-24], among which CD is being most extensively researched. The above mentioned GWAS found several genetic variants linked to CD onset, such as *NOD2* and *ATG16L1*. A summary of these variants is given below (Table 1).

NOD2, ATG16L1

NOD2, located on chromosome 16q12.1, was the first disease-susceptibility gene discovered for CD. Its genetic variants are common in European and American patients, but have not been found in Asian patients. *NOD2* is a pattern-recognition receptor that is involved in the homeostasis of intestinal immunity. It acts through mechanisms like autophagy, intracellular bacterial sensing, controlling the expression of the antibacterial peptide α-defensin in the Paneth cells of the small intestine, and improving immune tolerance by suppressing toll-like receptor (TLR) signals[25]. *NOD2* recruits the autophagy protein *ATG16L1* to the plasma membrane at the bacterial entry site; mutant *NOD2* failed to recruit *ATG16L1* to the plasma membrane and wrapping of invading bacteria by autophagosomes was impaired. Therefore, patients with CD with *NOD2* variants are considered to exhibit disorders of autophagy[26-28]. When the mechanism of autophagy is impaired, lipopolysaccharides and damage-associated molecular patterns trigger signaling by stimulating TLR and NOD-like receptors, tumor necrosis factor (TNF), and other inflammatory cytokines. They also stimulate caspase-1 causing interleukin (IL)-1β and IL-18 cleavage from precursors, which promotes extracellular secretion (inflammasomes). In an experiment using mice knocked out for *ATG16L,* which encodes *ATG16L1*, the protein necessary for the autophagic recruitment, TLR and TNF stimulation led to abnormal inflammasome activity in macrophages and other innate immunity cells[29].

ATG16L1 is a homolog of *ATG16* that was first reported by Mizushima *et al*[30,31]. Along with *AT5* and *ATG12*, this molecule is required to form autophagosomes. Prescott *et al*[32] reported that the incidence of

CD was likely to be two times higher in people with the T300A variant, an *ATG16L1* variant with a threonine-to-alanine substitution at amino-acid position 300. Later, a meta-analysis of 25 studies showed that T300A caused disease susceptibility to CD[33]. However, no significant difference was observed in an analysis of patients from Japan, South Korea, and China from 25 studies. This suggests that European and American patients exhibit different genetic factors compared to Asian patients, as is seen with *NOD2*. Moreover, a meta-analysis of 14 studies on UC reported an odds ratio of 1.06, or almost no difference[33].

The report that *ATG16L1* is a CD-susceptibility gene was a groundbreaking discovery suggesting a role for autophagy in the onset of IBD. Since then, several researchers have published studies on the link between *ATG16L1* and IBD.

Paneth cells are a specialized type of epithelial cell that are involved in innate immunity in the small intestine. When they come into contact with bacteria or other antigens, these cells release secretory granules containing antimicrobial peptides and a variety of proteins. In 2008, Cadwell *et al*[34] engineered a mouse with low expression of *ATG16L1* (Atg16L1^{HM} mouse). Tissue analysis did not find lysozymes that are normally seen in the ileal mucosa, but found abnormal Paneth cell granule secretion. Moreover, they analyzed Paneth cells in non-inflamed areas of the ileum in patients with CD homozygous for the *ATG16L1* variant T300A, and found abnormal Paneth cells that strongly resembled those observed in Atg16L1^{HM} mice. This suggests that *ATG16L1* may also play an important role by suppressing Paneth cells in humans. In a relatively recent study, Lassen *et al*[35] generated a knock-in mouse model expressing ATG16L1^{T300A}. Such mice do not develop spontaneous inflammation, although they exhibit morphological defects in both Paneth cells and goblet cells. Furthermore, the presence of the T300A mutation in *ATG16L1* leads to aberrant functionality of Paneth cells. These findings indicate the reason there is believed to be a close relationship between *ATG16L1* variants and Paneth cells.

Further, Murthy *et al*[36] reported that *ATG16L1* amino-acid positions 296 to 299 form a caspase cleavage motif, which greatly increases *ATG16L1* sensitivity when the cellular stress response activates caspase-3 in the presence of the T300A variant. This may result in impaired autophagy, leading to CD onset, and suggests that *ATG16L1* plays a role at the molecular level in CD onset.

In 2010, Cadwell *et al*[37] reported interesting data on role of *ATG16L1* by using Atg16L1^{HM} mice infected with MNV CR6, a species of mouse norovirus. MNV CR6-infected Atg16L1^{HM} mice showed abnormal secretion of Paneth cell granules, similar to that described above. This was not observed in wild-type mice without an *ATG16L1* variant, or in mice infected with a different MNV strain or with inactivated MNV. Administration of dextran sulfate sodium (DSS) to these infected mice led to pathology similar to that observed in human patients with CD: inflammation extending to the muscle layer and mesentery, and atrophy of the ileal villi, neither of which has been previously reported with DSS colitis. These symptoms were significantly suppressed by administering TNF-α antibodies or antibiotics. A recent report suggested that *ATG16L1* polymorphisms promote disease through defects in "sensing" protective signals from the microbiome, defining a potentially critical gene-environment etiology for IBD[38].

These data suggest that in addition to *ATG16L1* variants, CD onset is influenced by a complex variety of environmental factors, including viral infections and enterobacteria.

IRGM

In a 2007 GWAS, Parkes *et al*[39] reported that the *IRGM* gene on chromosome 5q33.1 was a CD-susceptibility gene. In humans, *IRGM* is a 20 kDa protein formed from 181 amino acids that is expressed in the large intestine, small intestine, and lymphocytes. *IRGM* is related to bacterial killing, vacuolar trafficking and acidification, phagosome maturation, and virus-induced autophagy. Moreover, it is known to be involved in controlling intracellular *Mycobacterium tuberculosis* by autophagy in macrophages[40]. A small nuclear polymorphism (SNP) with susceptibility is adjacent to *IRGM*, but detailed sequencing of *IRGM* did not reveal any CD-related variants with modified amino acids. This suggests the possibility that changes of *IRGM* expression, transcript splicing, or the ratio of translation of the protein are related to the development of CD.

In 2008, McCarroll *et al*[41] discovered a 20 kb deletion polymorphism upstream from *IRGM* that was linked to an SNP correlating with CD. In addition, they reported that the expression of *IRGM* suppressed autophagy of intracellular bacteria, which has been linked to CD, suggesting a role in the pathology of CD.

Recently, Rufini *et al*[42] reported that *IRGM* polymorphisms were important for CD susceptibility and phenotype modulation (fibrostricturing behavior, ileal disease, perianal disease, and intestinal resection).

IL-23R

IL-23 is a heterodimeric cytokine produced by activated macrophages and dendritic cells. It consists of two subunits, a p40 subunit, shared with IL-12, and a specific IL-23 subunit called p19[43,44]. It has been shown that IL-23 is involved in the initiation of the innate and adaptive immune activation that characterizes IBD. It binds a complex of IL-23 receptor (IL-23R) and IL-12Rβ subunits. *IL-23R* is predominantly expressed on activated/memory T cells, T-cell clones, natural killer cells and, at low levels, in monocytes, macrophages, and dendritic cell populations[45,46].

Recent studies have shown association of the *IL-23R* gene with chronic inflammatory diseases, especially IBD[47,48]. It is also reported that autophagy regulates IL-23 secretion and innate T cell responses through effects on IL-1 secretion[49].

XIAP

XIAP (X-linked inhibitor of apoptosis) is one of several inhibitor of apoptosis proteins (IAPs). IAPs were initially identified in baculoviruses, where they prevent defensive apoptosis of host cells[50]. Among the mammalian IAPs, *XIAP* is the most extensively studied and best characterized. *XIAP* has the most potent anti-apoptotic ability[51], which is believed to be primarily related to direct binding and inhibiting of caspases, the apoptotic proteases that are responsible for the initiation and execution of apoptosis[52]. Huang *et al*[53] showed that *XIAP* is a physiological inhibitor of autophagy, and has been associated with a variety of diseases that have been linked to autophagy. *XIAP* is related to X-linked lymphoproliferative syndrome type 2 (XLP2), a type of primary immunodeficiency. However, a genetic analysis performed by Zeissig *et al*[54] found *XIAP* variants in only 4% of male patients with childhood-onset CD. Recently, Schwerd *et al*[55] showed impaired antibacterial autophagy links granulomatous intestinal inflammation in Niemann-Pick disease type C1 and *XIAP* deficiency with *NOD2* variants in CD.

LRRK2

LRRK2 (leucine-rich repeat kinase 2) is a large multidomain protein belonging to the ROCO family of proteins, which are characterized by the presence of leucine-rich repeats, a Ras of complex (ROC) GTPase domain, a C-terminal ROC linker region, and a kinase domain. *LRRK2* localizes to specific membrane subdomains, including endolysosomal structures in many kinds of cells. Studies showed that *LRRK2* KO mice displayed an increase in the number and size of secondary lysosomes and autolysosome-like structures. Abnormal accumulation of undigested material indicates an impairment in the autophagosomal-lysosomal degradation system (autophagy-lysosomal clearance pathway).

LRRK2 has been identified as a disease-susceptibility gene for Parkinson's disease, leprosy, and CD. The CD-associated SNP is located upstream of the coding sequence of *LRRK2*[56,57]. It was reported that *LRRK2* expression levels were found to be significantly upregulated in colonic biopsy specimens from inflamed tissues of patients with CD[58]. *LRRK2* is known to be expressed only in mucosal lymphocytes in the colonic mucosa, but little else is known about it.

ULK1

ULK1 (Unc-51 like autophagy activating kinase 1) is one of the key regulators of autophagy initiation and progression. Mammals have two homologs of the yeast autophagy-initiating ATG1 kinase, *ULK1* and *ULK2*. *ULK1* is regulated by the nutrient- and energy-sensitive kinases TORC1 and AMPK. The tight regulation of ULK activity by intracellular energy and nutrient levels is in keeping with a central role for autophagy in the protection of cells from starvation.

Henckaerts *et al*[59] selected human homologs of 12 yeast autophagy genes, known to be found in IBD-related loci from GWAS, and conducted a meta-analysis of these searches. An analysis of correlations with CD identified *ULK1* as a CD-susceptibility gene. *ULK1* activity is regulated by a complex array of multiple phosphorylation and dephosphorylation events that influence the binding of regulatory and effector autophagy proteins[60,61]. However, little is known about the action of *ULK1* in association with IBD, and further research is necessary.

VDR

VDR (the vitamin D receptor) regulates the expression of *NOD2*, and it has been suggested that it controls the mechanism of autophagy. Unlike other genes, *VDR* has been shown to be a UC-susceptibility gene, not only among Europeans and Americans, but also in Asian and Middle Eastern populations[62]. Vitamin D deficiency increases the risk of CD onset[63]; thus, analyzing its signaling pathways could help elucidate the pathology of this disease.

Recently, Wu *et al*[64] showed a fundamental relationship between the *VDR*, autophagy, and gut microbial assemblage that is essential for maintaining intestinal homeostasis, but also contributes to the pathophysiology of IBD. Furthermore, Abreu-Delgado *et al*[65] reported that levels of serum vitamin D correlate positively with colonic *VDR* expression in visually normal mucosa; whereas inflammation correlates negatively with colonic *VDR* expression in visually diseased mucosa. The *VDR* needs further research.

MTMR3

MTMR3 (myotubularin-related protein 3) plays a role in autophagosome formation[66]. The myotubularin family is a class of PI3-phosphatases that regulate several physiological and pathophysiological phenomena, including endosomal trafficking, apoptosis, autophagy, and muscle development. As a member of this family, *MTMR3* has been considered to play a negative role in the initiation stage of autophagy. Recent reports indicate that *MTMR3* has at least two opposite functions in the autophagy pathway, inhibition of mechanistic target of rapamycin complex 1 (mTORC1) and reduction of local PI3P levels[67,68]. In this regard, the function of *MTMR3* in autophagy remains unclear.

ROLE OF AUTOPHAGY IN IBD THERAPY AND FUTURE PROSPECTS

Widely used therapeutic agents for IBD include

Table 2 Therapeutic agents for inflammatory bowel disease related to autophagy

Drug	Influence on autophagy	Mechanism related to autophagy
5-ASA	Promotion	Through NF-κB signaling pathway
Corticosteroid	Promotion	Through NF-κB signaling pathway Through mTORC1 signaling pathway Through overexpression of Bcl-2 in immature T-lymphocytes Osteocyte viability
Thiopurine (AZA, 6-MP)	Promotion	Clearance of TPMT*3A aggregates and/or aggregate precursors Protective role in hepatocytes
Immunomodulatory drugs (CsA, FK506)	Promotion	Response to toxicity Through mTORC1 signaling pathway
Biological drugs (IFX, ADA, *etc.*)	Inhibition	Anti-TNF agents inhibit autophagy (not yet clear)

5-ASA: 5-aminosalicylic acid; mTORC1: Mechanistic target of rapamycin complex 1; AZA: Azathioprine; 6-MP: 6-Mercaptopurine; TPMT: Thiopurine S-methyltransferase; CsA: Cyclosporine A; IFX: Infliximab; ADA: Adalimumab; TNF: Tumor necrosis factor.

steroids and 5-aminosalicylic acid (5-ASA), as well as immunoregulatory drugs such as azathioprine, and biologicals such as anti-TNF-α formulations. The process of autophagy is closely related to each of these existing therapeutic agents. The following sections summarize these relationships (Table 2).

5-ASA

The mechanism of action of 5-ASA has been described in several studies. The suppression of peroxisome proliferator-activated receptor gamma (PPARγ) due to the production of inflammatory cytokines is said to contribute to the intestinal inflammation seen in patients with IBD[69]. 5-ASA is considered to exert its anti-inflammatory action by acting on PPARγ in epithelial cells, and by regulating signal transmission from NF-κB and TLR[70]. Considering that NF-κB signaling is associated with autophagy[71], it might be that 5-ASA indirectly regulates autophagy.

Corticosteroids

The first-line treatment to induce remission for CD and UC is often corticosteroids. Corticosteroids downregulate proinflammatory cytokines, including IL-1, IL-6, and TNFα. Furthermore, inflammatory signaling induced by NF-κB is decreased by interaction with corticosteroid receptors[72], and, as noted above, NF-κB signaling regulates autophagy[71]. It has also been shown that corticosterone treatment affects mTORC1 signaling pathways[73]. It was reported that mTORC1 pathways and autophagy play an important role in the response to treatment with corticosteroids[74]. Corticosteroids are able to induce apoptosis in immature T lymphocytes, as these cells lack the inhibitor of apoptosis protein Bcl-2. It has been shown that overexpression of Bcl-2 in immature T lymphocytes can increase autophagy levels, presumably due to inhibition of apoptosis[75].

A relationship between corticosteroids and autophagy has been observed, not only for their therapeutic effects, but also for the adverse effects that accompany treatment. It has been shown, both *in vitro* and *in vivo*, that low doses of prednisolone and dexamethasone induce autophagy in osteocytes, and this is associated with osteocyte viability[76,77]. However, higher doses of corticosteroids induce apoptosis, suggesting that autophagy may act as a protective mechanism against the cytotoxic effects of corticosteroids[76].

Thiopurines (azathioprine and 6-mercaptopurine)

Thiopurines, including azathioprine and 6-mercaptopurine, are immunosuppressant drugs used to maintain remission in patients with IBD[78]. Thiopurines and autophagy have also been shown to be correlated by the adverse effects of treatment. The thiopurine S-methyltransferase (*TPMT*) genetic polymorphism is important for thiopurine metabolism. Individuals with inherited decreases in TPMT activity, mainly as a result of the effects of the TPMT*3A allele (minor allele frequency in Caucasians of approximately 5%)[79], are at greatly increased risk for severe life-threatening myelosuppression when treated with "standard" doses of thiopurine drugs[80-83]. It was shown that autophagy might represent an important route for the clearance of TPMT*3A aggregates and/or aggregate precursors[84]. Due to the severe adverse effects of thiopurines, a potential protective role for autophagy in hepatocytes has been investigated; it has been shown that autophagy has a protective role in hepatocytes during thiopurine therapy[78].

Immunomodulatory drugs (cyclosporine A, FK506, methotrexate)

Cyclosporine A (CsA), FK506, and methotrexate (MTX) are immunomodulatory drugs used mainly as second-line treatments to induce and maintain remission in severe, steroid-refractory CD[85], with more recent evidence suggesting a role for FK506 in UC[86]. Although some evidence suggests that CsA and FK506 are involved in autophagy, no relationship has been identified between MTX and autophagy.

Several studies have shown that treatment with CsA can induce autophagy in response to toxicity (such as CsA-induced nephrotoxicity), either as a survival process or as part of a cell death mechanism[87-89].

FK506 inhibits calcineurin by forming a complex with the immunophilin FK506 binding protein 12 (FKBP12), which is involved in immunoregulation[90]. FKBP12 is also the direct target of rapamycin, an inhibitor of mTORC1. The molecular mechanism by which mTORC1 regulates autophagy in mammals is being investigated[91,92], while future research is expected to help understand the relationship between FK506 and autophagy.

Biological drugs (infliximab, adalimumab, etc.)

The most commonly used biological drug for IBD is the anti-TNFα antibody infliximab. Other anti-TNFα treatments approved for treatment of patients with IBD patients include adalimumab, golimumab for UC only, and certolizumab pegol. Anti-TNFα biosimilars have also recently been developed[93]. The relationship between TNFα and autophagy has been confirmed in synovial fibroblasts[94], skeletal muscles[95], and trophoblastic cells[96]. These studies suggest that anti-TNF agents would inhibit autophagy, and while the mechanism of action is not yet completely clear, it has been the subject of extensive research lately.

The above data summarizes the relationship between autophagy and various drugs. However, existing medical therapies do not relieve the symptoms in many patients, and surgical intervention is often necessary. There is, therefore, a pressing need to develop new therapeutic agents. As seen in this review, autophagy plays an important role in controlling the immune system; hence drugs that regulate autophagy have received much attention as potential new therapeutic targets for IBD[97]. Further investigation of the role of autophagy in existing IBD therapies, and development of new therapeutic agents regulating autophagy, are the needs of the hour.

CONCLUSION

GWAS has identified several disease-susceptibility genes, and studies on the pathology and etiology of IBD are being regularly published; however, more aspects of IBD pathogenesis should be clarified. As the number of patients with IBD is still increasing around the world, particularly among the young, it is essential that the mechanism of IBD is elucidated and treatments based on this mechanism are developed. A better understanding of the relationship between autophagy and IBD will result in better IBD therapy in future.

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REVIEW

Gastrointestinal bleeding in patients on novel oral anticoagulants: Risk, prevention and management

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Abstract

Novel oral anticoagulants (NOACs), which include direct thrombin inhibitor (dabigatran) and direct factor Xa inhibitors (rivaroxaban, apixaban and edoxaban), are gaining popularity in the prevention of embolic stroke in non-valvular atrial fibrillation as well as in the prevention and treatment of venous thromboembolism. However, similar to traditional anticoagulants, NOACs have the side effects of bleeding, including gastrointestinal bleeding (GIB). Results from both randomized clinical trials and observations studies suggest that high-dose dabigatran (150 mg b.i.d), rivaroxaban and high-dose edoxaban (60 mg daily) are associated with a higher risk of GIB compared with warfarin. Other risk factors of NOAC-related GIB include concomitant use of ulcerogenic agents, older age, renal impairment, *Helicobacter pylori* infection and a past history of GIB. Prevention of NOAC-related GIB includes proper patient selection, using a lower dose of certain NOACs and in patients with renal impairment, correction of modifiable risk factors, and prescription of gastroprotective agents. Overt GIB can be managed by withholding NOACs followed by delayed endoscopic treatment. In severe bleeding, additional measures include administration of activated charcoal, use of specific reversal agents such as idarucizumab for dabigatran and andexanent alfa for factor Xa inhibitors, and urgent endoscopic management.

Key words: Warfarin; Endoscopy; Apixaban; Edoxaban; Gastrointestinal bleeding; Dabigatran; Rivaroxaban; Novel anticoagulants

Core tip: Although effective in the prevention and

treatment of thromboembolism, novel oral anticoagulants (NOACs) are still associated with bleeding complications including gastrointestinal bleeding (GIB). Physicians should exercise caution in the prescription of these drugs with careful review of the indications and appropriate dosage, as well as balancing the risks and benefits. Nonetheless, patients perceived to have an increased risk of GIB should not be precluded from taking NOACs, if they are also at a high risk of stroke. Instead, physicians should recognize the risk factors associated with NOAC-related GIB in order to undertake preventive measures to reduce the risk of GIB.

Cheung KS, Leung WK. Gastrointestinal bleeding in patients on novel oral anticoagulants: Risk, prevention and management. *World J Gastroenterol* 2017; 23(11): 1954-1963 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i11/1954.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i11.1954

INTRODUCTION

Novel oral anticoagulants (NOACs) include direct thrombin inhibitor (dabigatran) and direct factor Xa inhibitors (rivaroxaban, apixaban and edoxaban) are increasingly favored over conventional oral anticoagulants such as warfarin. NOACs are often used in the prevention of embolic stroke in non-valvular atrial fibrillation (AF) as well as in the prevention and treatment of venous thromboembolism (VTE). Compared with warfarin, NOACs have rapid onset and offset of action, predictable pharmacodynamics obviating regular therapeutic monitoring, and fewer food-drug or drug-drug interactions[1]. Although NOACs have been shown to have a favorable safety profile from multiple meta-analyses and phase IV studies, the risk of bleeding, particularly gastrointestinal bleeding (GIB), is still a concern in high-risk patients. This review will discuss the risk of GIB related to commonly prescribed NOACs with special focus on the dosing of different indications and in patients with impaired renal function, differences in bleeding risk between randomized clinical trials (RCTs) and observational studies, risk factors for GIB, prevention and management strategies.

NOMENCLATURE FOR ORAL ANTICOAGULANTS

Apart from "NOACs", other terms that have been used to describe these new oral anticoagulants include direct oral anticoagulants (DOACs) and target-specific oral anticoagulants. There are also calls to restrict the term "NOACs" to "non-vitamin K oral anticoagulants" instead of "novel oral anticoagulants"[2], as this terminology is well established in the medical literature. Recently, it has also been advocated that term "DOACs" should be adopted as the name implies similar pharmacological actions of this class of oral anticoagulants that inhibit a single target[3]. In this review, we use the term "NOACs" to refer to novel oral anticoagulants inclusive of dabigatran, rivaroxaban, apixaban and edoxaban.

PHARMACOLOGY OF NOAC

The characteristics and dosing of different NOACs are summarized in Tables 1 and 2. Dabigatran directly inhibits thrombin activity in a reversible manner. It is administered as a prodrug known as dabigatran etexilate and after absorption in the proximal small bowel, dabigatran etexilate is cleaved by serum and hepatic esterases to the active form[4]. The bioavailability of dabigatran etexilate is 7% with the majority of non-absorbed drug being excreted in the stool. Absorbed drug is mainly excreted unchanged by the kidneys. The half-life of dabigatran ranges from 9 to 17 h, depending on individuals' age and renal function. Dabigatran can be administered at a dose of 150 mg b.i.d or 110 mg b.i.d, and 75 mg b.i.d in the presence of renal insufficiency [creatinine clearance (CrCl) < 50 mL/min][5]. The drug is contraindicated in patients with severe renal impairment (CrCl < 30 mL/min) or advanced liver disease[6].

Rivaroxaban is a direct inhibitor of factor Xa, which reduces thrombin production. It has a bioavailability of 66%[7,8]. One-third of the absorbed drug is excreted by the kidney and two-thirds is metabolized by the liver into inactive forms. The half-life ranges from 6 to 13 h[8]. Rivaroxaban is administered at a dose of 20 mg daily, and 15 mg daily if the CrCl is < 50 mL/min[5]. The drug is also contraindicated in severe renal impairment (CrCl < 15 mL/min) or advanced liver disease[6].

Similar to rivaroxaban, apixaban directly inhibits factor Xa. It has a bioavailability of 50%[9]. Around 25% of the absorbed drug is excreted by the kidney with a half-life of around 12 h. Apixaban is administered at a dose of 5 mg b.i.d, and 2.5 mg b.i.d if patients has at least 2 of the following features: age 80 years or older, body weight 60 kg or less, or serum creatinine 1.5 mg/dL or more[5].

Edoxaban is also a direct factor Xa inhbitior, with a bioavailability of 60% and a half-life of around 12 h. Renal clearance accounts for 50% of the total clearance[6]. Edoxaban can be administered at a dose of 60 mg daily, and 30 mg daily if the CrCl is < 50 mL/min or body weight is less than 60 kg. The drug is contraindicated in severe renal impairment (CrCl < 15 mL/min) and advanced liver disease. Of note, serum levels of NOACs can be increased by potent inhibitors of p-glycoprotein and CYP3A4 (*e.g.*, azole antifungal agents, protease inhibitors), while serum levels can be decreased by strong inducers of p-glycoprotein and CYP3A4 (*e.g.*, rifampicin)[10].

MECHANISMS OF NOAC-RELATED GIB

There are several mechanisms by which NOAC causes

Table 1 Characteristics of different novel oral anticoagulants

	Dabigatran	Rivaroxaban	Apixaban	Edoxaban
Mechanism of action	Anti-thrombin	Anti-factor Xa	Anti-factor Xa	Anti-factor Xa
Bioavailability	7%	66%	50%	60%
Tmax (h)	1.5	2.5	3	1-5
T½ (h)	9-17	6-13	12	12
Dosing	b.i.d	once daily	b.i.d	once daily
Renal excretion	High	Moderate	Moderate	Moderate
Hepatic metabolism	Low	Moderate	Moderate	Moderate
Reversal agents	Idarucizumab[1] Aripazine	Andexanet alfa Aripazine	Andexanet alfa Aripazine	Andexanet alfa Aripazine

[1]Idarucizumab is the only FDA-approved specific reversal agent currently. Tmax: Time to peak plasma level; T½: Half-life; GIB: Gastrointestinal bleeding.

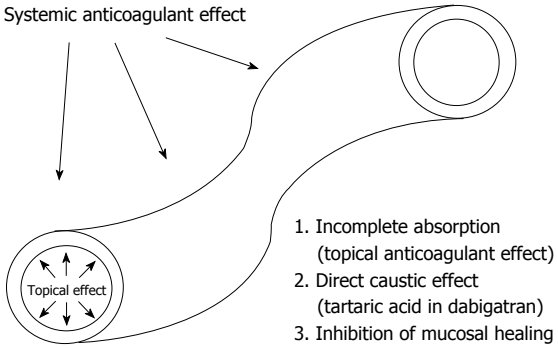

Figure 1 Pathogenesis of novel oral anticoagulant-related gastrointestinal bleeding. NOAC: Novel oral anticoagulant; GIB: Gastrointestinal bleeding.

GIB[1] (Figure 1). The anticoagulant effect can be local and/or systematic, and NOACs may inhibit GI mucosal healing. In addition, the tartaric acid in dabigatran etexilate is postulated to cause direct caustic injury. When compared with warfarin, dabigatran and rivaroxaban are associated with an increased risk of GIB only, but have not been demonstrated to increase bleeding in other organs including intracranial hemorrhage[1,10]. Of note, the sites of GIB differ for individual NOACs. In contrast to the usual pattern observed with warfarin, aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) where upper GIB predominates[11], lower GIB accounted for 53% of major GIB seen in dabigatran users in the RE-LY trial[12]. This has been hypothesized to be related to the incomplete absorption of the active NOACs in the upper GI tract with resulting increasing availability of dabigatran to the lower GI tract which exert topical effect on the mucosa leading to bleeding, especially in the presence of pre-existing lesions like angiodysplasias and erosions[4]. On the other hand, the bioavailability of warfarin is more than 95%, and non-absorbed warfarin does not have any topical effect[1,10]. Notably, upper GIB appeared to be more common than lower GIB among rivaroxaban users (76 % *vs* 24%)[13], while the risks of upper and lower GIB were comparable with high-dose edoxaban (60 mg daily)[6,14].

The dosing of NOACs may also affect the risk of GIB[1,10]. Both rivaroxaban and apixaban are factor Xa inhibitors, administered in active form, and have similar bioavailability. However, these two agents differ in the risk of GIB, which may be related to the higher peak level of once-daily dosing of rivaroxaban than the twice-daily dosing of apixaban. Similarly, the once-daily dosing of rivaroxaban may also account for the higher GIB risk observed in the head-to-head study of rivaroxaban and dabigatran[15].

RISK OF NOAC-RELATED GIB IN RCTS

Holster *et al*[16] summarized the risk of GIB associated with NOACs in a recent meta-analysis, which included 17 RCTs with a total of 75081 patients who received either NOACs or standard care (defined as either low-molecular-weight heparin, vitamin K antagonist, antiplatelet therapy or placebo). During a follow-up period ranging from 3 wk to 31 mo, there was a 1.5% GIB event, with 89% being major GIB (defined as GIB leading to a decrease in hemoglobin $\geqslant$ 2 g/dL within 24 h, a transfusion of $\geqslant$ 2 units of packed red cells, necessitating intervention including surgery, or fatal bleeding). The number needed to harm was 500. Overall, there was an increased risk of GIB among NOAC users, compared with standard care [pooled odds ratio (OR) 1.45], though significant heterogeneity existed regarding drug choices and the indications of anticoagulation.

Among different NOACs, both dabigatran and rivaroxaban were associated with a higher risk of GIB (OR 1.58 and 1.48, respectively), but not apixaban and edoxaban. However, since there are still no direct head-to-head comparisons of GIB risks among various NOACs in RCTs, it is difficult to conclude which drug has the lowest GIB risk. As patient characteristics differed across studies, indirect comparisons can be misleading[17].

For various indications of NOACs, the highest risk of GIB was seen in patients with acute coronary syndrome (OR 5.21), in whom NOACs were co-prescribed with antiplatelet agents. Patients prescribed NOACs for deep vein thrombosis and pulmonary embolism also had an increased risk of bleeding (OR 1.59). However, the GIB risk was not significantly increased in patients

Table 2 Dosing of different novel oral anticoagulants according to indications and renal function

	Dabigatran	Rivaroxaban	Apixaban	Edoxaban
Non-valvular AF				
United States	150mg b.i.d 75 mg b.i.d if CrCl 15-30 mL/min	20 mg daily 15 mg daily if CrCl 15-50 mL/min	5 mg b.i.d 2.5 mg b.i.d if Cr 15-29 mL/min OR two out of the following: age ⩾ 80 years, BW ⩽ 60 kg, Cr ⩾ 1.5 mg/dL	60 mg daily 30 mg daily if CrCl 15-50 mL/min
	Avoid if CrCl < 15 mL/min	Avoid if CrCl < 15 mL/min	Avoid if CrCl < 25 mL/min or Cr > 2.5 mg/dL	Avoid if CrCl < 15 mL/min
Europe	150 mg b.i.d 110 mg b.i.d if age ⩾ 80 years (may consider 110 mg b.i.d also if increased risk of bleeding)	20 mg daily -	5 mg b.i.d 2.5 mg b.i.d if Cr 15-29 mL/min OR two out of the following: age ⩾ 80 years, BW ⩽ 60 kg, Cr ⩾ 1.5 mg/dL	60 mg daily 30 mg daily if one out of the following: CrCl 15-50 mL/min, BW ⩽ 60 kg, concomitant use of p-gp inhibitors
	Avoid if CrCl < 30 mL/min	Avoid if CrCl < 15 mL/min	Avoid if CrCl < 15 mL/min	Avoid if CrCl < 15 mL/min
Postoperative DVT / PE thromboprophylaxis (hip or knee replacement)				
United States	Initial dose of 110 mg 1-4 h after operation, then 220 mg daily -	Initial dose of 10 mg 6-10 h after operation, then 10 mg daily -	Initial dose of 2.5 mg 12-24 h after operation, then 2.5 mg b.i.d -	- -
	Avoid if CrCl < 30	Avoid if CrCl < 30 mL/min	Avoid if CrCl < 30 mL/min	-
Europe	Initial dose of 110 mg 1-4 h after operation, then 220 mg daily Initial dose of 75 mg 1-4 h after operation, then 150 mg daily if CrCl 30-50 mL/min	Initial dose of 10 mg 6-10 h after operation, then 10 mg daily -	Initial dose of 2.5 mg 12-24 h after operation, then 2.5 mg b.i.d -	60 mg daily after 5 d of initial therapy with a parenteral anticoagulant 30 mg daily after 5 d of initial therapy with a parenteral anticoagulant if one out of the following: CrCl 15-50 mL/min, BW ⩽ 60 kg, concomitant use of p-gp inhibitors
	Avoid if CrCl < 30 mL/min	Avoid if CrCl < 15 mL/min	Avoid if CrCl < 15 mL/min	Avoid if CrCl < 15 mL/min
Treatment and prevention of recurrent DVT/PE				
United States	150 mg b.i.d after 5-10 d of initial therapy with a parenteral anticoagulant -	15 mg b.i.d for 3 wk, then 20 mg daily -	10 mg b.i.d for 1 wk, then 5 mg b.i.d -	60 mg daily after 5-10 d of initial therapy with a parenteral anticoagulant 30 mg daily after 5-10 d of initial therapy with a parenteral anticoagulant if one out of the following: CrCl 15-50 mL/min, BW ⩽ 60 kg, concomitant use of p-gp inhibitors
	Avoid if CrCl < 30 mL/min	Avoid if CrCl < 30 mL/min	Avoid if CrCl < 25 mL/min or Cr > 2.5 mg/dL	Avoid if CrCl < 15 mL/min
Europe	150 mg b.i.d after 5 d of initial therapy with a parenteral anticoagulant 110 mg b.i.d after 5 d of initial therapy with a parenteral anticoagulant if age ⩾ 80 years (may consider 110 mg b.i.d also if increased risk of bleeding)	15 mg b.i.d for 3 wk, then 20 mg daily -	10 mg b.i.d for 1 wk, then 5 mg b.i.d -	60 mg daily after 5 d of initial therapy with a parenteral anticoagulant 30 mg daily after 5 d of initial therapy with a parenteral anticoagulant if one out of the following: CrCl 15-50 mL/min, BW ⩽ 60 kg, concomitant use of p-gp inhibitors
	Avoid if CrCl < 30 mL/min	Avoid if CrCl < 15 mL/min	Avoid if CrCl < 15 mL/min	Avoid if CrCl < 15 mL/min

NOACs: Novel oral anticoagulants; AF: Atrial fibrillation; CrCl: Creatinine clearance; BW: Body weight; Cr: Creatinine; DVT: Deep vein thrombosis; PE: Pulmonary embolism; p-gp inhibitors: p-glycoprotein inhibitors.

receiving NOACs for prevention of VTE after orthopedic surgery and in medically ill patients. Although there was no significant increase in the overall risk of GIB among all patients receiving NOACs for AF, subgroup analysis showed an increase in risk among dabigatran and rivaroxaban users. The increased GIB risk in AF (but not with thromboprophylaxis after orthopedic surgery) among dabigatran and rivaroxaban users is likely explained by the duration effect, as orthopedic patients usually receive NOACs for a short, finite period (few weeks only)[18]. It has also been shown that among patients receiving dabigatran, only the higher dose (150 mg b.i.d) was associated with a higher GIB risk when compared with warfarin, indicating a dose-related effect[12,19-22]. The risk of GIB was also increased with high-dose edoxaban of 60 mg daily (HR 1.23), but was reduced with low-dose edoxaban of 30 mg daily (HR 0.89)[14]. However, subsequent systematic reviews and

Table 3 Risk factors for novel oral anticoagulant-related gastrointestinal bleeding

Risk factors	Definition
Higher dose of dabigatran and edoxaban	Dabigatran: a dose of 150 mg b.i.d Edoxaban: a dose of 60 mg daily
Concomitant use of ulcerogenic agents	Antiplatelet agents, NSAIDs or steroid
Older age	Age ⩾ 75 years
Renal impairment	Creatinine clearance < 50 mL/min
Prior history of peptic ulcers or GIB	
Helicobacter pylori infection	
Pre-existing GI tract lesions	Examples like diverticulosis, angiodysplasias
Ethnicity	Western population
HAS-BLED score	Score of ⩾ 3
Protective factors	Definition
Gastroprotective agents	Proton pump inhibitors or histamine H2-receptor antagonists

NOAC: Novel oral anticoagulant; GIB: Gastrointestinal bleeding; NSAIDs: Non-steroidal anti-inflammatory drugs.

meta-analyses which included more trials with different inclusion and exclusion criteria yielded conflicting results, showing either no or only a marginal increase in the risk of GIB[23-29].

RISK OF NOAC-RELATED GIB IN OBSERVATIONAL STUDIES

As most RCTs adopted stringent inclusion and exclusion criteria to enroll patients with relatively low risk of GIB, the results may not be generalizable to the general population. It was initially estimated that when NOACs were marketed, the risk of bleeding could be increased up to 15-fold as a result of prescription of these drugs to patients with higher risk of GIB[30]. In addition, RCTs that separately reported on GIB were usually limited to major GIB only, leading to an underestimation of the risk of all GIB[16].

To determine the association between NOACs and GIB in real-life settings, a recent meta-analysis[19] included 8 cohort studies with a total of 117339 NOAC users of either dabigatran or rivaroxaban. The pooled incidence rates of GIB were 4.5 per 100 patient-years and 7.18 per 100 patient-years for patients receiving dabigatran and rivaroxaban, respectively. Compared with warfarin, dabigatran was associated with an increased risk of GIB [relative risk (RR) 1.21], but rivaroxaban did not confer a significant increase in risk. However, one could not conclude that rivaroxaban had a lower GIB risk compared with dabigatran, as the non-significant association with GIB risk for rivaroxaban may be related to fewer observational studies, a shorter follow-up duration and an overall younger age of rivaroxaban users. In fact, in a recent head-to-head comparative observational study that recruited 118891 patients with non-valvular AF who were aged 65 years or older[15], rivaroxaban was found to be associated with a higher risk of major GIB compared with dabigatran (150 mg b.i.d) [hazard ratio (HR) 1.40]. Although the differences in baseline characteristics were adjusted by propensity scores in this study, the result should still be interpreted with caution due to the possibility of residual confounding by unmeasured factors.

Contrary to previous postulation[30], the risk of GIB was slightly lower in observational studies when compared with that reported in RCTs[16]. Several factors may account for this finding. Firstly, RCTs recruited older patients who had higher risk of stroke. In the four landmark phase III RCTs (RE-LY, ROCKET-AF, AIRSOTLE, ENGAGE AF-TIMI 48 trials)[14,20,31,32], the mean or median age of NOAC users was 70 years or above, while subjects from 5 studies included in the meta-analysis of cohort studies had a mean age below 70 years[33-37]. Secondly, patients from observational studies had fewer comorbidities, as reflected by a lower CHADS2 (congestive heart failure, hypertension, age of 75 years or above, diabetes mellitus, history of stroke, transient ischemic attack or thromboembolism) score[19]. Thirdly, physicians may avoid prescribing NOACs to high-risk patients in real-life practice.

One major concern about the meta-analysis of observational studies is that the results may be biased due to confounding factors. Nonetheless, major confounding factors including age, use of gastroprotective agents and ulcerogenic agents (including antiplatelet agents, NSAIDs, steroid and selective serotonin reuptake inhibitors), as well as indications of anticoagulation were evaluated by subgroup and sensitivity analysis. As for apixaban and edoxaban, observational studies comparing their risk of GIB with that of warfarin are currently lacking.

PREVENTION OF NOAC-RELATED GIB

Factors associated with the NOAC-related GIB are summarized in Table 3. Older age (⩾ 75 years) was associated with an increased risk of NOAC-related GIB. Among patients receiving dabigatran, there is a 2.5-fold increase in risk of GIB[12,34,36,38]. A greater risk of major GIB was also observed in older patients receiving rivaroxaban[34,39]. As elimination of NOACs depends on

Table 4 Components of HAS-BLED bleeding risk score

Clinical characteristics	Definition	Points
Hypertension	Systolic blood pressure > 160 mmHg	1
Abnormal liver or renal function	Chronic liver disease (*e.g.*, cirrhosis) or biochemical evidence of significantly impaired liver function (*e.g.*, bilirubin > 2 times the ULN plus one or more liver enzymes > 3 times the ULN Chronic dialysis, renal transplantation, or serum creatinine ⩾ 200 micromol/L	1 or 2
Stroke	Previous history of stroke	1
Bleeding tendency or predisposition	Bleeding disorder or previous bleeding episode requiring hospitalization or transfusion	1
Labile INRs	Labile INRs in patients taking warfarin (failure to maintain a therapeutic range at least 60% of the time)	1
Elderly	Age > 65 years	1
Drugs	Concomitant antiplatelet agents or NSAIDs Excessive alcohol use (⩾ 8 units per week)	1 or 2

Maximum score is 9. ULN: Upper limit of normal; INR: International normalized ratio; NSAIDs: Non-steroidal anti-inflammatory drugs.

renal excretion, patients with impaired renal function are more likely to have drug accumulation and hence higher bleeding risk[10]. A prior history of peptic ulcer disease or GIB was associated with a 2.3-fold increased risk of GIB[38]. Concomitant antiplatelet therapy is also a well-recognized risk factor[12,16,38,40]. For example, among dabigatran users, concomitant antiplatelet use was associated with a 30% to 50% higher risk of GIB[12,38].

Ethnicity was another risk factor. Chinese patients receiving dabigatran were observed to have a higher incidence rate of GIB (4.2 per 100 person-years)[38], compared with the western population (1.2 to 1.5 per 100 person-years in Denmark and 0.6 to 3.4 per 100 person-years in the United States)[22,41]. Genetic factors, particularly factor V-Leiden mutation which is exceedingly rare in the Asians, may account for this difference[42-44].

The HAS-BLED score (hypertension, abnormal liver/renal function, history of stroke, bleeding tendency, labile INRs, elderly aged ⩾ 65 years, drug/alcohol use) was initially derived to predict bleeding risk of warfarin in patients with AF (Table 4)[45]. A score of ⩾ 3 is considered high-risk, with a score of 3 conferring a risk of 3.74 bleeding events per 100 patient-years[45,46]. However, it should be acknowledged that patients with an increased risk of thromboembolism usually have one or more of the comorbidities included in the HAS-BLED score, which predispose them to bleeding. Other risk factors for GIB [*e.g.*, *Helicobacter pylori* (HP) infection, colonic diverticulosis, or presence of angiodysplasias] should also be considered in the decision making when prescribing NOACs[47]. Concurrent use of antiplatelet agents and NSAIDs also increases the bleeding risk and should factor into the decision making.

Co-administration of gastroprotective agents [either proton pump inhibitors (PPIs) or histamine H2-receptor antagonists (H2RAs)] was found to be associated with a 50% reduction in the risk of GIB[38]. This protective effect seems to be confined to the upper GI tract among those who had history of peptic ulcer disease or GIB. PPIs offered a slightly better protective effect than H2RAs, with the largest effect seen in patients using both PPIs and H2RAs (85% reduction in the risk of GIB)[38]. The protective effect may be mediated by the reduction of bleeding from pre-existing peptic ulcers. In addition, acid suppression may reduce the absorption of dabigatran[20,48]. While the impact of gastroprotective agents is strong for dabigatran-related GIB, the effect appears to be modest for rivaroxaban[19].

Prevention of GIB relies on first reviewing the indications of NOACs and avoiding NOACs in patients with contraindications[49], including renal impairment (CrCl less than 30 mL/min for dabigatran and CrCl less than 15 mL/min for other NOACs), and advanced liver disease with coagulopathy. Other preventive measures include the use of appropriate dosage with reference to the CrCl, institution of renal protective measures (*e.g.*, avoiding NSAIDs and herbs), correction of modifiable risk factors (*e.g.*, HP eradication, alcohol abstinence, avoidance of co-administration of antiplatelet agents or NSAIDs). For patients identified to be at high risk for GIB (*e.g.*, HAS-BLED score ⩾ 3, history of previous GIB), prescription of gastroprotective agents[1,10,47] and use of apixaban or low-dose dabigatran (110 mg b.i.d) are recommended[6,50]. However, it is also important to note that lower dose of NOACs is less efficacious in stroke prevention[26]. For the same reason, the use of HAS-BLED score is meant to "flag up" patients at high risk for GIB rather than to withold anticoagulation[51]. For those who have experienced GIB while on warfarin, dabigatran (150 mg b.i.d) or rivaoxaban, reducing the dose of dabigatran to 110 mg b.i.d or switching to apixaban may be considered[1,50]. Screening and surveillance colonoscopy has been advocated to detect occult tumors before initiation of NOACs, hence reducing the incidence of tumor-associated GIB[6,52]. This practice, however, is not the standard of care recommended by the American Heart Association and American Stroke Association (AHA/ASA) or the CHEST guidelines. Interestingly, NOACs may lead to an earlier diagnosis of GI tract malignancies as they may provoke bleeding of these lesions[52].

MANAGEMENT OF OVERT GIB

Specific management of patients taking NOACs who present with overt, non-major GIB involves cessation of the drug and endoscopic management[1,10,49]. Owing to the short half-life, cessation of drug will lead to a rapid return of the coagulant function within 12-24 h and near complete recovery after five half-lives in patients with normal renal function[5,10]. In the case of severe bleeding and/or hemodynamic instability, activated charcoal, hemodialysis/hemoperfusion and reversing anticoagulation can be considered. Activated charcoal can be given to reduce intestinal absorption of residual drug if the last dose of the NOAC is taken within 2 h. This potential benefit needs to be weighed against subsequent impairment of endoscopic visualization[53]. Hemodialysis or hemoperfusion may also be considered for dabigatran in the case of life-threatening GIB or renal failure[54], but not for direct factor Xa inhibitors as they are highly protein-bound[1].

Non-specific reversal agents include prothrombin complex concentrates (PCCs) (either weight-based 3-factor or 4-factor PCCs), activated PCCs and recombinant factor VIIa (rFVIIa). However, well-designed clinical trials are not available to prove their efficacy, and their use was also infrequent in the phase III trials and observational studies[5,49]. Moreover, their use has been associated with a risk of thromboembolism[55-57], although it has been shown in a recent study that 4-factor PCCs actually had a similar safety profile compared with fresh frozen plasma in terms of thromboembolic events (around 7%) and deaths[58]. Given the uncertain efficacy and the potential risk of thromboembolism, these agents should only be considered in the following situations: life-threatening GIB, ongoing bleeding despite standard measures, or delayed clearance of NOACs in patients with renal failure[49]. Anti-fibrinolytic agents (tranexamic acid) have been used in the management of NOAC-related GIB, but the experience is still limited[49,59]. FFP and cryoprecipitate are not effective in reversing the anticoagulant effect[49].

Specific reversal agents have also been developed recently. Idarucizumab, a humanized monoclonal antibody fragment (Fab) against dabigatran, is shown to be able to completely reverse anticoagulation in around 90% of patients[60], and has been approved in the United Sates. Andexanet alfa is a recombinant modified human factor Xa decoy protein which binds to the factor Xa inhibitors, and therefore is a universal factor Xa reversal agent[49]. It has been shown to substantially reverse anti-factor Xa activity, with hemostasis achieved in around 80% of patients presenting with acute major bleeding[61]. Aripazine is a cationic small molecule that can prevent NOACs (including dabigatran and factor Xa inhibitors) from binding with their targets through non-covalent binding and charge-charge interaction[62]. It is still in the developmental phase, but has shown promising results. However, these agents are also associated with thromboembolic risks. For instance, around 5% and 18% developed thromboembolism after receiving idarucizumab and andexanet alfa, respectively[60,61].

The timing of endoscopy depends on the severity of GIB and hemodynamic status of the patients. In patients with mild GIB, endoscopic evaluation can be deferred for 12-24 h[10,63]. Advantages of this delayed approach are increased effectiveness of endoscopic intervention as the drug effects have worn off, increased safety in a non-emergency setting, and improved endoscopic visualization due to attenuation/cessation of bleeding and better colonic cleansing. On the other hand, if patients present with severe GIB or are hemodynamically unstable, emergency endoscopy should be performed promptly after resuscitation. Radiological and/or surgical interventions will be the last resort if repeated endoscopic management fails.

CONCLUSION

Compared with warfarin, there is a higher risk of GIB for high-dose dabigatran (150 mg b.i.d), rivaroxaban and high-dose edoxaban (60 mg daily). Reviewing the indications of NOACs and prescribing a particular NOAC on an individual basis are therefore of utmost importance. In addition, physicians should be aware of the risk factors for NOAC-related GIB and adopt preventive measures accordingly. In the majority of cases, overt GIB can be handled by withholding NOACs combined with delayed endoscopic treatment.

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REVIEW

Traditional Chinese herbal extracts inducing autophagy as a novel approach in therapy of nonalcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is one of the leading causes of chronic liver diseases around the world due to the modern sedentary and food-abundant lifestyle, which is characterized by excessive fat accumulation in the liver related with causes other than alcohol abuse. It is widely acknowledged that insulin resistance, dysfunctional lipid metabolism, endoplasmic reticulum stress, oxidative stress, inflammation, and apoptosis/necrosis may all contribute to NAFLD. Autophagy is a protective self-digestion of intracellular organelles, including lipid droplets (lipophagy), in response to stress to maintain homeostasis. Lipophagy is another pathway for lipid degradation besides lipolysis. It is reported that impaired autophagy also contributes to NAFLD. Some studies have suggested that the histological characteristics of NAFLD (steatosis, lobular inflammation, and peri-sinusoid fibrosis) might be improved by treatment with traditional Chinese herbal extracts, while autophagy may be induced. This review will provide insights into the characteristics of autophagy in NAFLD and the related role/mechanisms of autophagy induced by traditional Chinese herbal extracts such as resveratrol, Lycium barbarum polysaccharides, dioscin, bergamot polyphenol fraction, capsaicin, and garlic-derived S-allylmercaptocysteine, which may inhibit the progression of NAFLD. Regulation of autophagy/lipophagy with traditional Chinese herbal extracts may be a novel approach for treating NAFLD, and the molecular mechanisms should be elucidated further in the near future.

Key words: Traditional Chinese herbal extracts; Non-alcoholic fatty liver disease; Autophagy

Core tip: Due to the modern sedentary and food-abundant lifestyle, the incidence of non-alcoholic fatty liver disease (NAFLD) has doubled during the past years, and its prevalence ranges from 20% in China and 27% in Hong Kong to 30% in Western countries. Although NAFLD is a major cause of chronic liver diseases, a satisfactory treatment targeting one or several pathological mechanisms of NAFLD has yet to be identified. Recent studies have suggested that Chinese herbal extracts (resveratrol, Lycium barbarum polysaccharides, dioscin, bergamot polyphenol fraction, capsaicin, garlic-derived S-allylmercaptocysteine) may inhibit NAFLD progression by inducing autophagy, the role and mechanisms of which are summarized in this review.

Liu C, Liao JZ, Li PY. Traditional Chinese herbal extracts inducing autophagy as a novel approach in therapy of nonalcoholic fatty liver disease. *World J Gastroenterol* 2017; 23(11): 1964-1973 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i11/1964.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i11.1964

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the leading causes of chronic liver diseases around the world, and is characterized by an excessively high accumulation of fat deposits in the liver resulting from causes other than chronic alcohol abuse[1,2]. The incidence of NAFLD has doubled during the past years, and its prevalence ranges from 20% in China and 27% in Hong Kong to 30% in Western countries, primarily due to the modern sedentary and food-abundant lifestyle in those regions[3,4]. The spectrum of NAFLD extends from non-alcoholic simple steatosis (NAS) to non-alcoholic steatohepatitis (NASH) and liver cirrhosis. Furthermore, NAFLD can progress to liver cancer without fibrosis[5-7].

NAFLD is often accompanied by obesity, diabetes and hyperlipidemia, and therefore closely associated with insulin resistance and lipid metabolism dysfunction, both of which can lead to the excessive accumulation of lipid droplets in hepatocytes (the first hit). Such lipid accumulation makes hepatocytes particularly vulnerable to internal and external stimuli during the first hit. As a result, lipid peroxidation, oxidative stress, cytokines, endoplasmic reticulum (ER) stress and endotoxins can all further aggravate any pre-existing liver injury, induce inflammation, impair autophagic flux and activate Kupffer cells. These types of cellular responses lead to lobular inflammation, Mallory-Denk bodies, NASH, fibrosis, and finally liver cirrhosis[8-10]. Moreover, a small percentage of such patients develop hepatic carcinoma[11]. Although NAFLD is a major cause of chronic liver diseases, a satisfactory treatment targeting one or several pathological mechanisms of NAFLD has yet to be identified.

Autophagy is a self-digestion process that occurs in all cells. Basal autophagy in eukaryotic cells is a protective response to stress resulting from internal and external stimuli such as injury, infection, *etc*. Double membrane fragments derived from intracellular organelles, such as mitochondria, pieces of ER and Golgi apparatus, can enfold damaged organelles and misfolded or unfolded proteins, which are then transported to lysosomes for degradation. The degradation products are finally recycled as substrates to be used for new cell formation[12,13].

Three main types of cellular autophagy have been identified: macroautophagy, chaperone-mediated autophagy, and microautophagy. Autophagy is a multi-step process including initiation, elongation, enclosure, maturation and degradation. It is widely acknowledged that about 30 mammalian homologs of yeast autophagy-related proteins (Atg) have been identified which are involved in initiation and elongation of the isolation membrane[14]. The initiation step requires the ULK1-Atg13-Atg101-FIP200 complex and Beclin1-Vps34-Vps15-Atg14L complex[14,15]. Under starvation stress, mTOR is inactivated, resulting in ULK1 activation and phosphorylation of Atg13, Atg101 and FIP200. The above two complexes recruit two conjugation systems including Atg12 conjugation system (including Atg5, Atg12, Atg7, Atg10 and Atg16L1) and LC3 conjugation system (including LC3, Atg4, Atg7 and Atg3), which are essential for elongation and enclosure steps[15].

Basal level of autophagy in a cell helps to maintain its homeostatic state and normal function, and promote its survival under stressful conditions. However, constant stimulation can still lead to autophagic cell death[16,17]. It is well documented that aging, neurodegeneration, tumors, immunological diseases, diabetes and NAFLD have an intertwined relationship with autophagic disorders[18-20]. Thus, maintenance of autophagy balance is important for good health.

NAFLD AND AUTOPHAGY

NAFLD is always accompanied by the combined comorbidities of obesity, diabetes and dyslipidemia, otherwise described as metabolic syndrome[21]. The basic pathogenesis of NAFLD is an excessive accumulation of lipid droplets in hepatocytes, resulting from dysfunctional lipid metabolism combined with insulin resistance[22]. The lipid droplets are accumulations of triglyceride that can be easily identified by staining with hematoxylin and eosin or Oil Red O. A therapeutic approach that induces lipid degradation and simultaneously inhibits fat synthesis while maintaining a normal level of lipid metabolism may represent the proper strategy for treating NAFLD.

Two major lipid metabolism pathways have been identified in human: the lipolysis pathway and the lipophagy pathway[23-25] (Figure 1). Lipolysis refers to the gradual degradation of intracellular lipid droplets

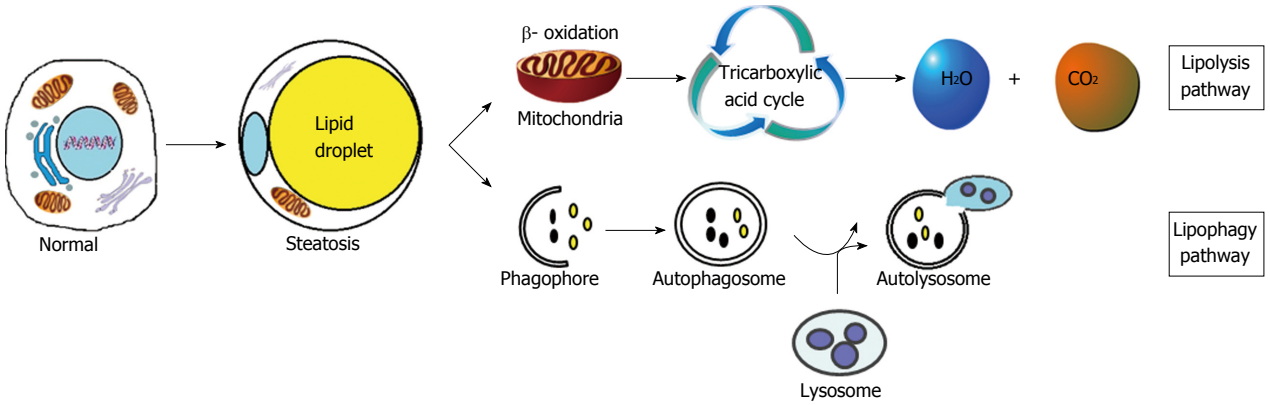

Figure 1 Two major lipid metabolism pathways have been identified in human: the lipolysis pathway and the lipophagy pathway.

into free fatty acids and glycerol by the activity of cytoplasmic lipases. These newly released free fatty acids are then transported into mitochondria, where they undergo β-oxidation to form acetyl-CoA, which in turn, is finally converted to carbon dioxide and water *via* the Krebs cycle. As the major pathway of lipid degradation in eukaryotic cells, lipolysis is a complex multi-step process that also plays a significant role in maintaining energy balance[26].

Another method used by cells to degrade lipids is the lipophagy pathway, by which the double membrane wraps lipid droplets and sends them to lysosomes as autolysosomes for degradation[27]. Lipophagy ensures the degradation of excessive lipid droplets deposited in cells, and the maintenance of cellular "steady state". Lipolysis and lipophagy both play important roles in the degradation of lipid droplets.

It is widely accepted that autophagy is up-regulated during the early stage of NAFLD as an attempt to prevent lipid accumulation[28]. However, as NAFLD progresses, the autophagy process is blocked[29]. Singh and his research team[30] were the first to identify a relationship between autophagy and lipolysis. They found that mice fed with a high-fat diet or methionine choline-deficient (MCD) diet had significantly decreased levels of autophagy. Treating the mice with 3-maleimidopropionic acid or silencing the ATG5 gene with siRNA sharply increased the accumulation of lipid droplets in liver cells. Furthermore, rapamycin (mTOR inhibitor) was found to promote autophagy and also to alleviate lipid deposition both *in vivo* and *in vitro*[31]. Pretreatment with rapamycin (25 ng/mL) resulted in increased autophagy, while the levels of ER stress, apoptosis and lipid droplets decreased in palmitic acid-induced fatty hepatocytes[32]. These findings indicated that autophagy might negatively regulate lipid deposition and ER stress.

In cultured cells and mouse models, knockdown of Atg5 or Atg7 led to increased levels of both ER stress and insulin resistance[33]. Double immunofluorescence studies confirmed that lipid breakdown occurred partially *via* the autophagy-lysosome pathway, and inhibitors of autophagosome formation or autophagosome-lysosome fusion could markedly reduce such degradation[30]. Furthermore, autophagy was also found to help regulate the inflammatory response. Knockout of Atg5 in mouse macrophages blocked autophagy and increased IL-1β levels following administration of D-galactosamine/lipopolysaccharide[34]. Moreover, several studies conducted with animal models of NAFLD and actual NAFLD patients have reported that autophagy flux was suppressed, and that restoring autophagy balance could alleviate the histologic signs of fatty liver disease[32,33].

Briefly, short-term inhibition of autophagy in NAFLD could be induced through the mTOR complex, while long-term inhibition could be regulated *via* the transcription factors FoxO and TFEB, which control the transcription of autophagic genes and are inhibited by insulin-induced activation of Akt/PKB and mTOR, respectively. mTOR could be over-activated in the liver, presumably as a result of over-nutrition and/or hyperinsulinemia. Calcium-dependent protease calpain-2 induced by obesity could also lead to the degradation of Atg7, with impaired autophagy. A reduction in expression of cathepsins B, D and L and a defect in lysosomal acidification could impair substrate degradation in autolysosomes. Finally, high-fat diet could lead to impaired autophagosome-lysosome fusion. In turn, decreasing hepatic autophagy and the associated lysosomal degradation could increase the ER stress in NAFLD[15].

In conclusion, the progression of NAFLD is closely associated with impaired autophagy flux, and restoring autophagy balance improves NAFLD.

ROLE OF AUTOPHAGY INDUCED BY TRADITIONAL CHINESE HERBAL EXTRACTS IN TREATING NAFLD

Traditional Chinese herbal extracts, usually extracted from native plants, have been used in various clinics for thousands of years. In the past, due to the lack of advanced analytical technologies, people had little understanding of the mechanisms by which

certain Chinese herbal extracts might treat or cure certain diseases. However, due to recent advances in technologies used in biochemistry and pharmacology, more and more people have become aware of the strong and lasting efficacy of the Chinese herbal extracts used to maintain health. Moreover, several such medicines are now used in the clinical treatment of NAFLD[35,36]. Recent studies have suggested that Chinese herbal extracts function by inducing autophagy, which may inhibit NAFLD progression (Table 1).

Resveratrol

Resveratrol (trans-3,4,5-trihydroxystilbene) is a naturally polyphenolic compound found in edible plants, such as grapes, peanuts and berries. Due to its anti-inflammatory, antioxidant and anti-cancer effects, resveratrol is widely used to help prevent cardiovascular and cerebrovascular diseases, treat cancer, and reduce steatosis[37-39]. Several clinical trials have reported that orally administered resveratrol inhibits the progression of NAFLD[40-42].

In a randomized, double-blind, controlled clinical trial, 50 NAFLD patients were given one 500 mg capsule of resveratrol per day for 12 wk while eating an energy-balanced diet. Some parameters, such as anthropometric measurements (weight, body mass index and waist circumference), liver enzymes (ALT and AST), and biomarkers of inflammation (hs-CRP, TNF-α and IL-6) and hepatocellular apoptosis (cytokeratin-18 fragment M30), as well as the histological characteristics (steatosis and fibrosis) of the patients, were significantly improved compared with the patients who received a placebo capsule[41]. These data suggest that resveratrol prevents NAFLD by inhibiting the inflammatory response, apoptosis and fibrotic process.

Other studies have shown that resveratrol decreases lipogenesis by suppressing expression of acetyl-CoA carboxylase (ACC), peroxisome proliferator-activated receptor γ (PPAR-γ) and sterol regulatory element-binding protein-1 (SREBP-1)[43,44]. Additionally, resveratrol was reported to reduce the levels of proinflammatory cytokines TNF-α, IL-6 and IL-1β in mice fed a high-fat diet by affecting the NF-κB pathway[43,45]. Finally, results of another study suggested that resveratrol could significantly increase autophagy and SIRT1 activity, and might improve the symptoms of NAFLD partially by inducing autophagy *via* the cAMP-PRKA-AMPK-SIRT1 signaling pathway[46].

When male C57BL/6 mice fed with MCD diet were administered resveratrol (100 mg/kg or 250 mg/kg) or AML12 cells cultured with MCD medium were treated with resveratrol (50 μmol/L or 100 μmol/L), certain autophagic markers (LC3II) became significantly up-regulated while certain autophagic negative regulators (p62) became down-regulated; steatosis and inflammatory response (IL-6, IL-1β and TNFα) also became down-regulated. Then, AML12 cells treated with chloroquine showed blockade of autophagy, with inflammatory response (IL-6, IL-1β and TNFα) and oxidative stress (reactive oxygen species, ROS) being accumulated in the cells. However, autophagy was up-regulated while inflammatory response and oxidative stress were attenuated when the AML12 cells were further treated with resveratrol[47]. These facts indicate that resveratrol protects against NAFLD partially through regulating autophagy[46,47].

Lycium barbarum polysaccharides

The Lycium barbarum polysaccharides (LBPs) consist of fibrous-like proteoglycan molecules that are extracted from the rare but traditional medicinal herb, Chinese wolfberry (*Lycium barbarum L.*). Recent evidence has confirmed that LBPs are composed of arabinose, glucose, galactose, mannose, xylose and rhamnose. Due to their antioxidant, anti-cancer, anti-aging, neuroprotective, anti-hyperlipemia and anti-hyperglycemia properties, LBPs are increasingly consumed by elderly individuals[48,49]. In a NASH rat model, LBPs displayed therapeutic effects when used to treat steatosis, inflammation and hepatic fibrosis. Moreover, LBPs were shown to reduce steatosis by reducing mRNA expression of SREBP-1c while regulating inflammatory cytokines (TNF-α, IL-1β and MCP-1), partially by inhibiting activation of the NF-κB pathway[50,51]. LBPs were also shown to alleviate hepatic fibrosis by affecting the TGF-SMAD signaling pathway. Finally, when LBPs were administered to female Sprague-Dawley rats fed with a high-fat diet, certain autophagic markers (Atg5 and LC3II) became significantly up-regulated while certain autophagic negative regulators [phosphorylated (p)mTOR and p62] became down-regulated[51]. Rat obesity, insulin resistance, hepatic injury (inflammatory foci and cellular necrosis), oxidative stress (antioxidant enzymes CAT and GPx) were also improved. Autophagy was reported to have the effects of improving insulin resistance and oxidative stress. We speculate that LBPs improve NAFLD/NASH *via* several different mechanisms, including autophagy[51].

Dioscin

Dioscin is a natural steroidal saponin compound found in dietary foods especially Dioscoreaceae (*Dioscorea opposite Thunb*), which is widespread throughout Asian countries such as China, North Korea and Japan. Pharmacological studies have confirmed that dioscin can reduce inflammation, decrease blood sugar and lipid levels, protect hepatocytes and promote digestion[52,53]. Due to these effects, dioscin is now widely consumed in China.

Dioscin was found to promote β-oxidation of fatty acids by up-regulating ACADM, ACADS, PPARα, ACSL1, ACSL5, CPT1 and ACO expression. It can also inhibit triglyceride and cholesterol synthesis by down-regulating SREBP-1c, FAS, ACC1 and SCD1 expression, which

Table 1 The beneficial properties of traditional Chinese herbal extracts in non-alcoholic fatty liver disease

Chinese herbs	Model		Treatment		Pharmacological mechanisms	Ref.
	Animal model	Cell model	Animal model	Cell model		
Resveratrol (RSV)	ULK1 heterozygous knockout mice were fed with high-fat diet for 12 wk	-	Oral feeding with 50 mg/kg per day RSV from week 9 to week 12	-	Improved NAS score, insulin resistance, oxidative stress, inflammation, glucose tolerance and modulated autophagy	[45]
	4-wk induction of NAFLD with high-fat diet (60% fat) in 129/SvJ mice	Steatosis was induced by incubating HepG2 cells with palmitate acid (0.2 mmol/L) for 24 h	Diet containing RSV (0.4%) for 4 wk	Treated with RSV at various concentrations (10, 20, 40, 80 μmol/L) for a further 24 h	Reduced lipid accumulation, stimulated β-oxidation and induced autophagy through cAMP-PRKA-AMPK-SIRT1	[46]
Lycium barbarum polysaccharides (LBPs)	NASH induced by high-fat diet for 12 wk in adult female Sprague-Dawley rats	Steatosis was induced by incubating BRL-3A cells with sodium palmitate acid	Oral gavage feeding with 1 mg/kg per day LBPs once a day from week 9 to week 12	Treated with LBPs for 24 h	Reduced insulin resistance, serum aminotransferases, inflammatory responses, apoptosis and induced autophagy	[51]
Dioscin	NAFLD induced by high-fat diet (45% kcal fat) for 10 wk in C57BL/6J mice and ob/ob mice	-	Oral feeding with different dioscin concentrations (20, 40, 80 mg/kg per day)	-	Reduced body weight, lipid accumulation, inflammation oxidative damage and induced β-oxidation, autophagy, energy expenditure	[55]
Bergamot polyphenol fraction (BPF)	NAFLD induced by cafeteria diet (15% fat) every other day in addition to standard chow diet ad libitum for 14 wk in male Rcc:Han WIST rats	-	Drinking water containing 50 mg/kg per day BPF for 3 mo	-	Reduced serum triglyceride, blood glucose, hepatic steatosis and induced autophagy	[59]
Capsaicin	NAFLD induced by high-fat diet (49% fat) for 24 wk in TRPV1$^{-/-}$ and C57BL/6 wild-type mice	Steatosis was induced by incubating HepG2 cells with 1 mmol/l oleate/palmitate (2:1)	Diet containing 0.01% capsaicin for 24 wk	Treated by various capsaicin concentrations (0.1-10 μmol/L)	Reduced lipogenesis (FAS, SREBP-1, LXR, PPARα) and induced lipolysis (phospho-HSL, CPT1), autophagy through PPARδ-dependent manner	[65]
Garlic-derived S-allylmercaptocysteine (SAMC)	NAFLD induced by high unsaturated fat diet (30% fish oil) for 8 wk in female Sprague-Dawley rats	-	Intraperitoneal injection of 200 mg/kg SAMC, 3 times per week for 8 wk	-	Reduced lipogenesis (SREBP-1c), fibrosis (TGF-β1, α-SMA, PC-1), oxidative stress (CYP2E1), inflammation (TNF-α, IL-1β, iNOS, COX-2, MCP-1, MIP-2, KC) and induced lipolysis (adiponection), antioxidative stress (CAT, GPx)	[67]
	NAFLD induced by high unsaturated fat diet (30% fish oil) for 8 wk in female Sprague-Dawley rats	-	Intraperitoneal injection of 200 mg/kg SAMC, 3 times per week for 8 wk	-	Reduced intrinsic apoptosis (Bcl-2, Bcl-XL, Bakl, Bax) and extrinsic apoptosis (Fas, TRAIL, FADD, cleaved caspase-8), induced autophagy (vps34, beclin1, Atg12, LC3II, phosphorylated mTOR and p62)	[68]

NAFLD: Non-alcoholic fatty liver disease.

might help to prevent lipid deposition[54,55]. Dioscin was also shown to increase oxygen consumption and energy expenditure. The levels of HO-1, Nrf2, GSS and SOD2 expression were found to be up-regulated and KEAP1 expression was down-regulated in a dose-dependent manner in ob/ob and C57BL/6J mice pretreated with dioscin, strongly suggesting that dioscin has an anti-oxidative effect. Additionally, the levels of p-mTOR/mTOR, Beclin-1, Atg5 and LC3 II/I protein expression were all up-regulated by dioscin[55]. Dioscin might regulate autophagy through the mTOR-independent pathway. These findings indicate that dioscin protects against NAFLD, partially by inducing autophagy[54,55].

Bergamot polyphenol fraction

The bergamot polyphenol fraction (BPF) consists of bioactive molecules extracted from Bergamot (Citrus bergamia Risso Poiteau), which is like Buddha's-hand. While bergamot is native to Italy, it is now widely distributed throughout the subtropical regions of China, including Guangdong, Guangxi, Fujian and Yunnan. Bergamot has anti-inflammatory, anti-hypertensive and hepatic protective effects, and also promotes digestion[56,57]. A clinical study found reduced total low-density lipoprotein, cholesterol, triglyceride and blood glucose levels in 237 patients who had taken oral BPF for 30 d[58].

Due to its pharmacological profile, BPF may be useful for treating hyperlipemic and hyperglycemic disorders. In a cafeteria diet-induced rat model of metabolic syndrome, BPF significantly reduced steatosis by decreasing total serum lipid levels. Moreover, the expression levels of two autophagy markers (LC3 II/I and Beclin-1) were increased while SQSTM1/p62 expression was reduced, indicating that BPF could stimulate autophagy[59]. The specific mechanism by which BPF prevents NAFLD remains unclear. However, enhancement of lysosomal function *via* transcription factor EB, and activation of ULK1 kinase by AMPK might help to up-regulate autophagy[59].

Capsaicin

Capsaicin (8-methyl-N-vanillylnonanamide) is a major chemical component of hot peppers (*Capsicum annuum L.*), which is originally from Mexico but has become a favorite seasoning food in China. Odorless and colorless dietary capsaicin is a potent agonist of transient receptor potential vanilloid 1 (TRPV1), which is a non-selective cation channel with a preference for positive ions that transmit sensations of pain[60]. Long-term intake of dietary capsaicin can lower blood pressure, reduce cholesterol accumulations, and accelerate the decomposition and excretion of cholesterol[61,62].

Furthermore, appropriate amounts of dietary capsaicin have beneficial effects on obesity and NAFLD[63,64]. A survey indicated that dietary capsaicin could reduce lipid accumulation and triglyceride levels in mice fed with a high-fat diet by up-regulating the levels of uncoupling protein 2 (UCP2)[64]. UCP2 was thought to play an important role in mitochondrial lipolysis and oxidative stress. Another study showed that capsaicin-activated TRPV1 raised the levels of hepatic phosphorylated hormone-sensitive lipase (phospho-HSL) and carnitine palmitoyl transferase 1 (CPT1), which were critical regulators of lipolysis. This effect may be TRPV1-dependent because it was absent in TRPV1 (-/-) mice. At the same time, the levels of hepatic FAS, SREBP-1, PPARα and liver X receptor remained unchanged, which was important for lipogenesis. These findings suggest that capsaicin promotes lipolysis without inhibiting fat synthesis in NAFLD patients.

On the other hand, capsaicin was shown to enhance the expression levels of PPARδ and several autophagy-related proteins, including LC3 II, Beclin1, Atg5 and Atg7 in HepG2 cells, which had been pretreated with free fatty acids (oleate/palmitate, 2:1). Furthermore, autophagy induced by capsaicin was further increased by PPARδ agonist (GW0742) in steatosis HepG2 cells. Autophagy inhibited by capsazepine (inhibition of capsaicin) was further reduced by PPARδ antagonist (GSK0660) in steatosis HepG2 cells. It is suggested that chronic dietary capsaicin appears to prevent NAFLD by enhancing PPARδ-dependent autophagy[65].

Garlic-derived S-allylmercaptocysteine

S-allylmercaptocysteine (SAMC) is the major active component of garlic (*Allium sativum L.*), which is one of the most favorite seasonings of food in China. Garlic is originally from the western plateau of Asia, but is now widely planted in low-wet areas of China, including Henan, Shandong, Jiangsu, *etc*. Garlic has the effects of sterilization, antioxidant and anti-cancer. A randomized, double-blind, controlled clinical trial found that body weight and body fat mass were decreased in 55 NAFLD patients who had orally taken two garlic tablets per day (containing 400 mg of garlic powder)[66]. Furthermore, some pharmacological studies had confirmed that SAMC could ameliorate NAFLD. A survey indicated that SAMC could reduce steatosis, fibrosis, oxidative stress and inflammation in female rats fed with a highly unsaturated fat diet (30% fish oil) by up-regulating the levels of lipolysis markers (adiponectin), antioxidative stress markers (CAT and GP_X), and down-regulating the levels of lipogenesis markers (SREBP-1c), fibrosis markers (TGF-β_1, α-SMA and PC-1), oxidative stress markers (CYP2E1) and inflammatory markers (TNF-α, IL-1β, iNOS, COX-2, MCP-1, MIP-2 and KC). The protective effect of SAMC was partly through regulation of p38 MAPK, NF-κB and AP-1 signaling pathways[67]. Another survey suggested that hepatic autophagic negative regulators (phosphorylated mTOR and p62), intrinsic apoptotic markers (phosphorylated p53, Bcl-2, Bcl-XL, Bakl and Bax) and extrinsic apoptotic markers (Fas, TRAIL, FADD and cleaved caspase-8) were reduced

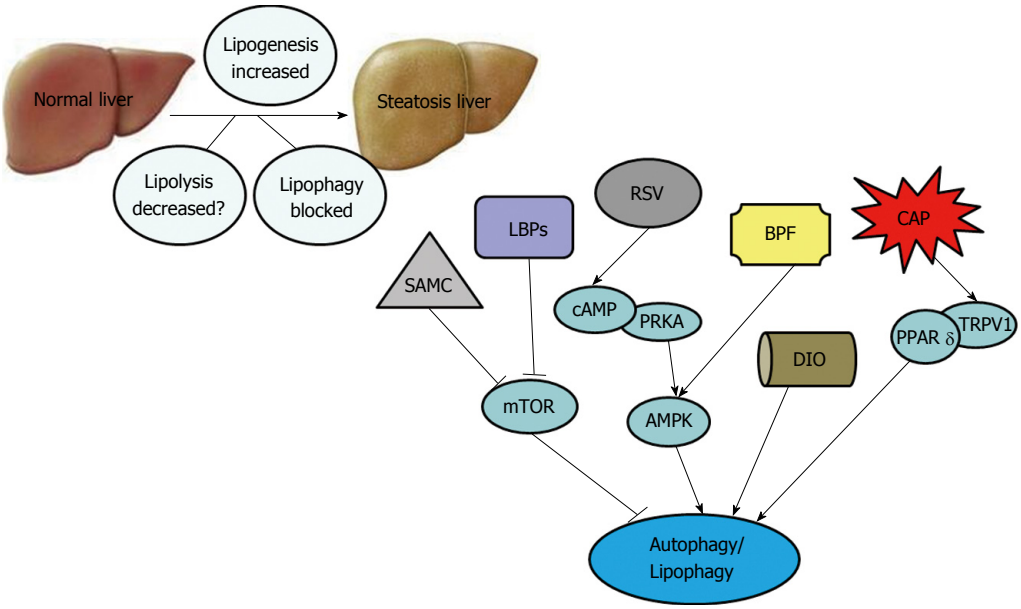

Figure 2 The role of autophagy induced by traditional Chinese herbal extracts in treating non-alcoholic fatty liver disease. SAMC: Garlic-derived S-allylmercaptocysteine; LBPs: Lycium barbarum polysaccharide; RSV: Resveratrol; BPF: Bergamot polyphenol fraction; DIO: Dioscin; CAP: Capsaicin; PPAR: Peroxisome proliferator-activated receptor; TRPV1: Transient receptor potential vanilloid 1.

while hepatic autophagic markers (vps34, beclin1, Atg12 and LC3II) induced in NAFLD rat models after intraperitoneal injection of SAMC (200 mg/kg 3 times per week)[68]. These findings indicate that SAMC prevents NAFLD, partially by inducing autophagy[68].

CONCLUSION

Traditional Chinese herbal extracts are widely used to prevent cancer, neurodegeneration and metabolic syndrome, as well as cardiovascular and cerebrovascular diseases. Furthermore, many people use them as "first choice" medications for maintaining health[69,70]. Traditional Chinese herbal extracts have beneficial effects in treating NAFLD, as they could reduce steatosis and inhibit inflammation and oxidative stress[71-73]. In addition, these medicines appear to reverse histologic changes in the livers of NAFLD patients, which may prevent NAFLD from progressing to hepatic cirrhosis and even carcinoma.

Autophagy is a protective response that helps to maintain homeostasis and to promote survival. Lipophagy is a special kind of autophagy by which the double membrane wraps lipid droplets and sends them to lysosomes for degradation. The fundamental function of lipophagy is the degradation of abnormal lipid droplets deposited in cells and the maintenance of steady state. But, autophagy is partially suppressed in NAFLD/NASH patients and animal models, and restoring autophagy may slow the progression of NAFLD. Moreover, autophagy is a double-edged sword. It protects hepatocytes by inhibiting oxidative stress and inflammation[74,75]; yet, its over-stimulation may result in autophagic cell death that aggravates any existing liver damage[76].

As we have discussed above, it is strongly suggested that some traditional Chinese herbal extracts, such as resveratrol, LBPs, dioscin, BPF, capsaicin and SAMC, should have beneficial effects on NAFLD/NASH, partially due to their ability to activate autophagy (Figure 2). However, additional studies are needed to elucidate the molecular mechanisms by which traditional Chinese herbal extracts protect from NAFLD. Finally, prospective, randomized, double-blind, controlled clinical trials should be conducted to evaluate the specific therapeutic effects and safety of traditional Chinese herbal extracts for NAFLD patients.

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MINIREVIEWS

Clinical translation of bioartificial liver support systems with human pluripotent stem cell-derived hepatic cells

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Abstract

There is currently a pressing need for alternative therapies to liver transplantation. The number of patients waiting for a liver transplant is substantially higher than the number of transplantable donor livers, resulting in a long waiting time and a high waiting list mortality. An extracorporeal liver support system is one possible approach to overcome this problem. However, the ideal cell source for developing bioartificial liver (BAL) support systems has yet to be determined. Recent advancements in stem cell technology allow researchers to generate highly functional hepatocyte-like cells from human pluripotent stem cells (hPSCs). In this mini-review, we summarize previous clinical trials with different BAL systems, and discuss advantages of and potential obstacles to utilizing hPSC-derived hepatic cells in clinical-scale BAL systems.

Key words: Artificial liver; Clinical trial; Hepatocytes; Pluripotent stem cells; Bioreactors

Core tip: The current lack of transplantable donor livers in the world has led to the development of extracorporeal liver support systems as one possible approach to overcome this problem. Bioartificial liver (BAL) support systems require a cell source to replicate human liver function, yet the ideal cell source for this purpose has yet to be determined. Highly-functional hepatocyte-like cells have recently been generated from human pluripotent stem cells, which show promise as a potential cell source in BAL support systems for the treatment of liver failure in the future.

Sakiyama R, Blau BJ, Miki T. Clinical translation of bioartificial liver support systems with human pluripotent stem cell-derived hepatic cells. *World J Gastroenterol* 2017; 23(11): 1974-1979 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i11/1974.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i11.1974

INTRODUCTION

Needs for bioartificial liver systems in clinical practice

Liver disease is one of the most prevalent medical conditions in the world today, affecting hundreds of millions of people worldwide[1-3]. Many of these diseases, such as end-stage liver diseases and some inherited liver diseases, can only be treated successfully with a liver transplant[4]. Although 11606 patients were added to the liver transplant waiting list in the year 2015, only 7127 patients received a liver transplant in that same year[4]. This discrepancy demonstrates the profound shortage of transplantable donor livers. This shortage of livers resulted in a high waiting list mortality, with 1423 patients dying in 2015 while waiting for a transplant[4]. Therefore, it is imperative that new therapies are developed to provide an alternative to liver transplantation.

Extracorporeal liver support systems were developed with the aim of stabilizing a patient long enough for his or her own liver to regenerate or for physicians to procure a transplantable liver. Early support systems functioned to supplement liver function by removing toxins from the blood through non-biological hemofiltration[5]. These non-biological type extracorporeal liver support systems have been clinically established and are widely used in countries where liver transplantation is limited[5]. However, it became apparent that non-biological hemofiltration devices were incapable of adequately replicating liver function[5]. In order to overcome the limitations of non-biological devices, live cells that possess liver function were incorporated into the development of bioartificial liver support (BAL) systems[5]. There are several types of BAL systems that have been proposed which differ in their cell housing mechanism, including hollow fiber-based[6-10], multilayer membrane-based[11], sponge/scaffold-based[12-14], and floating/encapsulated-based systems[15] (Figure 1). Although most of these housing mechanisms have successfully cultured cells on the small experimental scale, hollow fiber-based BAL systems are widely used in clinical trials.

SOURCES OF HEPATOCYTES FOR BAL SYSTEMS

Several types of cells may be selected for use in a BAL system. These include primary hepatocytes isolated from human livers, human hepatoblastoma cell lines, and primary animal hepatocytes[16]. Human primary hepatocytes are ideal for the BAL system[16]. However, the low availability and inconsistent quality of primary human hepatocytes prevent their use in clinics[16]. Although human hepatic cancer cell lines and animal liver cells are readily available, they are less metabolically active than primary human hepatocytes[17]. In addition, the risk of zoonoses precludes the use of animal cells. For example, it has been shown that porcine endogenous retroviruses are capable of infecting human cells *in vitro*[18].

Recent advancements in stem cell research have demonstrated that hepatocyte-like cells can be derived from human pluripotent stem cells (hPSCs)[19]. hPSCs can be generated from a patient's own cells by introducing several transcription factors[20]. They are capable of differentiating into cells from all three germ layers, including neural cells[21-23], osteogenic cells[24], cardiac cells[25] , adipogenic cells[26], pancreatic cells[27,28], vascular cells[29], hematopoietic cells[30], endothelial cells[30], and hepatocytes[31,32]. hPSC-derived hepatic cells have been shown to express hepatocyte marker genes and proteins[33]. They also demonstrate hepatic functions including albumin secretion, urea synthesis, cytochrome P450 enzyme induction[31], and glycogen storage[34].

hPSC-derived hepatic cells possess minimal risk when used in a BAL system, but are unsuitable for other applications due to their risk of tumorigenicity. The genetic instability of hPSCs results in an underlying uncertainty of transplanting large quantities of hPSC-derived hepatic cells directly into a patient[35]. On the other hand, the risk of tumorigenicity is minimized in a BAL system, as the hPSC-derived hepatic cells would be isolated from the patient's blood stream by multiple layers of filter membranes (Figure 2). Therefore, while hPSC-derived hepatic cells may not be ideal for cell transplantation, they are viable candidates for a BAL system.

SUCCESSES AND CHALLENGES OF DEVELOPING CLINICAL BAL SYSTEMS

Several BALs have been evaluated in clinical trials, as previously explored in van de Kerkhove *et al*[13,14] (Table 1)[6-10,13,14]. The Extracorporeal Liver Assist Device (ELAD) utilizes the human hepatoblastoma cell line HepG2/C3A (100 g) in hollow fiber-based dialysis cartridges. A phase III trial treated 96 patients with alcohol-induced liver decompensation. In subjects age < 50 years, creatinine < 1.3 mg/dL, bilirubin $\geqslant$ 16 and international normalized ratio (INR) $\leqslant$ 2.5, the 91-d survival rates were 93.9% for ELAD-treated subjects and 68.4% for control subjects (P = 0.006)[10]. A second BAL design, the Modular Extracorporeal Liver Support (MELS) system, consists of interwoven hollow fiber membranes, creating a three-dimensional

Table 1 Bioartificial liver devices used in clinical trials

Bioreactor device	Ref.	Cells	Mass (g)[1]	Bioreactor design	Scaffold	Fluid	Separation	Treatment time (h)	Phase	Indication (*n*)	Effect
HepatAssist	Demetriou *et al*[9]	Cryopreserved porcine hepatocytes	50-70	Hollow fiber	Microcarrier + external inoculation	Plasma	3000 kDa cut-off	6	Ⅲ	ALF (147), PNF (24)	HepatAssist survival of 71.0% *vs* control survival of 62.0% $P = 0.28$, (NS)
Vitagen ELAD	Reich *et al*[10]	HepG2/C3A	200-400	Hollow fiber	External inoculation	Plasma	70 kDa cut-off	Up to 168	Ⅲ	AILD (96)	ELAD survival of 80.4% *vs* control survival of 65.2% $P = 0.068$, (NS)
LSS	Mundt *et al*[7]	Primary porcine hepatocytes	up to 500	Hollow fiber	External inoculation	Plasma	300 kDa cut-off	7-46	Ⅰ / Ⅱ	ALF (8)	Bridged to OLT 8
MELS	Sauer *et al*[8]	Primary human hepatocytes	up to 600	Hollow fiber	External inoculation	Plasma	400 kDa cut-off	7-74	Ⅰ	ALF (2), PNF (2), AOC(4)	Bridged to OLT 6, Survival without OLT 1, Died without OLT 1
Excorp Medical BLSS	Mazariegos *et al*[6]	Primary porcine hepatocytes	70-120	Hollow fiber	Collagen + external inoculation	Whole blood	100 kDa cut-off	12	Ⅰ	ALF (2), AOC (2)	Bridged to OLT 1, Died without OLT 3
AMC-BAL	van de Kerkhove *et al*[13,14]	Primary porcine hepatocytes	100	Nonwoven	Spiral membrane + polyester matrix	Plasma	None	24	Ⅰ	ALF (12)	Bridged to OLT 11, Survival without OLT 1

[1]100 million cells/gram of liver. AILD: Alcohol-Induced Liver Decompensation; AOC: Acute-on-chronic liver failure; ALF: Acute liver failure; PNF: Primary graft nonfunction; OLT: Orthotopic liver transplantation.

framework utilizing primary human hepatocytes. In one trial, eight patients (two with acute liver failure, four with acute-on-chronic liver failure, and two with primary nonfunction) were successfully bridged to liver transplantation[8]. Several other trials have yielded similar results regarding degree of effectiveness.

Despite the effectiveness of BAL systems in clinical trials, their translation from the laboratory bench to the patient's bedside has been hindered by three obstacles. Firstly, it is necessary to prepare a sufficient quantity of hPSC-derived hepatic cells for clinical applications. It has been widely suggested that approximately 30% of the total liver volume is required for survival. Considering that the average mass of a human liver is 1.5 kg, and that 100 million hepatocytes are contained in 1 g of liver tissue, a minimum of 45 billion hPSC-derived hepatic cells would be required to produce a clinical-scale BAL device[36] (Figure 3). Secondly, the operation cost of a BAL device is currently too expensive for widespread clinical use. The process of culturing 45 billion hPSCs and inducing hepatic differentiation consumes large quantities of culture medium and supplements including recombinant growth factors[37]. As the length of treatment increases, the cost of operating a BAL device accumulates significantly. Thirdly, it has not been well investigated whether hPSC-derived hepatic cells maintain their liver functions over a long period of time in BAL devices. The loss of cell viability and functionality throughout the course of treatment may be problematic[38].

The most critical factor for large-scale cell culture is oxygen and nutrient supply. The oxygen and nutrients must be uniformly supplied to a large number of cells. It is well known that the anchorage-dependent hepatocytes easily form aggregates, and if the diameter of the aggregates exceeds 100μm at atmospheric concentrations, central necrosis occurs resulting from lack of oxygen and nutrition[39]. This fact indicates that the organization of the cell culture space in the large-scale BAL system must allow for sufficient oxygen and nutrient penetration of the cell aggregates. A sophisticated controlling system and well-engineered bioreactor will be required to monitor oxygen and nutrient supply. In addition, since hPSCs are sensitive to environmental factors, the shear stress from the culture medium must be minimized[40]. Ideally, the bioreactor should mimic the structure within the liver, which provides appropriate pressure and shear stress similar to the Space of Disse.

CONCLUSION

BAL systems have demonstrated a potential to treat patients with liver failure by providing temporary support for them to recover their own hepatocytes or to bridge them to liver transplantation. Early BAL systems have encountered significant limitations due to the low functionality and availability of cells for this application. With emerging stem cell technology, hepatocyte-like cells can be differentiated from hPSCs. Due to their functional similarity to primary human hepatocytes and minimal risk of use, these

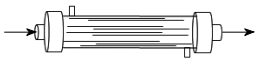
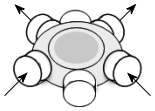
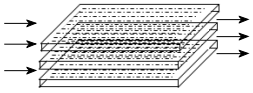
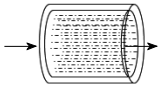
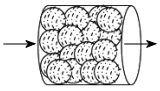
Type	Pros	Cons
Hollow fiber (tubular) 	Simple structure, divertible from dialyzer, minimal shear stress, immunoisolation	Uneven gas-liquid mass transfer, no intrinsic oxygen supply
Hollow fiber (interwoven) 	Ease of scale-up, efficient and uniform mass transport, minimal shear stress, immunoisolation, good oxygen and nutrient supply	Complex structure
Multilayer membrane 	Uniform cell distribution and microenvironment	Limitations to scale-up, cells exposed to direct shear stress, low surface area-to-volume ratio, no intrinsic oxygen supply
Sponge/scaffolds 	Ease of scale-up, minimal barrier to nutrient/metabolite transport	Non-uniform cell distribution, cells exposed to shear stress, no intrinsic oxygen supply
Floating/encapsulated 	Ease of scale-up, uniform microenvironment	Poor cell stability, barrier to nutrient/metabolite transport due to encapsulation, degradation of microcapsules over time, no intrinsic oxygen supply

Figure 1 Artificial liver device designs.

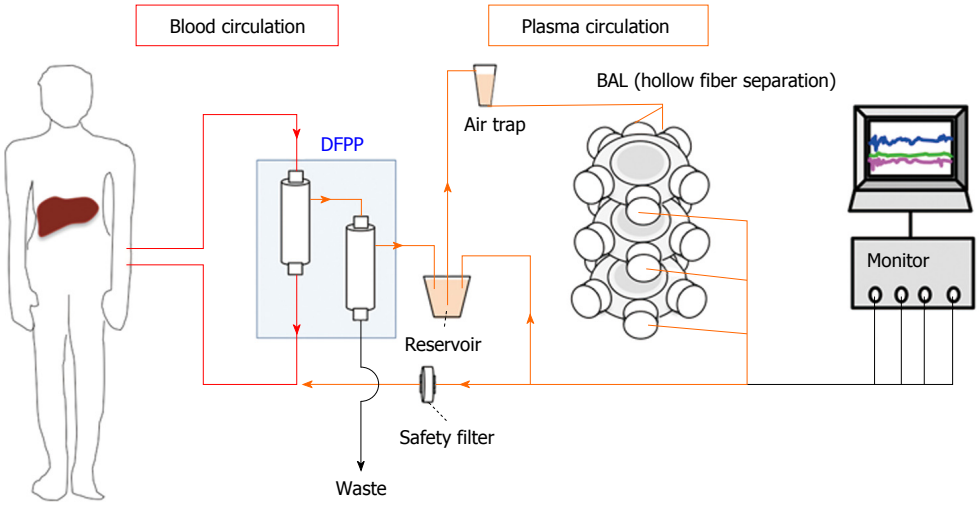


Figure 2 Bioartificial liver system with human pluripotent stem cells-derived hepatic cells using double filtration plasmapheresis. In a bioartificial liver (BAL) system, patient plasma is first separated from whole blood by double filtration plasmapheresis (DFPP). Plasma then perfuses a bioartificial device using hydrophilic hollow fibers. The human pluripotent stem cells (hPSCs)-derived hepatic cells are inoculated at the outside of the hollow fibers. The detoxified patient plasma is filtered once more before returning to the patient's blood stream. The hollow fiber membranes and safety filter provide two layers of separation between the patient's blood stream and the hPSC-derived hepatic cells.

hPSC-derived hepatic cells will be the ideal cell source to develop clinical-grade bioartificial devices. Further clinical translational studies will be required to overcome the obstacles to developing large-scale BAL devices with hPSC-derived hepatic cells. If successful, these readily available and highly functional extracorporeal liver support systems will be a feasible alternative for the treatment of liver failure in the near future.

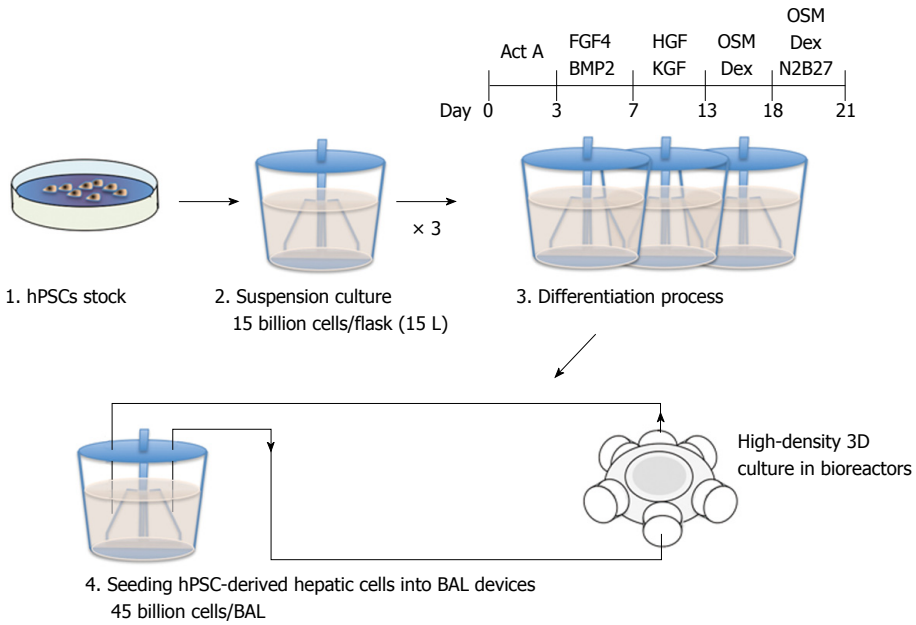


Figure 3 A strategy and cell number estimate of human pluripotent stem cells-derived hepatic cells in the mass production of bioartificial liver devices. Undifferentiated human pluripotent stem cells (hPSCs) can be expanded in a 15 L suspension culture system up to a maximum of 15 billion cells[37]. Three of these suspension culture flasks will be required to prepare 45 billion cells for a clinical-scale bioartificial liver (BAL) device. After inducing hepatic differentiation, the hPSC-derived hepatic cells will be cultured at high density in bioreactors to generate a BAL device.

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ORIGINAL ARTICLE

Basic Study

Effect of treatment failure on the CagA EPIYA motif in *Helicobacter pylori* strains from Colombian subjects

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Abstract

AIM

To evaluate effect of treatment failure on *cagA* and *vacA* genotypes in *Helicobacter pylori* (*H. pylori*) isolates from Colombia.

METHODS

One hundred and seventy-six participants infected with *H. pylori* from Colombia were treated during 14 d with the triple-standard therapy. Six weeks later, eradication was evaluated by ^{13}C-Urea breath test. Patients with treatment failure were subjected to endoscopy control; biopsies obtained were used for histopathology and culture. DNA from *H. pylori* isolates was amplified using primers specific for *cagA* and *vacA* genes. The phylogenetic relationships among isolates obtained before and after treatment were established by conglomerate analysis based on random amplified polymorphic DNA (RAPD) fingerprinting.

RESULTS

Treatment effectiveness was at 74.6%. Of the par-

ticipants with treatment failure, 25 accepted subjected to a second endoscopy. Prevalence of post-treatment infection was 64% (16/25) and 40% (10/25) by histology and culture, respectively. Upon comparing the *cagA* and *vacA* genotypes found before and after therapy, multiple *cagA* genotypes (*cagA*-positive and *cagA*-negative) were found before treatment; in contrast, *cagA*-negative genotypes decreased after treatment. *vacA s1m1* genotype was highly prevalent in patients before and after therapy. The 3'*cagA* region was successfully amplified in 95.5% (21/22) of the isolates obtained before and in 81.8% (18/22) of the isolates obtained after treatment. In the isolates obtained from patients with treatment failure, it was found that 72.7% (16/22) presented alterations in the number of EPIYA motifs, compared to isolates found before treatment.

CONCLUSION

Unsuccessful treatment limits colonization by low-virulence strains resulting in partial and selective eradication in mixed infections, and acts on the *cagA*-positive strains inducing genetic rearrangements in *cagA* variable region that produces a loss or gain of EPIYA repetitions.

Key words: *Helicobacter pylori*; RAPD-PCR; Treatment failure; EPIYA motifs; 3' *cagA* variable region

Core tip: This study evaluated the effect of treatment failure on *cagA* and *vacA* genotypes in *Helicobacter pylori* (*H. pylori*) isolates. It was found, that unsuccessful treatment of *H. pylori* limits the colonization by low-virulence strains, resulting in partial and selective eradication in mixed infections. Also, acts on the *cagA*-positive strains inducing genetic rearrangements (deletion or acquisition of EPIYA motifs) that could alter the adherence of CagA protein to the epithelial cell membrane, the level of tyrosine phosphorylation and CagA multimerization, impacting its effects on cellular signaling. Finally, in some cases, may lead to the divergence of *H. pylori cagA*-positive sub-clones.

Bustamante-Rengifo JA, Matta AJ, Pazos AJ, Bravo LE. Effect of treatment failure on the CagA EPIYA motif in *Helicobacter pylori* strains from Colombian subjects. *World J Gastroenterol* 2017; 23(11): 1980-1989 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i11/1980.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i11.1980

INTRODUCTION

Helicobacter pylori (*H. pylori*) colonize the gastric mucosa of over 50% of the population in the world[1-3]. This bacteria is generally acquired during childhood and persists throughout life[4]. Although most *H. pylori*-positive persons are asymptomatic, only a small set of infected individuals will progress to severe gastrointestinal diseases such as non- Hodgkin's lymphoma of the stomach or distal gastric adenocarcinoma[1,2,4]. The clinical outcome of the infection is influenced by immune mechanisms in the host, environmental factors[3,5-7], and genetic heterogeneity of the strain[8]. To date several genes in the genome of *H. pylori* have been identified and associated with disease[9], however, the two genes best understood in terms of structure and function are *cagA* and *vacA* genes.

The *cagA* gene is an important constituent of *cag* pathogenicity island, present in 50%-60% of the Western *H. pylori* strains[8,10], and in more than 90% of the strains isolated in East Asia, encodes a bacterial oncoprotein (CagA)[2,11,12], which is directly translocated within the epithelial cells *via* a type Ⅳ secretion system; CagA undergoes tyrosine phosphorylation by host cell kinases within repeated sequences of five amino acids (glutamic acid-proline-isoleucine-tyrosine-alanine), called EPIYA motifs[1,11,13]. These motifs show variation in the number of repetitions present in the carboxyl-terminus region of the protein, and based on the sequences surrounding them, they are defined as EPIYA-A, -B, -C, and -D[1]. EPIYA-A and EPIYA-B motifs are typically present in the CagA proteins of all *cagA*-positive isolates, followed by one to three EPIYA-C motifs, or by an EPIYA-D motif in Western and East Asian-type isolates, respectively[12,13]. Phosphorylated CagA interacts with the SHP-2 phosphatase and the Crk protein[14], resulting in reorganization of the cytoskeleton, cell elongation (hummingbird phenotype), and abnormal proliferation[11]. Hence, the sequence polymorphisms and duplications shown by CagA protein in their C-terminal region can modify the risk of disease by *H. pylori*[13]. Due to this, characterization of the number and type of EPIYA motifs in clinical isolates provides an additional value to the detection of the *cag* island[15].

Unlike *cag* PAI, all the *H. pylori* strains carry the *vacA* gene[2,10], but this is only expressed in about half of all the strains[16,17], it encodes an vacuolating toxin known as VacA that exerts multiple effects in the epithelial cells, resulting in cell damage, and inhibits activation and proliferation of T cells[2]. *vacA* is a polymorphic gene that exhibits two major regions of sequence diversity: signal (s) and median (m) region. There are two types of signal sequence of *vacA* (s1 or s2) and two types of median region (m1 or m2) of *vacA*[8,17,18]. The combination of *s* and *m* alleles results in different degrees of cytotoxicity and influences the pathogenicity of bacteria[10,11]. The *vacA s1m1* and *s1m2* strains produce large and moderate amounts of vacuolating toxin, respectively, and are strongly associated with gastric adenocarcinoma and peptic ulcer[10,19], while the *vacA s2m2* strains are virtually not toxic and rarely associated to disease[20].

This extraordinary genetic diversity is generated

through a high rate of point mutations, slipped-strand mispairing, and frequent intra-genomic and inter-genomic recombination[2,4,19], it is creating a non-linear system for diversification[19]. Thus, *H. pylori* probably uses its genetic plasticity to adapt physiologically to changing conditions in its host through of the selection of clonal variants of the same strain with changes in the surface molecules or variations in factors of interaction with the cell (*e.g.*, loss all or part of *cag*-PAI, change number of EPIYA repetitions of CagA protein), contributing to maintaining of host-pathogen equilibrium that promotes persistence.

From this point view, the eradication of *H. pylori* has been proposed as a promising measure in the prevention of gastric lesions associated with infection. Nevertheless, an ideal treatment is not yet available[21]; in practice, 20%-30% of the therapies fail[22]. In most cases, this failure is attributed to the acquired resistance of strains to antibiotics[23]. Other factors poorly understood may also influence treatment failure[24,25]. Previous studies suggest that eradication rates are associated with the genetic characteristics of *H. pylori*[8], thus the *cagA*-positive/*vacA* s1 genotypes show a higher sensitivity than *cagA*-negative/*vacA* s2 genotypes to eradication treatment[24,26]. These findings are consistent with the observations of Correa *et al*[22], who observed that unsuccessful treatment is associated to increased prevalence of less virulent genotypes. Therefore, for the purpose of this study, we will shift the focus of how the *H. pylori* genotype influences the outcome of eradication therapy to who the treatment failure modifies the genotype of the infecting strains. We evaluate here the effect of the triple-standard therapy on *cagA* and *vacA* genotypes in *H. pylori* strains from Colombian subjects with unsuccessful treatment.

MATERIALS AND METHODS

Subjects and samples

In 2009, 206 adults were voluntarily recruited with symptoms of dyspepsia (91 male, 115 female, mean age of 40.5 ± 0.8 years), in Túquerres Colombia, a population with high prevalence of *H. pylori* and preneoplastic lesions[3,27]. The participants underwent to upper gastrointestinal tract endoscopy. Antrum and gastric body biopsies were obtained for histopathological evaluation and *H. pylori* culture. Informed consent was obtained from all participants, and the study was approved by the Human Ethics Committee at Universidad del Valle (CIREH), certificate of approval No I073-07.

Histopathology

Expert pathologists in gastric mucosa biopsies performed the histopathological diagnosis, according Sydney's classification system[28]. The categories used were non-atrophic gastritis (NAG), multifocal atrophic gastritis without intestinal metaplasia (MAG), intestinal metaplasia (IM), and dysplasia (DYS). The Steiner silver stain allowed to evaluate the presence of *H. pylori*.

Treatment of H. pylori infection

The 176 patients positive for *H. pylori* through histology were treated with Clarithromycin 500 mg, amoxicillin 1000 mg, and omeprazole 20 mg (Genfar laboratories, Bogotá, Cundinamarca, Colombia) twice daily for 14 d. Resolution of the infection was evaluated through a ^{13}C-Urea breath test (UBT), six weeks later. Participants with treatment failure were subjected to a control endoscopy, the gastric mucosa fragments obtained were again used for histopathological diagnosis and culture.

H. pylori culture, DNA extraction and genotyping

H. pylori were cultured from biopsies of antrum and body gastric obtained before (first endoscopy) and after (second endoscopy) anti-*H.pylori* treatment in patients with treatment failure. Chromosomal DNA was extracted from lysis of pure *H. pylori* cultures following a protocol of digestion with Proteinase K and later steps of precipitation with ethanol as previously described[29]. The *vacA* and *cagA* status was evaluated using the primers described by van Doorn *et al*[30].

Amplification of cagA 3' region harboring EPIYA motifs

Primers *cagA*2530S and *cagA*3000AS previously described by Panayotopoulou *et al*[13], allowed to characterize the number of EPIYA repetitions present on *H. pylori* isolates obtained before and after treatment, resulting in the generation of several fragments separated equidistantly by 100 bp. The PCR amplicons ranged in the range of 390 bp (2 repetitions), 490 bp (3 repetitions), 570 bp (4 repetitions), and 670 bp (5 repetitions).

RAPD-PCR

To study the DNA sequence diversity among *H. pylori* strains obtained at baseline and post-treatment, two random primers were used: 1254 and 1281[31]. The RAPD-PCR conditions employed were previously described[29]. Each strain was amplified by duplicate under the same conditions.

Statistical analysis

For categorical variables, the McNemar test on paired data was used to determine the significance of the differences in the proportions of *cagA* and *vacA* genotypes observed before and after treatment in patients with unsuccessful treatment. The clustering analysis and its association with anatomic location of the isolates, exposure to anti-*H. pylori* treatment, and histopathological diagnosis were evaluated using the χ^2 test. All data were analyzed with the statistical software (SPSS version 15). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Overall results

Of the 206 patients initially recruited, 176 (85.4%) and 149 (72.3%) participants were *H. pylori*-positive by histopathology and culture, respectively. The isolates obtained were characterized by virulence factors and antibacterial susceptibility. Subsequently, 176 participants were treated with 14-d standard therapy. Six weeks after, it was possible to contact 174 participants to conduct the [^{13}C]-Urea breath test, nine of these participants were excluded because they were pregnant or had changed their home address. With the 165 participants in which was possible to conduct post-treatment control, it was found that 11 cases were ambiguous and 31 participants were UBT-positive. The eradication rate was 74.6% (123/165). Of the participants with treatment failure, only 25 accepted to undergo a second endoscopy. Prevalence post-treatment infection was at 64% (16/25) and 40% (10/25) by histopathology and culture, respectively. Once again, the strains obtained were characterized by virulence markers.

Histopathology analysis

Of sixteen subjects with treatment failure diagnosed by histopathology, thirteen (81.3%) presented chronic NAG and three (18.7%) presented IM. When comparing the histopathological diagnosis of each of the 10 patients with treatment failure in which it was possible to isolate *H. pylori*, with histopathological diagnosis before treatment, it was found that diagnosis did not coincide in only one case (SV512) (data not shown).

Antibiotic susceptibility and treatment failure

Of the 149 isolates obtained before treatment, 136 (91.3%) isolates showed *in vitro* sensitivity to the two antibiotics used in standard triple therapy, whereas 4% and 2.7% of isolates were resistant to amoxicillin and clarithromycin, respectively. The remaining isolates presented double resistance (data not shown). After of the treatment, it was observed that the isolates obtained from the 10 patients with treatment failure, only one of them (SV415) had previously shown isolates resistant to clarithromycin (MIC > 4.0 mg/L), while the remaining participants presented prior to treatment, isolates sensitive to amoxicillin and clarithromycin according to antibiotic susceptibility testing.

Analysis of H. pylori genotypes

The *cagA* and *vacA* status of *H. pylori* strains obtained from antrum and gastric body, before and after treatment in the 10 patients with treatment failure, are shown in Table 1. In 90% (9/10) of the patients before treatment, the infection with multiple *H. pylori* strains within and among anatomical sites was observed. In contrast, in only 60% (6/10) of the patients after the treatment presented mixed colonization within and among anatomical locations. When comparing the *cagA* and *vacA* genotypes found in each patient before and after therapy, only one case was found (SV444) in which these were identical and a specific site within the stomach was confirmed, the remaining nine patients presented differences. Multiple *cagA* (*cagA*-positive and *cagA*-negative) genotypes were found in these nine participants before therapy, but in four of them, just *cagA*-positive genotype was found after intervention, differences that were significant (P = 0.000). The *vacA s1m1* genotype was highly prevalent, present in isolates of eight patients before and after treatment; the remaining patients (SV377 and SV480) presented a *vacA s2m2* genotype prior to treatment that then varied with administration of anti-*H. pylori* treatment toward a *vacA s1m1* genotype, nevertheless, these findings were not significant (P = 0.125) (Tables 1 and 2). None of patients harbored isolates with *vacA s1m2* or *s2m1* genotypes. In general, for most subjects, virulent strains were present before and after therapy, nevertheless, the antibiotic treatment limited colonization by multiple strains among and within the anatomic sites evaluated for each patient.

Amplification of cagA 3' variable region

The 3' end of *cagA* was successfully detected in 95.5% (21/22) of the isolates obtained before treatment and in 81.8% (18/22) of the isolates obtained after treatment in the 10 patients with therapeutic failure, which allowed corroborated the existence of *cag* locus and besides predicted the number of EPIYA repetitions (Table 1). The size of most PCR amplicons varied between 495 to 695 bp. However, reproducible bands with unexpected molecular weights of 200 bp were observed (Figure 1A and Table 1). A single-band was found in 59% (26/44) of the isolates obtained among antrum and body samples, before and after treatment, while 29.5% (13/44) of the isolates presented a double-band with different molecular weight, which confirms the presence of multiple *cagA*-positive strains in these subjects. All negative isolates for EPIYA-PCR (n = 5) were confirmed by *cag* empty-site, in two cases the test positivity (SV377 antrum greater curvature pre-treatment and SV526 antrum lesser curvature post-treatment) did not permit to establish the *cag* status by effect of multiple colonization. In the three remaining cases the amplification was not obtained. Finally, no statistical differences were observed when comparing the proportions of amplification of the EPIYA-PCR *vs cagA*-PCR in detecting of the locus *cag* in strains obtained before and after treatment (P > 0.05).

Interpretation of RAPD fingerprint

A reproducible RAPD pattern was observed using primers 1281 and 1254. These could discriminate 44 fingerprints. With primer 1281, well-defined profiles of one to 15 fragments of 74 to 1341 bp were observed (Figure 1B). It was evaluated whether exposure to anti-*H. pylori* treatment, anatomic location of the isolates within the stomach, and the histopathological

Table 1 Comparison of the *cagA* (EPIYA motifs) and *vacA s* and *m* genotypes found before and after anti-*Helicobacter pylori* treatment in isolates from patients with treatment failure

Patient (n = 10)	Anatomic location	Anti-*H. pylori* treatment			
		Genotype		EPIYA motifs (bp)	
		Before	After	Before	After
SV314	Antrum lesser curvature	*cagA+/vacA s1m1*[1]	*cagA+/vacA s1m1*	495	200
	Antrum greater curvature	*cagA+/vacA s1m1*[1]	*cagA+/vacA s1m1*	495/200	200
SV318	Antrum lesser curvature	*cagA+/vacA s1m1*[1]	*cagA+/vacA s1m1*	495	595/495
	Antrum greater curvature	*cagA+/vacA s1m1*[1]	No change	200	595/495
	Body greater curvature	*cagA+/vacA s1m1*[1]	*CagA+/vacA s1m1*	495/200	NA
SV377	Antrum lesser curvature	*cagA+/vacA s2m2*[2]	*cagA+/vacA s1m1*	695/595/495/200	200
	Antrum greater curvature	*cagA+/vacA s2m2*[2]	*cagA+/vacA s1m1*	NA	200
	Body greater curvature	*cagA+/vacA s2m2*[2]	Undefined	200	495
SV415	Antrum lesser curvature	*cagA+/vacA s1m1*[1]	*cagA+/vacA s1m1*	495	495/200
	Body greater curvature	*cagA+/vacA s1m1*[1]	*cagA+/vacA s1m1*	495/200	495
SV444	Body greater curvature	*cagA+/vacA s1m1*	No change	595	595
SV471	Antrum greater curvature	*cagA+/vacA s1m1*[1]	*cagA+/vacA s1m1*	495	595
	Body greater curvature	*cagA+/vacA s1m1*[1]	*cagA+/vacA s1m1*	495/200	595
SV480	Antrum lesser curvature	*cagA+/vacA s2m2*[2]	*cagA+/vacA s1m1*[1]	595/495	595/200
	Body greater curvature	*cagA+/vacA s2m2*[2]	*cagA+/vacA s1/m1*	200	595
SV509	Antrum lesser curvature	*cagA+/vacA s1m1*[1]	*cagA+/vacA s1m1*	200	NA
	Antrum greater curvature	*cagA+/vacA s1m1*[1]	*cagA+/vacA s1m1*	200	495
SV512	Antrum greater curvature	*cagA+/vacA s1m1*[1]	*cagA+/ vacAs1m1*	495	NA
	Body greater curvature	*cagA+/vacA s1m1*[1]	No change	495/200	595
SV526	Antrum lesser curvature	*cagA+/vacA s1m1*	*cagA+/vacA s1m1*[1]	595	NA
	Antrum greater curvature	*cagA+/vacA s1m1*[1]	*cagA+/vacA s1m1*	595/200	595
	Body greater curvature	*cagA+/vacA s1m1*[1]	*cagA+/vacA s1m1*	595/200	595

[1]Concurrent infection with *cagA-/vacA s1m1* isolate; [2]Concurrent infection with *cagA-/vacA s2m2* isolate. NA: Did not amplify.

Table 2 Effect of the antibiotic pressure on the virulence-associated genotypes of *Helicobacter pylori* isolates obtained from 10 Colombian patients with treatment failure

Genotype	Before treatment (n = 22)	After treatment (n = 22)	P vaule
cagA			
Positive	2 (9.1)	17 (77.3)	0.000
Mixed	20 (90.9)	5 (22.7)	
vacA			
s1m1	17 (22.7)	21 (95.5)	0.125
s2m2	5 (77.3)	1 (4.5)	

result was associated to the RAPD conglomerates. Isolates obtained before treatment in patients with treatment failure (12/22; 54.5%) were included in conglomerate I, while isolates obtained after treatment (10/22; 45.5%) were included in conglomerate II (P = 0.042). In contrast, no significant differences were found in the segregation of the isolates by anatomic site and histopathological diagnosis (P = 0.414 and P = 0.339, respectively).

In the case of primer 1254, well-resolved fingerprints were also observed, the number and size oscillated from three to 30 and 87 to 1187 bp, respectively (Figure 1C). The dendrogram obtained includes anatomic location of the isolates, exposure to anti-*H.pylori* treatment, and histopathological result (Figure 2). The conglomerate analyses showed three principal conglomerates in a parsimonious arrangement. In no conglomerate, the distribution of the strains by anatomic site and exposure to treatment showed significant differences (P = 0.700 and P = 0.851, respectively). In contrast, significant differences were observed in the segregation of the histopathological diagnosis associated to each isolate (P = 0.006). Conglomerate I included 19/36 (52.8%) isolates associated to chronic NAG, and conglomerate II grouped 7/8 (87.5%) isolates associated to IM.

Impact of treatment on the 3' region of cagA

Analyzing the number of EPIYA motifs in *H. pylori* isolates obtained from patients with treatment failure with respect to isolates found before treatment in each of them according to the anatomic location. It was found an alteration in the number of EPIYA repeats in 72.7% (16/22) of the isolates due to the action of the antibiotics used. In six of these (SV377 BGC, SV471 AGC, SV509 AGC, SV512 BGC, SV318 ALC and AGC) the variation consisted in the gain of repeats, but in the last two isolates a divergence of *cagA*-positive subclones was observed. In contrast, in two isolates (SV314 ALC and SV480 ALC) the change consisted in the loss of EPIYA repetitions. While in SV377 ALC, SV415 ALC, and BGC isolates, the change evidenced after antibiotic pressure was the product of the replacement of the initial isolate with another isolate from a different region of the stomach of the same patient. Only in one isolate (SV480 BGC), the change in the number of EPIYA repetitions post-treatment was attributed to the re-infection of this patient with new *H. pylori* strain based on differences in genotyping of isolates and RAPD profiles. In the remaining four isolates (SV314 AGC, SV471 BGC, SV526 AGC and BCG), one of the CagA species initially present was

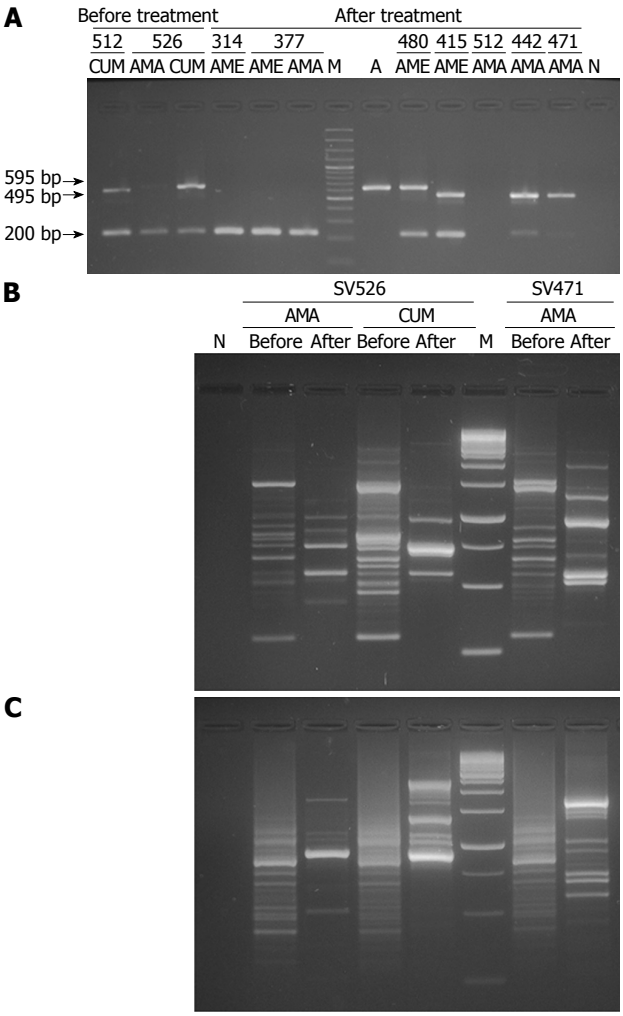

Figure 1 Electrophorectic analyses. A: EPIYA-PCR products from DNA of *H. pylori* isolates obtained before (first endoscopy) and after (second endoscopy) treatment in patients with treatment failure, the PCR products were analyzed in agarose gel at 2%. Line A positive control, clinical isolate of *H. pylori* (PZ5085) with EPIYA motif ABCC (570 ± 25 bp) confirmed through sequencing; line M, 100-bp weight marker; line N, negative reaction control. The distribution of the different molecular weights among isolates is an indication of the presence of multiple EPIYA repetitions; B and C: RAPD patterns generated with primer 1281 and 1254, respectively, in *H. pylori* isolates obtained before and after anti-*H. pylori* treatment; line N: Negative reaction control; line M: 100-bp weight marker. ALC: Antrum lesser curvature; AGC: Antrum greater curvature; BGC: Body greater curvature; *H. pylori*: *Helicobacter pylori*.

lost, reflecting the co-infection by *cagA*-positive strains, and the subsequent selection of the resistant strain after treatment. Finally, one isolate (SV444 BGC) did not evidence any alteration, and in the five remaining isolates, the alteration could not be observed due to problems of amplification with the EPIYA-PCR. In all cases, the RAPD fingerprints generated by both primers supported the findings.

DISCUSSION

To remove *H. pylori* from the gastric mucosa, standard 14-d triple-drug including PPI-clarithromycin and amoxicillin is one of the most effective first-line therapy and best tolerated for patients[24]. Although high success rates have been obtained in clinical trials[32], the effectiveness of triple-therapy has decreased over time, achieved with its implementation eradication rates of up to 70%, less than 80% rate expected, and below what should be expected for an infectious disease[33], which is consistent with the 74.6% eradication rate found. This is worrisome because every failure to eradicate the infection can result in the emerging resistance of the microorganism to antibiotics employed, being unknown other possible implications on the genome of the strains. Thus, it is important to evaluate the effect of treatment failure on *cagA* and *vacA* genes in *H. pylori* isolates from Colombia.

As previously documented, the most important factors for treatment failure are pre-existing antibiotic resistance[33] and lack of compliance to treatment[34]. However, in this study, antimicrobial susceptibility testing performed on isolates obtained prior to treatment showed low rates of resistance to clarithromycin (2.7%) and amoxicillin (4%)[29], besides the compliance was strictly monitored during the treatment, without clinically important adverse sequelae during the treatment administration. Therefore, it seems that both factors are unlikely explanations for the results of our study. Thus, other factors such as increased free and prolonged use of PPIs[16], genetic polymorphisms of the *CYP2C19*[35], cigarette smoking, increased acidity[25], high bacterial load[36], and genotype of infecting strain[22,24] could be associated with treatment failure.

In this study, we found that 90% of the patients with treatment failure, before treatment carried multiple strains of *H. pylori* within and among the anatomic sites evaluated; after treatment, only 60% of the patients showed multiple colonization. One hypothesis is that presence of multiple *H. pylori* strains in an individual probably represents a stable association during the establishment of infection in the long-time[37]. When comparing *cagA* and *vacA* genotypes found in each patient, within the intragastric locations evaluated before and after therapy, it was observed that in several patients, virulent genotypes (*cagA*-positive; *vacA s1m1*) predominated before and after treatment; on the contrary, the low-virulence genotypes found before treatment were almost undetectable after therapy. These preliminary findings contrast with that reported by Correa *et al*[22] who suggest that failure to remove this bacteria can lead in some individuals to the survival of low-virulence strains (*cagA*-negative; *vacA s2m2*) because these strains seem to be more resistant to treatment.

The difference between these findings can be explained from the use in the present research of multiple biopsies (antrum and body) by patient to establish the genotypes, minimizing the effect of sampling error, without overestimating multiple colonization. Additionally, the results obtained could be reflecting a possible association between virulent genotypes and antibiotic resistance, which has been previously described in several studies[16,37,38]. In fact,

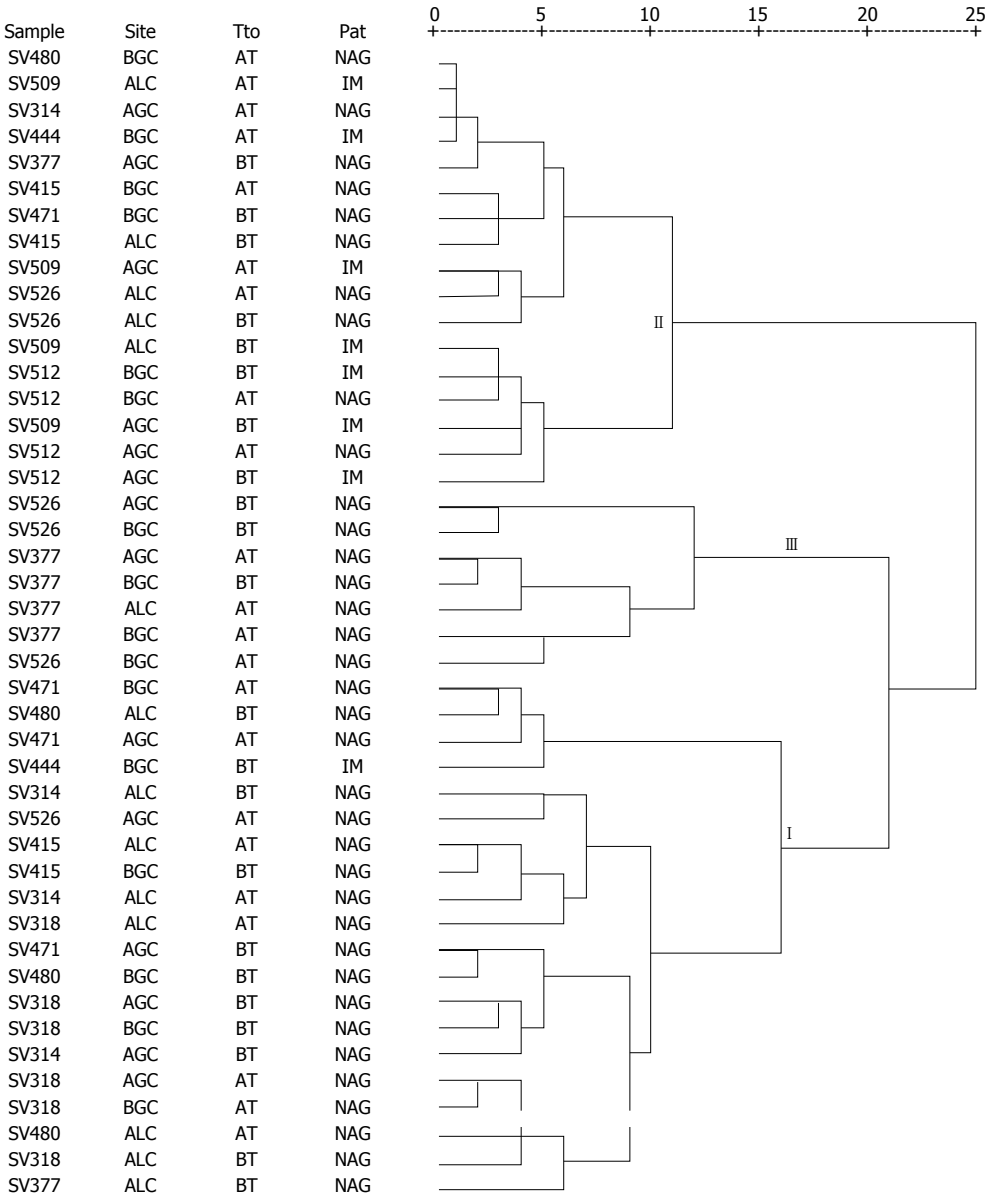

Figure 2 Dendogram of the random amplified polymorphic DNA profile generated with primer 1254 in *Helicobacter pylori* isolates obtained before (first endoscopy) and after (second endoscopy) treatment in patients with treatment failure. Three separate conglomerates (Ⅰ, Ⅱ, and Ⅲ) are indicated. Most of the *H. pylori* isolates associated to chronic non-atrophic gastritis (19/36) and intestinal metaplasia (7/8) were included in conglomerate I and II, respectively (P = 0.006). No significant differences were noted in the segregation of isolates according to the anatomical site and exposure to treatment (P = 0.700 and P = 0.851, respectively). Conglomerate analyses were designed following Ward's conglomeration method and estimation of distances between each pair or group of isolates were calculated with the squared Euclidean distance. Distances between isolates are given in a 25-point standardized scale, where the fingerprints with distances below or equal to 5 were considered related and distances greater than 5 were considered unrelated. AT: After treatment; BT: Before treatment; NAG: Non-atrophic gastritis; IM: Intestinal metaplasia; *H. pylori*: *Helicobacter pylori*; ALC: Antrum lesser curvature; AGC: Antrum greater curvature; BGC: Body greater curvature.

the *cagA*-positive strains have a higher replication rate than the equivalent *cagA*-negative strains[39], thus, increasing the possibility of acquiring mutations that can be evolutionarily advantageous, supporting the hypothesis of treatment failure in these patients by acquisition of mutations that possibly modified the site of the antibiotic action (secondary antibiotic resistance). However, the presence of point mutations associated with resistance in the *H. pylori* isolates was not confirmed by sequencing, being a limitation of the study.

Additionally, the characterization of the number of EPIYA repetitions present between isolates obtained before and after treatment in patients with treatment failure, showed that antibiotic pressure in some strains induces genomic rearrangements inside the 3' end of *cagA* gene that generate a non-directed alteration (gain or loss) of EPIYA repeats, agreeing with findings reported previously in *in-vitro* experiment[29]. It is hypothesized that these genetic changes could be directed through intergenomic and intragenomic recombination, processes that are improved through a second-order selection[13,19,40], allowing bacteria to confront variable and stressful environments within its

current host[41].

In this case, the ability of *H. pylori* to edit particular immunostimulatory genetic regions (3' end of *cagA* gene), it could lead to: (1) the synthesis of a non-phosphorylatable form of CagA by loss of all EPIYA motifs, which compromises their ability to interact with SHP-2, minimizing the alteration of signal transduction pathways of the cell, supported by the bands obtained with molecular weight not expected (200 bp); or (2) the alteration of number of EPIYA motifs impacting the adherence of CagA protein to the epithelial cell membrane[42]; level of tyrosine phosphorylation, the induction of IL-8 secretion[43], and multimerization of CagA[44]. Also, in some cases lead to differentiation of *cagA*-positive sub-clones with distinct numbers of EPIYA repeats (*e.g.*, SV318 ALC and AGC, SV314 ALC), each acting as possible source of genetic elements for other clones.

These clonal variants cooperate by quorum sensing and recombination to downregulate their interplay with the individual, and thus produce less damage[19]. However, some tissue damage and inflammation are unavoidable, because in these strains diverse CagA species are secreted, altering multiple signaling pathways and inducing various degrees of elongation in gastric epithelial cells[13]. This hypothesis may partly explain the findings of Mera *et al*[45] who in a randomized trial of 795 adults with preneoplastic gastric lesions for 3, 6, and 12 years, observed that patients with treatment failure at 12 years of follow-up had a modest decrease in their histopathological score compared with average histopathology at baseline.

In general, the RAPD profiles obtained were of great utility because they initially supported the close clonal association among the *H. pylori* strains present in present in different gastric localizations of the same patient; for this reason, in the clusters analysis for both primers no differences were found in the segregation of the strains by anatomical site. Subsequently, the profiles generated by primer 1254 clearly defined a pathological conglomerate for IM (P = 0.006), in agreement with the hypothesized by Kidd *et al*[46] who suggest that clonal grouping by RAPD patterns can be associated to disease. Likewise, Vega *et al*[16] demonstrated the usefulness of the DNA fingerprints to discriminate *H. pylori* isolates associated with peptic ulcer, nevertheless, in the present research this association was not evident with primer 1281.

Additionally, the DNA fingerprints generated by primer 1254 permitted evidencing the close clonal relationship among some isolates obtained before and after treatment (Recrudescence), not showing significant differences in segregation of isolates according to the exposure to treatment, agreeing with that expected and contrasting with the results obtained with primer 1281. In spite of these differences, the conglomerate analysis for primer 1281 also reflected the close relationship of some isolates obtained before and after treatment, although in lesser number. There was only one case (SV480 BGC) where the lack of proximity within the clusters formed together with the differences in genotyping for the basal isolate and post-treatment suggest reinfection of the patient with a new *H. pylori* strain.

Our study has some limitations. First, due to the absence of sequencing of bacterial genes encoding 16S rRNA and *pbp-1A* in isolates of patients with treatment failure, it was no possible to detect point mutations that explain the acquisition of secondary resistance to clarithromycin and amoxicillin, respectively. Second, although EPIYA PCR is a practical tool for amplify the *cagA* gene, and characterize the number and type of EPIYA motifs, in the bands with unexpected molecular weight (200 bp), the number of motifs is unknown because it was not sequenced. Third, the number of *H. pylori* isolates obtained from subjects with unsuccessful treatment in this study is small, so our findings must be confirmed in other studies, where it is also advisable to evaluate the effect of treatment failure on the signal and median region of the *vacA* gene that can equally present genetic variation through recombination. Future interventions may be designed based on this research.

In conclusion, the present research shows that although the anti-*H. pylori* treatment fails to eradicate the infection in some patients, it limits colonization by low-virulence genotypes leading to a partial and selective elimination in mixed infections, and it acts in some cases on *cagA*-positive strains inducing: (1) mutations that make it resistant to antibiotics; and (2) genetic rearrangements as deletion or acquisition of EPIYA motifs, decreasing or increasing the phosphorylation of CagA and its binding to SHP-2, respectively. All this leads to divergence of *cagA*-positive strains with implications for pathogenesis that are not yet well understood and need to be studied. Finally, it is possible that indiscriminate use of antibiotics to treat upper respiratory and intestinal infections also alter the genetic structure of *H. pylori* strains that coexist within the host.

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COMMENTS

Background

Infection with *Helicobacter pylori* (*H. pylori*) is the greatest risk factor for development of gastric adenocarcinoma, especially in individuals infected with *cagA*-positive strains. In developing countries, the colonization with multiple strains of high (*cagA*-positive) and low virulence (*cagA*-negative) is common, these *H. pylori* clones can co-exist in dynamic equilibrium within of host, cooperating through quorum sensing and recombination, which can lead to subregulation of its interaction to induce lower damage even when some damage is inevitable. In any case, the eradication of *H. pylori* with antibiotics constitutes an important primary prevention strategy of gastric lesions and

atrophy. Although major improvements have been made in the efficacy of treatment regimes, all of them result in failures to eradicate the infection. Few studies have focused on evaluating the effect of treatment failure on virulence factors of *H. pylori.*

Research frontiers

Virulence-associate genotypes of *H. pylori* are important determinants of the clinical outcome of the infection. In many series, patients with severe gastritis, atrophic gastritis, peptic ulcer disease and distal gastric cancer are predominantly infected with the *cagA-positive/vacA s1m1* strains, whereas the *cagA-negative/vacA s2m2* strains are more frequent in patients with non-ulcer dyspepsia and mild gastritis. That virulence factors are linked with disease implies that they are a fixed characteristic, but this is not the case because change in genotypes promoted by environmental pressures (*e.g.,* antibiotic treatment, high uptake salt and hyperchlorhydria) can occur through of intragenomic recombination (*e.g.*, number motif EPIYA in CagA) or recombination with clonal variants of the same strain or with other strains in cases of mixed infection that can lead to partial or complete loss of *cag* PAI and changes in *vacA* genotype, reflects local selection of *H. pylori* particular phenotypes that appears to be essential for persistent colonization of host.

Innovations and breakthroughs

A previous study indicates that the unsuccessful treatment of *H. pylori* results in a increase of less virulent genotypes in Colombian patients, it suggesting that the *cagA*-negative/*vacA s2m2* strains were less responsive to treatment or a possible loss of specific virulence factors (*cag* PAI) induced by antibiotic pressure. In contrast, in a recent study, where we evaluated the in vitro effect of antibiotics using in the standard triple therapy, we were found that antibiotic pressure does not induce loss of the cag pathogenicity island, but it can lead in most cases to genetic rearrangements within the 3' region *cagA*, these findings are consistent with the results found in this new study, it showing that the failure of anti-*H. pylori* treatment limits colonization by low-virulence strains resulting in partial and selective eradication of *H. pylori* in mixed infections, and acts on the *cagA*-positive strains inducing genetic rearrangements within the cagA variable region that produces a non-directed alteration like loss or gain of EPIYA motifs, which as a set could alter the pathogenic process induced by *H. pylori*. These events should be considered after failure of first-line treatment.

Applications

These findings have important implications for the treatment of gastro-duodenal diseases caused by *H. pylori*, suggesting that the failure of anti-*H.pylori* treatment results in survival of more virulent genotypes in mixed infections, this alters the dynamic balance between clones of *H. pylori* and host, leading to *H. pylori* to rapidly adapt to new conditions in the stomach by genetic rearrangements that favor the acquisition or deletion of EPIYA motifs, which may lead to upregulation of interaction with the host. In support of this, it has been reported that infection with a single antral-colonizing strain could lead to duodenal ulceration, while, the colonization with two different strains in antrum and corpus leads to lower physiological alterations. These determinants are important considerations in deciding who should be treated.

Terminology

CagA is recognized as a major etiologic determinant of *H. pylori*-associated gastric disease. This bacterial protein is translocate into the gastric epithelial cell cytoplasm via the type IV secretion system. Once injected, CagA localizes to the plasma membrane and undergoes tyrosine phosphorylation by multiple members of the Src family of kinases on specific tyrosine residues within repeating Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, encoded in the 3'variable region of *cagA* gene. These EPIYA motifs are defined as EPIYA-A, -B -C, and -D, according to the amino acid sequence that surrounds the EPIYA sequence. Phosphorylated CagA interacts with the SHP-2 phosphatase and the Crk protein resulting in reorganization of the cytoskeleton, cell elongation, and abnormal proliferation.

Peer-review

In this study, the authors investigated to identify effects of treatment failure on the CagA EPIYA motif in *H. pylori* isolates. This study was well written.

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ORIGINAL ARTICLE

Basic Study

Chemotherapy response evaluation in a mouse model of gastric cancer using intravoxel incoherent motion diffusion-weighted MRI and histopathology

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Author contributions: Cheng J performed the animal experiments, reviewed the experimental and histological data, and wrote the manuscript; Wang Y designed the entire scientific research, instructed manuscript writing, and revised the manuscript; Zhang CF instructed the statistical analysis; Wang H adjusted the parameters of IVIM-DWI scanning; Wu WZ performed the animal experiments and recorded the experimental data; Pan F performed the animal experiments and recorded the experimental data; Hong N instructed the animal experiments and revised the manuscript; Deng J wrote the Metlab program of the bi-exponential IVIM model and analyzed the IVIM parameter data.

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Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at wangyi@pkuph.edu.cn. Participants gave informed consent for data sharing.

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Abstract

AIM

To determine the role of intravoxel incoherent motion (IVIM) diffusion-weighted (DW) magnetic resonance imaging (MRI) using a bi-exponential model in chemotherapy response evaluation in a gastric cancer mouse model.

METHODS

Mice bearing MKN-45 human gastric adenocarcinoma xenografts were divided into four treated groups (TG1, 2, 3 and 4, n = 5 in each group) which received Fluorouracil and Calcium Folinate and a control group (CG, n = 7). DW-MRI scans with 14 b-values (0-1500 s/mm^2) were performed before and after treatment on days 3, 7, 14 and 21. Fast diffusion component (presumably pseudo-perfusion) parameters including the fast diffusion coefficient (D*) and fraction volume (f_p), slow diffusion coefficient (D) and the conventional apparent diffusion coefficients (ADC) were calculated by fitting the IVIM model to the measured DW signals. The median changes from the baseline to each post-treatment time point for each measurement (ΔADC, ΔD* and Δf_p) were calculated. The differences in the median changes between the two groups were compared using the mixed linear regression model by the restricted maximum likelihood method shown as z values. Histopathological analyses including Ki-67, CD31, TUNEL and H&E were conducted in conjunction with the MRI scans. The median percentage changes were compared with the histopathological analyses between the pre- and post-treatment for each measurement.

RESULTS

Compared with the control group, D* in the treated group decreased significantly (ΔD*$_{treated}$% = -30%, -34% and -20%, with z = -5.40, -4.18 and -1.95. P = 0.0001, 0.0001 and 0.0244) and f_p increased significantly (Δ$f_{ptreated}$% = 93%, 113% and 181%, with z = 4.63, 5.52, and 2.12, P = 0.001, 0.0001 and 0.0336) on day 3, 7 and 14, respectively. Increases in ADC in the treated group were higher than those in the control group on days 3 and 14 (z = 2.44 and 2.40, P = 0.0147 and P = 0.0164).

CONCLUSION

Fast diffusion measurements derived from the bi-exponential IVIM model may be more sensitive imaging biomarkers than ADC to assess chemotherapy response in gastric adenocarcinoma.

Key words: Xenografts; Intravoxel incoherent diffusion-weighted magnetic resonance imaging; Chemotherapy; Treatment response; Gastric adenocarcinoma

Core tip: In a mouse gastric cancer model, we demonstrated that the intravoxel incoherent motion (IVIM)-derived tissue perfusion coefficient (D*) decreased, whereas perfusion fraction (PF) increased immediately after chemotherapy and during the treatment course. No considerable overlaps were observed in D* and PF measurements between the treated and control groups. IVIM-derived perfusion measurements offer a potential accurate evaluation of chemotherapeutic efficacy. This imaging study is ready to be translated into a clinical study and may facilitate individualized treatment strategy and prompt treatment adjustment in gastric cancer patients.

Cheng J, Wang Y, Zhang CF, Wang H, Wu WZ, Pan F, Hong N, Deng J. Chemotherapy response evaluation in a mouse model of gastric cancer using intravoxel incoherent motion diffusion-weighted MRI and histopathology. *World J Gastroenterol* 2017; 23(11): 1990-2001 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i11/1990.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i11.1990

INTRODUCTION

Gastric cancer is the second leading cause of cancer-related death worldwide[1]. The prognosis of patients with advanced and unresectable gastric cancers is very poor with a median survival rate of approximately 10 mo[2]. Recently, fluorouracil and its derivatives, cisplatin, irinotecan, taxane and trastuzumab, have been recognized as first-line treatments for gastric cancer[2]. 5-Fluorouracil continuous infusion (5-FUci) is accepted as a standard chemotherapy regimen for advanced gastric cancer for its minimal toxicity as shown in clinical trials[2]. However, treatment responses vary in individual patients, mainly due to the heterogeneous and dynamic nature of tumor progression after sequential treatments. Thus, early detection and accurate assessment of tumor response to treatment is essential for individualized treatment planning[3].

Diffusion-weighted magnetic resonance imaging (DW-MRI) has emerged as a noninvasive imaging technique, which exploits tissue water mobility reflective of the microstructural properties of tumor tissue and detects tissue changes after treatment. DW-MRI uses motion-probing gradients to sensitize MRI signal loss arising from the Gaussian diffusion distribution. Higher tissue cellularity and cell membrane integrity of tumor malignancy are associated with more restricted diffusion, whereas tumor cell apoptosis and necrosis result in increased diffusivity of water molecules[4,5]. Tissue water mobility can be quantified by the apparent diffusion coefficient (ADC) using the conventional mono-exponential model to fit DW signals acquired at 2-3 b-values between 0 and 1000 s/mm^2. ADC primarily represents extracellular tissue diffusion with a mixed effect of fast diffusion component arising from microvascular blood flow. Previous studies have demonstrated that ADC values increase after chemotherapy, radiotherapy or administration of combined targeted medicine, indicating that ADC may serve as an imaging biomarker for the assessment of tissue microstructural changes prior to any changes in tumor size[6-8]. However, ADC values after treatment may not consistently increase because treatment may cause cellular swelling in the early phase of apoptosis

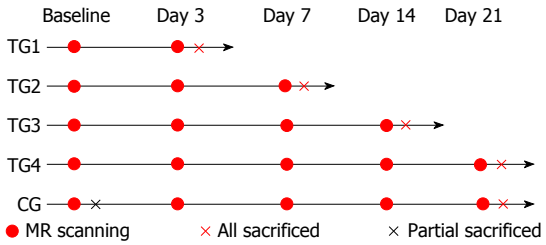

Figure 1 Schedule of magnetic resonance image acquisition and sacrifice of treated and control mice. TG: Treated group; CG: Control group.

and may cause the formation of fibrosis, both of which decrease the extracellular space and consequently decrease ADC values[6,8]. The complex interplay of biophysical processes including tissue cellularity, intracellular and extravascular/extracellular water diffusion, and tissue perfusion contributes to variations in post-treatment ADC values that have yet to be clarified[8].

With multiple b-values at lower range, the DW signal decay deviating from mono-exponential decay is not non-negligible due to the pseudo-perfusion effect at lower b-values. To more accurately evaluate post-treatment tumor tissue changes, extravascular diffusion and microvascular perfusion need to be distinguished from each other using an advanced DW-MRI method that takes into account the intravoxel incoherent motion (IVIM) phenomenon[9]. The IVIM DW-MRI may distinguish between multi-compartmental (*e.g.*, diffusion *vs* perfusion and intracellular *vs* extracellular) effects in biological tissues[9]. IVIM DW-MRI exploits the fact that, by using more diffusion weightings (b-values) at lower b-values (0-200 s/mm^2) with smaller intervals, DW signal decay can be used to quantify microvascular perfusion activity, whereas signal decay at higher b-values (> 200 s/mm^2) can be used to quantify tissue water molecular diffusion; therefore, IVIM DW-MRI can distinguish the microvascular perfusion effect from "pure" diffusion. Consequently, IVIM DW-MRI allows the following to be calculated noninvasively without contrast agent administration: the "pure" (slow) diffusion coefficient (D), pseudo-perfusion (fast) diffusion coefficient (D*), and fractional volume (f_p)[9,10]. IVIM measurements have been used to differentiate between benign and malignant tumors[11-13] and evaluate therapeutic responses to chemotherapy in different tumor types[14-17]. However, the variability of IVIM measurements in response to chemotherapy and/or radiotherapy in these studies remains controversial.

To the best of our knowledge, no previous studies have evaluated the therapeutic response to the standard chemotherapy 5-FU using IVIM DW-MRI in an animal model of gastric adenocarcinoma. The purpose of our study was to evaluate treatment response in tumor tissues by studying changes in diffusion and pseudo-perfusion properties based on ADC and IVIM measurements in a mouse model of gastric adenocarcinoma as early as 3 d after treatment, and to compare the imaging findings with histopathological results of tissue cellularity and microvascular properties at designated post-treatment time points.

MATERIALS AND METHODS

Animal model

All experimental procedures were approved by the Institutional Review Board of and Institutional Animal Care and Use Committee of Peking University People's Hospital, Beijing, China. Animals were housed in a pathogen-free facility at a temperature of 22 ℃, humidity of 61%, and a 12 h light–dark cycle. They were provided with food and water in accordance with the animal welfare guidelines established by our institution's Office of Laboratory Animal Welfare (OLAW). Twenty-seven BALB/c nude female mice, weighing 20-24 g and aged 6-8 wk, were obtained from the Vital River Laboratories (Beijing, China). They were acclimated to their new environment for one week before experimentation.

All animals were randomly divided into a control group (CG) and four treated groups (TG1-4) with five mice in each treated group and seven in the control group. All groups underwent MRI scans and were sacrificed for histopathological analysis based on the schedule shown in Figure 1. Two mice in the control group were sacrificed at baseline after MRI scanning. Five mice in the control group underwent MRI at each time point (day 0, day 3, day 7, day 14, and day 21) and were then sacrificed on day 21. All tumors were resected from each animal post-sacrifice for serial histopathological analyses and compared with the MRI findings.

Tumor model

Cells from the poorly differentiated human gastric adenocarcinoma cell line MKN-45 were obtained from the American Type Culture Collection (Rockville, MD, United States). MKN-45 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum and 1% nonessential amino acids. Concentrations of 95% O_2 and 5% CO_2 were maintained. Cells were passaged twice a week with a 1:2 split using 0.25% trypsin (HyClone, Ft. Collins, CO, United States). The tumor xenografts were developed by subcutaneously injecting approximately 1 × 10^6 cells suspended in 100 μL medium, into both flanks of the nude mice; therefore, each mouse developed two tumors. Tumors grew for 10-15 d until they reached 100-250 mm^3 in size prior to treatment.

Treatment protocol

Animals in the treated groups received 5-FU injections (40 mg/kg) (Shangdong Qilu Pharmaceutical Co., Ltd., China) in 0.2 mL 0.9% sodium chloride intraperitoneally

Table 1 Parameters of T2WI and intravoxel incoherent motion MR scanning

	T2WI	IVIM
Plane	Axial and coronal	Axial
TR (ms)	2800	2500
TE (ms)	72	42
Fat suppression	No	Yes
Matrix	256 × 192	64 × 64
FOV (mm^2)	70 × 35	70 × 35
Thickness (mm)	1.5	1.5
Gap (mm)	0.5	0.5
NEX	4	1 (b = 0-300) 2 (b = 400-600) 4 (b = 800-1500)
b value (s/mm^2)	NA	0, 10, 20, 30, 50, 80, 130, 200, 300, 400, 600, 800, 1000, and 1500

IVIM: Intravoxel incoherent motion.

once a day for 5 consecutive days starting on day 0. No medicine was given during the last two days of the week[18]. In addition, calcium folinate (Jiangsu Hengrui Medicine Co., Ltd., China) was injected (45 mg/kg) intraperitoneally twice a day; the first injection was given one hour before the 5-FU injection, and the second injection was given at the same time as the 5-FU injection. This procedure was repeated for three weeks. Animals in the control group received intraperitoneal injections of 0.1 mL sterile water at the same time points as the treated animals received 5-FU injections.

Image acquisition

MR images were acquired on a 3.0 Tesla MRI scanner (Discovery 750, GE Healthcare, Waukesha, WI, United States). Each mouse was anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). After anesthetization, the mouse was placed in a test tube filled with salt alginate impression gel. The gel had been dissolved in warm water before use to mitigate the possibility of artifacts arising from the tissue-air interface at each subcutaneous xenograft tumor area and to maintain the mouse's body temperature. The tube containing the mouse was then placed in a small animal birdcage coil (Magtron Inc., Jiangyin, China) in the supine position on the MR platform. Anatomic MR images for tumor volume measurements were acquired with a T2-weighted (T2W) spin-echo (SE) sequence with the parameters listed in Table 1.

DW-MR images were acquired with a single-shot SE echo-planar imaging (DW-SE-EPI) sequence with the parameters listed in Table 1. A spatial-spectral excitation pulse was used to excite a slice of magnetization from water protons while leaving the fat protons unaffected to achieve fat suppression. Parallel imaging using the array spatial-sensitivity encoding technique (ASSET) was employed to reduce the echo train length and thus mitigate image distortion. The total acquisition time for anatomic and DW-MRI scanning was approximately 15 min.

Post-processing

Anatomic and DW-MR images were transferred to a workstation (SW45; GE Healthcare, Waukesha, WI, United States). One radiologist with 5 years of experience in MRI interpretation performed tumor volume (V_T) measurements on the axial and coronal T2W MR images. He was blinded to the treatment conditions and histopathological results. The three-dimensional maximal diameters of each tumor were measured. Tumor volume was calculated as $4/3\pi \times$ (length × width × height)/2.

On each DW image slice with b-value = 0 s/mm^2, a region of interest (ROI) of the entire tumor was manually drawn and automatically copied onto DW images with other b-values. Within each ROI, the averaged signal intensity at each b-value was calculated. In the standard clinical protocol, conventional ADC was calculated by the linear fitting of the logarithm of signal intensities acquired at b-value = 0 and 1000 s/mm^2.

IVIM parameters, including the fast diffusion coefficient (D*) and corresponding fractional volume (V_{fast}), and the slow diffusion coefficient (D), and corresponding fractional volume (V_{slow}), were calculated using the bi-exponential model $S(b)/S(0) = V_{slow} \times e^{-bD} + V_{fast} \times e^{-b(D+D^*)}$ where $V_{fast} + V_{slow}$ approximately 1, and S(b) and S(0) represent the signal intensities at the corresponding b-value. The trust-region-reflective non-linear curve-fitting algorithm (Matlab, MathWorks Inc., Natick, MA, United States) was used for bi-exponential signal fitting to derive all four parameters. Prior to the non-linear fitting, the initial estimates of D and V_{slow} were derived by the linear fitting of DW signals with higher b-values (> 200 s/mm^2), where fast diffusion effects can be ignored. The initial estimate of D* was made by a linear fitting of signals with lower b-values (< 80 s/mm^2), where the fast diffusion effect dominated the signal decay. Finally, the fractional volume of the fast component that is presumably considered the pseudo-perfusion fractional volume (f_p) was calculated as $100\% \times V_{fast}/(V_{fast} + V_{slow})$. In order to compare the fitting behavior using the two-compartment bi-exponential IVIM and mono-exponential signal decay model, both methods were used to fit the signal decay with the full range of 14 b-values in a subset of animals.

Histopathological analysis

After image acquisition, animals in the treated groups and control group were sacrificed on day 3 (TG4), day 7 (TG3), day 14 (TG2), and day 21 (TG1 and CG). Two entire tumors from each sacrificed animal were excised, fixed in 10% formalin for 24 to 48 h, and immersed in 70% ethanol. Each tumor specimen was paraffin embedded and cut into 5-μm thick sections. Tissue sections were stained for immunohistochemistry on the Ventana Discovery XT Autostainer (Tucson, Arizona, United States). Immunohistochemical analyses including

Table 2 Median and interquartile range observed in intravoxel incoherent motion parameters, apparent diffusion coefficients and V_T values in the treated and control groups

		Day 0	Day 3	Day 7	Day 14	Day 21
V_T (mm^3)	Treated	219.96 (125.39-280.12)	356.88 (248.76-386.13)	368.33 (320.33-504.98)	559.3 (442.16-644.68)	439.7 (260.97-841.22)
	Control	157.1 (29.04-432.25)	281.71 (104.30-596.15)	476.34 (261.56-777.13)	938.64 (511.07-1345.82)	1515.24 (675.23-1856.48)
ADC (mm^2/s)	Treated	0.57 (0.56-0.59)	0.62 (0.60-0.64)	0.62 (0.58-0.69)	0.83 (0.75-0.94)	0.72 (0.64-0.86)
	Control	0.61 (0.56-0.68)	0.57 (0.52-0.67)	0.55 (0.50-0.70)	0.65 (0.54-0.83)	0.77 (0.59-1.11)
D* (mm^2/s)	Treated	1.99 (1.89-2.47)	1.4 (1.28-1.60)	1.58 (1.36-1.70)	1.68 (1.61-1.82)	1.68 (1.36-1.92)
	Control	2.07 (1.57-2.57)	2.23 (1.61-2.68)	1.97 (1.42-2.89)	2.25 (1.53-2.36)	1.81 (1.59-2.00)
fp (%)	Treated	19.15 (15.23-21.27)	32.90 (28.38-40.72)	37.19 (26.63-46.91)	49.95 (42.81-55.49)	42.56 (38.02-53.59)
	Control	22.38 (14.52-29.10)	22.98 (16.22-33.03)	22.47 (16.46-28.54)	27.87 (24.35-47.52)	40.2 (33.40-54.69)
D (mm^2/s)	Treated	0.39 (0.37-0.41)	0.34 (0.29-0.37)	0.33 (0.23-0.36)	0.33 (0.29-0.35)	0.32 (0.23-0.38)
	Control	0.40 (0.33-0.44)	0.41 (0.33-0.44)	0.39 (0.33-0.47)	0.39 (0.32-0.47)	0.35 (0.32-0.44)

IVIM: Intravoxel incoherent motion; ADC: Apparent diffusion coefficients.

Ki67, TUNEL and CD31 staining were performed on areas containing viable tumor tissues. Tumor cells, sufficiently stained with chromogen, were considered positive compared with the surrounding tissues. For each type of histopathological analysis, each stained slide was digitized with an optical magnification (× 200 or × 400) using a LEICA DFC 550 Digital Microscope Camera (Wetzlar, Germany). One pathologist (W.G.) with 10 years of experience defined the viable tumor areas by avoiding significant areas of necrosis on the slides. The microscopic images were then analyzed by Image J (version 1.42; National Institutes of Health, Bethesda, MD, United States) to automatically detect the difference between the target cells with positive staining and the background cells. The detected target cells were manually confirmed based on the image intensity and minimum particle size threshold[19]. At least three sections from each tumor were measured and averaged.

With regard to Ki67 staining (ab15580; Abcam, Cambridge Science Park, United Kingdom), the proliferating cell density was calculated as the ratio of cells stained by Ki-67 to the total number of background cells. In TUNEL staining (Roche, Basel, Switzerland), the apoptotic cell density was calculated as the ratio of cells with positive TUNEL expression to the total number of viable tumor cells. In CD31 staining (sc1506; Santa Cruz Biotechnology, Santa Cruz, CA, United States), microvessel endothelial cells were identified, and the microvessel density (MVD) was calculated as the total number of vessels divided by the viable tumor cells. In addition, each tumor section was stained with hematoxylin and eosin (H&E, Sigma-Aldrich; Ventana, Tucson, AZ, United States) to delineate tumor necrosis. The necrotic fraction was calculated as the ratio of the necrotic area to the total tumor area on at least three sections of each tumor; these ratios were then averaged.

Statistical analysis

The median and interquartile range of tumor volume (V_T), ADC, D, D*, and f_p were calculated for baseline and post-treatment scans. Medians were calculated due to the data skew distribution observed in the stem-leaf plot.

Median percentage changes (ΔV_T%, ΔADC%, ΔD*% and Δf_p%) between baseline and each post-treatment scan in each tumor were calculated. Logarithm transformation was used to meet the precondition of the normal distribution for mixed linear model analysis. After logarithm transformation, ΔV_T%, ΔADC%, ΔD*% and Δf_p% were compared between the control and treated groups using the mixed linear regression model with the restricted maximum likelihood method, and were recorded as z values. *P* values less than 0.05 were considered statistically significant. All statistical analyses were performed using the statistical software Stata (version 13.0; StataCorp LP, College Station, Texas, United States).

RESULTS

Two animals from TG2 and TG3 died on day 2 after treatment. One animal in TG1 was excluded due to severe ulceration on day 3 after treatment. Two animals from TG2 and TG3 were excluded due to severe image distortion and chemical shift artifacts present in IVIM DW images. After these exclusions, the mice were distributed throughout the study groups as follows: 7 (CG), 4 (TG1), 3 (TG2), 3 (TG3), and 5 (TG4).

Tumor volume measurement

Tumor volumes at baseline and at each post-treatment time point in the treated and control groups are shown in Table 2 and Figure 2A; percentage changes (ΔV_T%) in baseline and post-treatment time points were then calculated (Figure 3A). Differences in ΔV_T% between the treated group and the control group were not significantly different on day 3 ($\Delta V_{Ttreated}$% = 103% and $\Delta V_{Tcontrol}$% = 125%, z = -0.17, *P* = 0.8659) and day 7 ($\Delta V_{Ttreated}$% = 127% and $\Delta V_{Tcontrol}$% = 384%, z = -1.36, *P* = 0.1724). However, tumor volumes in the control group on day 14 ($\Delta V_{Tcontrol}$% = 890%) and day 21 ($\Delta V_{Tcontrol}$% = 1449%) were significantly higher than those in the treated group at the same time points ($\Delta V_{Ttreated}$% = 187%, z = -3.62,

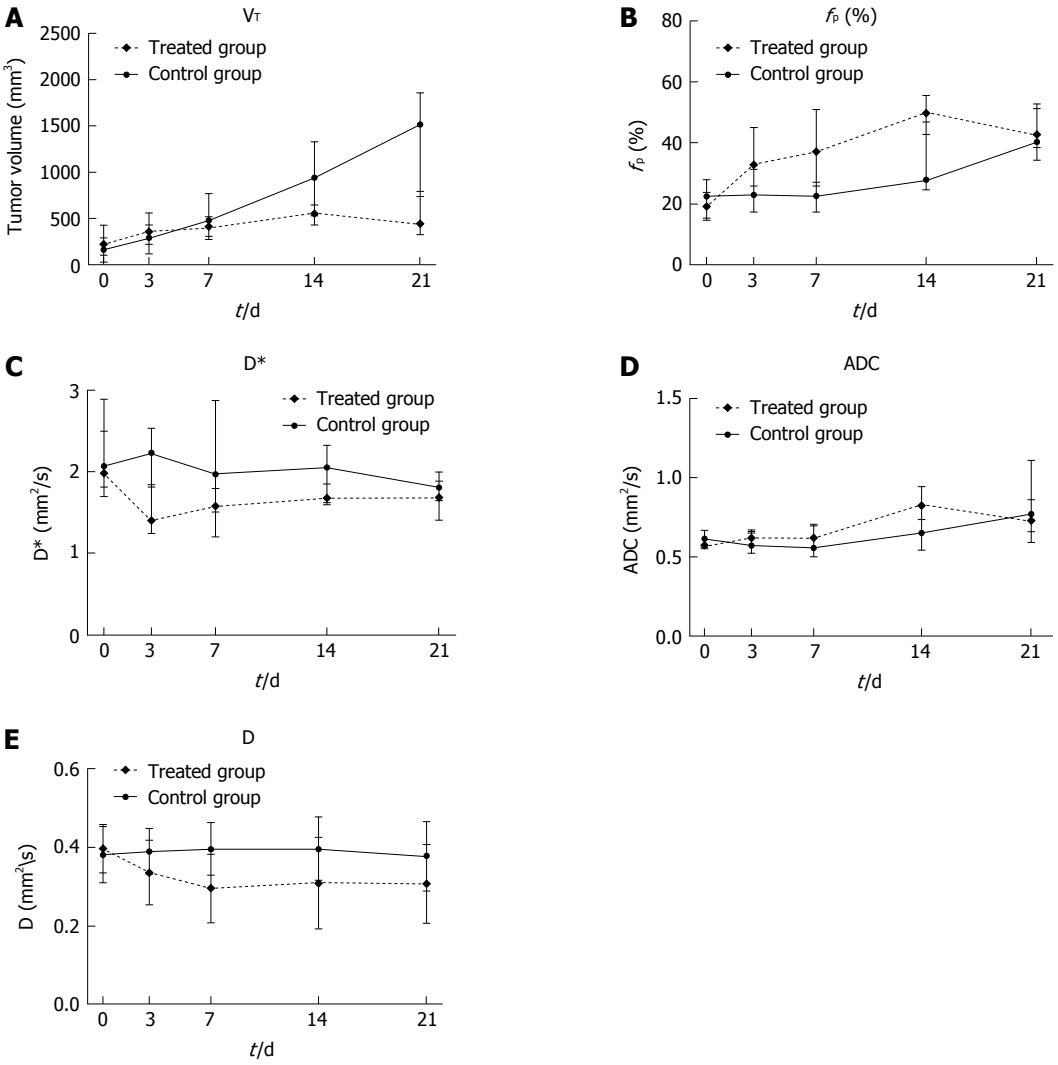

Figure 2 Median tumor volume (A), *fp* (B), D* (C), apparent diffusion coefficients (D) and D values (E) of all animals in the treated groups (dashed line) and control group (solid line). The vertical bars represent the interquartile ranges. ADC: Apparent diffusion coefficient.

P = 0.0003 and $\Delta V_{Ttreated}$% = 156%, z = -6.30, P = 0.0001), respectively. Tumor volume changes in two representative mice (treated and control) are shown on T2W images (Figure 4). Tumor volume in the control group increased significantly from day 14 to day 21, whereas no obvious tumor volume changes were observed in the treated group from day 3 to day 21.

Tumor IVIM measurement

IVIM measurements (D, D* and f_p) and conventional ADC values for all tumors in the treated and control groups at each time point are shown in Table 2 and Figure 2B-E. The median percentage changes in pre-treatment baseline and each post-treatment time point are shown in Figure 3B-D.

The IVIM derived perfusion measurements D* and f_p showed opposite trends with decreasing perfusion related diffusion coefficients (D*) and increasing perfusion fractions (f_p) after treatment. D* in the treated groups decreased on days 3, 7, 14, and 21 with a median percentage change ($\Delta D^*_{treated}$%) of -30%, -34%, -20% and -31%, respectively. In contrast, D* in the control group was close to baseline with a median percentage change ($\Delta D^*_{control}$%) of 3%, 1% and -1% on days 3, 7 and 14, which were significantly different from $\Delta D^*_{treated}$% at each time point (z = -5.40, -4.18 and -1.95, P = 0.0001, 0.0001 and 0.0244). D* in the control group decreased at the end of the experimental period on day 21 ($\Delta D^*_{control}$% = -9%), which was not significantly different from that in the treated group (z = -1.95, P = 0.0513). The median percentage increase in f_p in the treated groups ($\Delta f_{ptreated}$%) was as follows: 93% on day 3, 113% on day 7 and 181% on day 14, and the percentage change in f_p in the control group ($\Delta f_{pcontrol}$%) increased less during the course of the study (13%, 9% and 58% on days 3, 7 and 14). Significant differences in Δf_p% were found between the treated and control groups on day 3, 7 and 14 (z = 4.63, 5.52, and 2.12, P = 0.001, 0.0001, and 0.0336), respectively. At the end of the experimental period (day 21), the treated and control groups showed increases in f_p ($\Delta f_{ptreated}$%

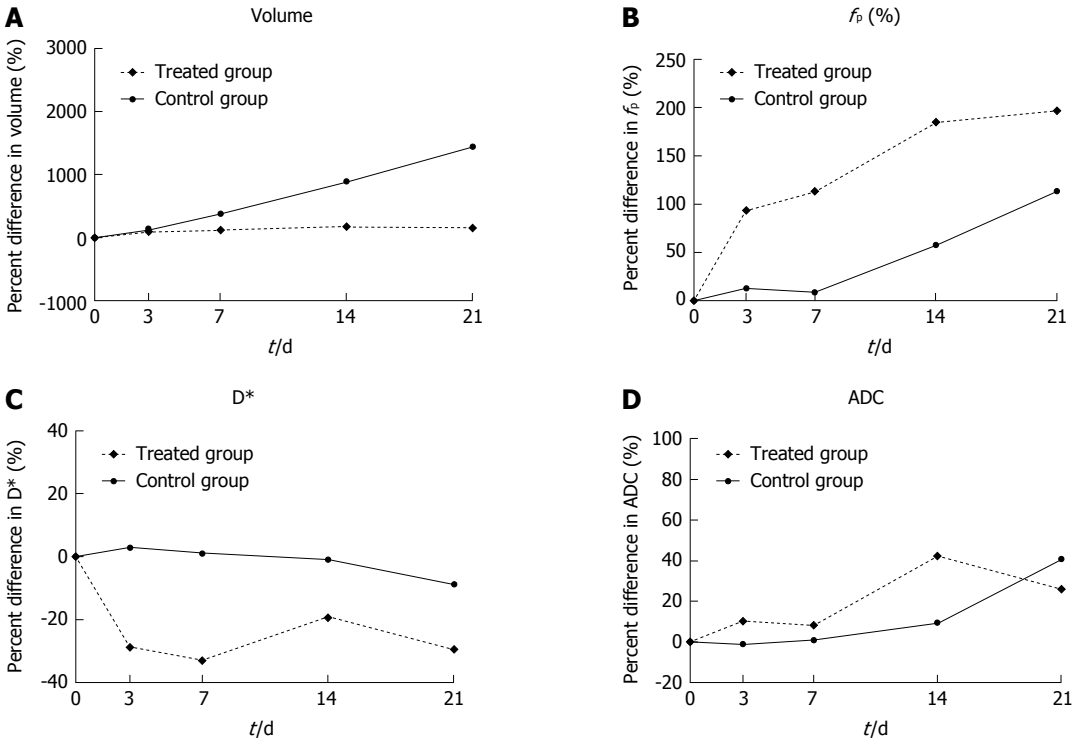

Figure 3 Median percentage change from baseline in tumor volume (A), *fp* (B), D* (C) and ADC (D) values of all animals in the treated groups (dashed line) and control group (solid line), $P < 0.01$.

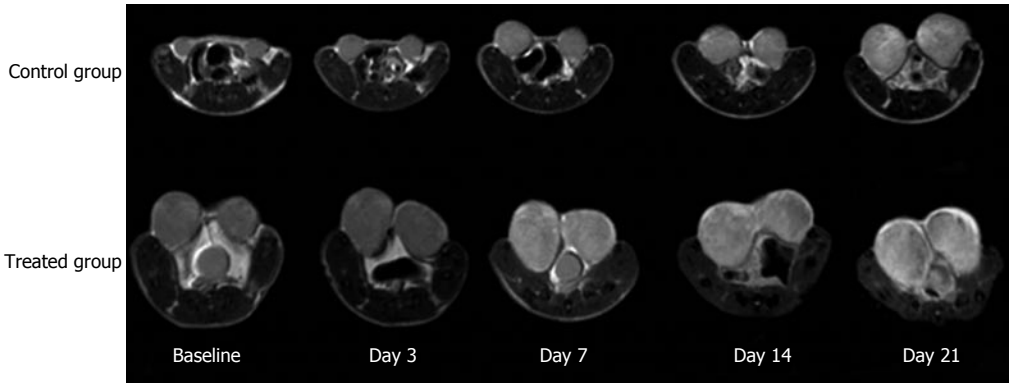

Figure 4 Representative axial T2-weighted images of a control mouse (first row) and a treated mouse (second row) before (baseline) and after treatment (day 3, day 7, day 14 and day 21). Tumor volumes in the control mouse markedly increased on day 14 and day 21. In comparison, tumor volume in the treated mouse did not show an obvious change after treatment.

= 179% and $\Delta f_{pcontrol}$% = 113%), but no significant differences were observed (z = 1.15, P = 0.2484).

The ADC value in the treated groups increased with median percentage changes ($\Delta ADC_{treated}$%) of 11%, 9%, 42%, and 26% on days 3, 7, 14 and 21 after treatment, whereas the ADC in the control group only increased slightly before day 14 ($\Delta ADC_{control}$% = -1%, 1% and 10% on days 3, 7 and 14, respectively), but with a marked increase of 41% on day 21. The difference in ΔADC% between the control and treated groups was significant on day 3 (z = 2.44, P = 0.0147) and day 14 (z = 2.40, P = 0.0164), but not on day 7 (z = 1.40, P = 0.1600) or day 21 (z = -0.10, P = 0.9213).

IVIMDW images and signal intensity decay with increasing b-values were observed in two representative mice in the control and treated group (Figure 5). Tumor areas were clearly delineated in the images without obvious artifacts. Tumor areas showed brighter signals due to more restricted tissue water diffusion compared to the surrounding tissues. The signal-to-noise ratio of the tumor ROI at the highest b value was beyond the background noise; therefore, it was sufficient for bi-exponential fitting (Figure 5B). Within each tumor ROI, DW signal decay was fitted more adequately with the bi-exponential model to calculate the IVIM-related parameters compared to the mono-exponential model (Figure 5B). Faster signal decay in the treated mouse indicated overall increased motion of water molecules

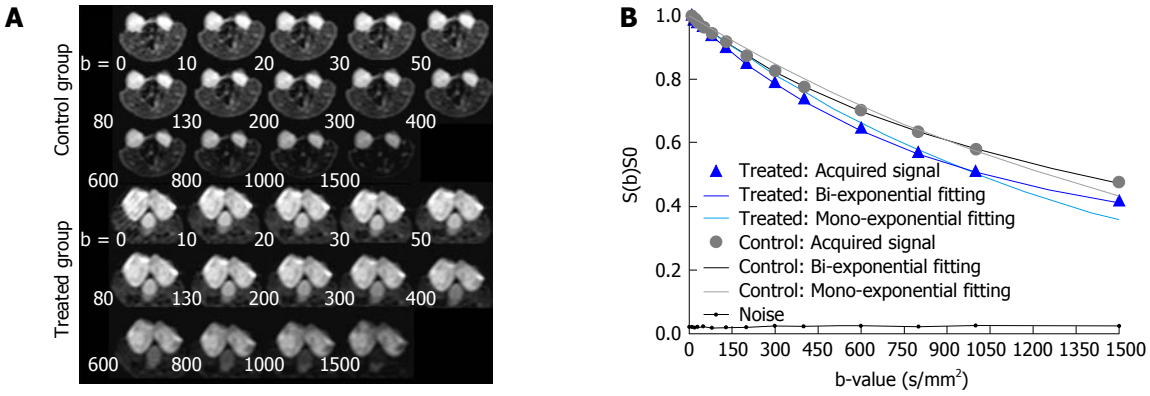

Figure 5 IVIMDW images and signal intensity decay with increasing b-values. A: IVIMDW images acquired at increasing b-values in a representative mouse from the treated group and one from the control group; B: Within the tumor ROI, the averaged signal intensity decay as a function of the b-value is plotted (day 7) by both the bi-exponential and mono-exponential model, respectively. For the treated mouse, D* = 1.27 × 10^{-3} mm^2/s, *fp* = 57.3% and ADC = 0.69 × 10^{-3} mm^2/s. For the control mouse, D* = 2.15 × 10^{-3} mm^2/s, *fp* = 17.2% and ADC = 0.55 × 10^{-3} mm^2/s.

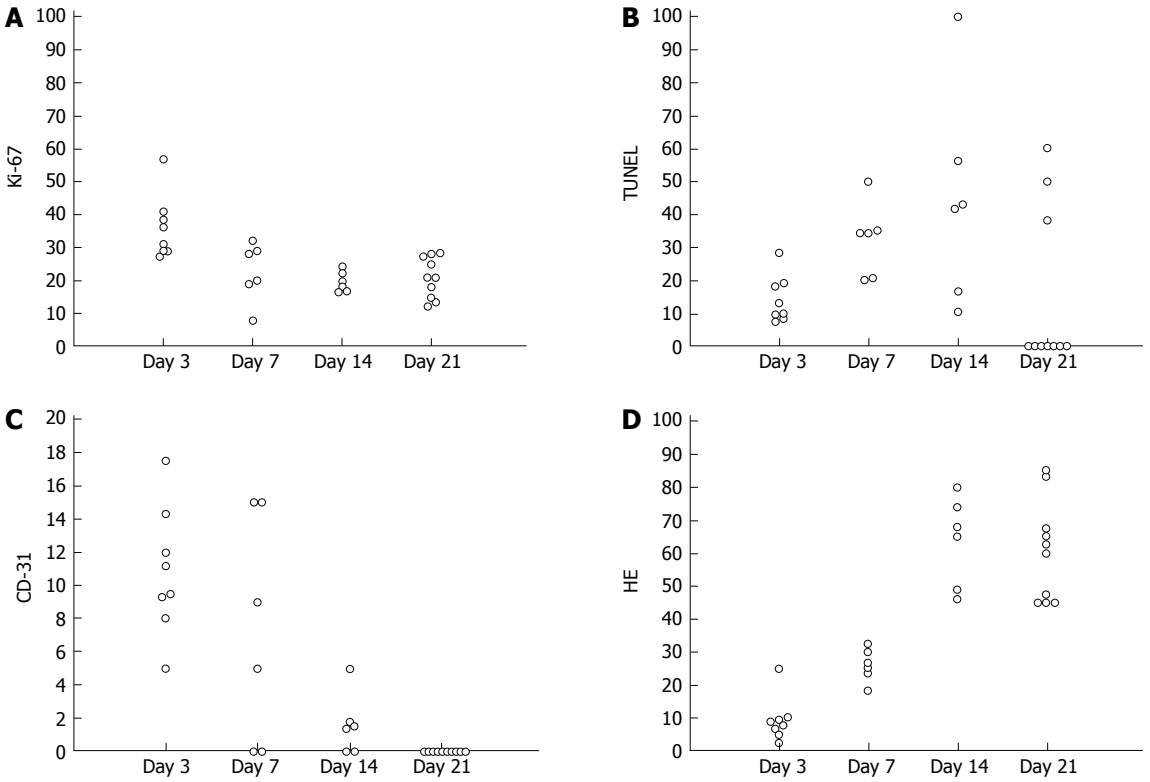

Figure 6 Change in histopathological examinations. A: Proliferating cell density (Ki67 staining); B: Apoptotic cell density (TUNEL staining); C: Microvessel density (CD31 staining); D: Necrosis fraction (HE staining), are plotted at each time point after treatment in the treated group.

and less organized tissue microstructure secondary to treatment.

Histopathological characteristics

The proliferating cells identified on Ki-67 staining showed an apparent decrease at each time point in the treated groups (Figure 6A). The apoptotic activity, quantified as the percentage of cells positive in TUNEL staining, demonstrated a marked increase after treatment (Figure 6B). Vascularity of the endothelium assessed by CD31 staining demonstrated a trend toward reduced MVD after treatment (Figure 6C). The tumor necrotic fraction identified by H&E staining increased markedly after treatment (Figure 6D). In tumor tissues replaced by significant necrosis during the treatment course, non-positive cells were identified in histopathological analyses and are labeled as 0 in Figure 6. Representative histopathological images (Ki-67, TUNEL, CD31 and H&E) of the treated animals revealed tumor tissue changes at each time point during treatment (Figure 7). At the end of the experimental period (day 21), extensive areas of tumor

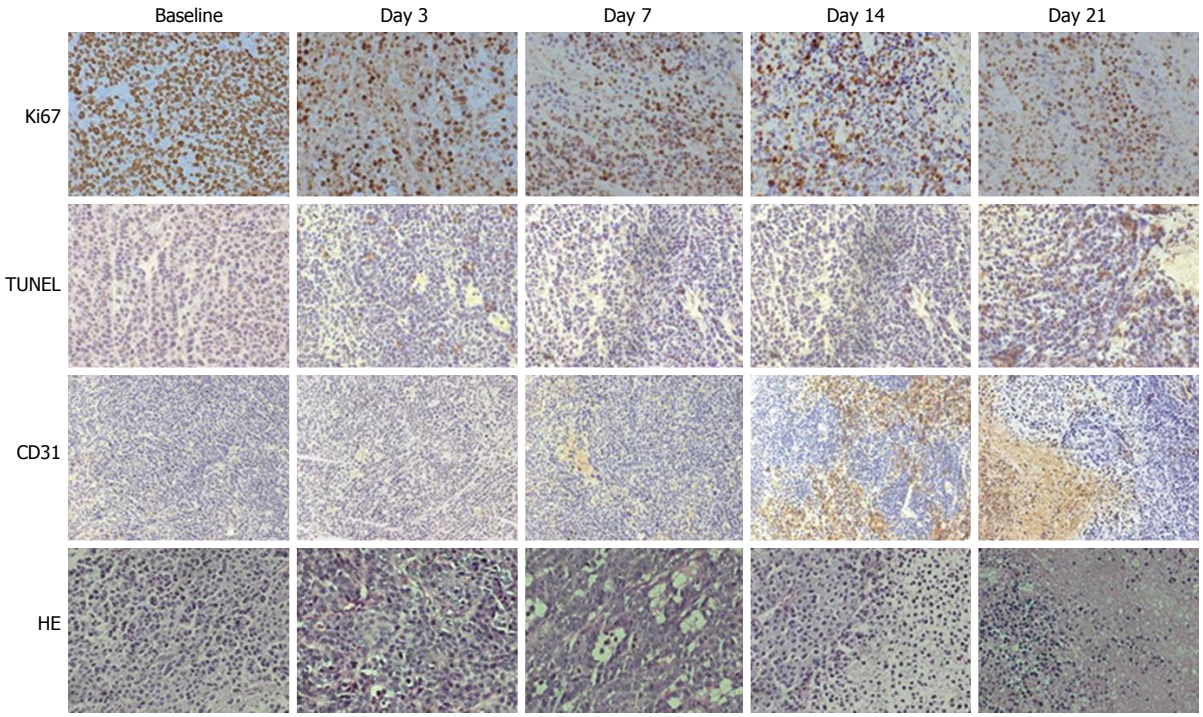

Figure 7 Representative histopathological images of a tumor specimen with Ki-67 staining (first row), TUNEL staining (second row), CD31 staining (third row) and hematoxylin and eosin staining (fourth row) at baseline, day 3, day 7, day 14 and day 21 after treatment in the treated group. Brown staining in the Ki-67 images indicates cells in the proliferative state. Brown staining in the TUNEL images indicates cells undergoing apoptosis. Brown staining in the CD31 images indicates endothelial cells. Viable tumor tissues and necrosis were identified on HE stainings. HE: Hematoxylin and eosin.

necrosis were present in both treated and control mice.

DISCUSSION

In the present DW-MRI study, we found significant changes in the fast diffusion coefficient (D*) and fractional volume (f_p) as early as three days after chemotherapy in a mouse model of gastric adenocarcinoma based on the bi-exponential IVIM model. We demonstrated that IVIM parameters provide critical information in addition to conventional apparent diffusion measurement for the assessment of treatment response. DW-MRI findings correlated well with histopathological changes showing a decrease in proliferating cells and MVD and an increase in apoptosis and tumor necrosis in the treated group.

The IVIM model developed by Le Bihan[8,20] suggests that at the macroscopic level, the capillary network is distributed in space in a pseudorandom manner, and the overall movement of the blood's water molecules within capillaries (*i.e.*, perfusion) mimics the diffusion model. The perfusion-related fast diffusion coefficient D* is considered proportional to the mean capillary segment length and average blood velocity[20]. As reported in previous studies, D* can be used to distinguish benign and malignant salivary gland tumors based on the fact that angiogenesis in malignant tumors leads to increased microscopic blood flow[13]. IVIM DW-MRI has also been used in previous clinical studies to investigate therapeutic responses in different tumor types. Lewin *et al*[17] demonstrated that f_p increased two weeks after treatment of advanced hepatocellular carcinoma with the anti-angiogenic drug sorafenib, whereas changes in the pure diffusion coefficient D and ADC values were not significant. Hauser *et al*[15] evaluated the therapeutic response to radiotherapy combined with chemotherapy and/or targeted therapy with cetuximab in patients with squamous cell carcinomas of the head and neck. Significant increases in f_p, D, and ADC values at 7.5 mo after therapy were demonstrated in patients based on standard clinical progression evaluation criteria. Ganten *et al*[14] reported an increase in D values at week 2 and 4 after treatment with no change in f_p in a rectal cancer study. However, in the aforementioned clinical treatment response assessment studies, a change in D* in response to treatment was not reported. A recent experimental study reported that D* significantly decreased only 4 h after treatment, but recovered to baseline at 24 h in a rabbit VX2 liver tumor model treated with the vascular disrupting agent CKD-516[9]. The variability of IVIM derived measurements in these studies may be attributed to inconsistent IVIM imaging protocols, the range of b-values, and multi-compartment diffusion signal models[4,9].

In our study, we found that D* decreased significantly after treatment, which significantly correlated with decreased MVD revealed by endothelial cell CD31 staining. In addition, increased cellular apoptosis and necrosis in response to treatment may contribute

to changes in both tissue diffusion and perfusion properties. As a consequence, the destruction of tumor microvasculature and decreased cellularity led to the mixture of a fast perfusion component and a slow diffusion component after chemotherapy, which potentially resulted in an increased f_p approaching 50% and indicated equilibrium between perfusion and diffusion components. In contrast, D* was similar to the baseline value, and f_p showed a smaller increase in untreated tumors in the control group.

ADC derived from conventional DW MRI has been widely accepted for evaluating the therapeutic efficacy of chemotherapy, radiotherapy or combined therapy with targeted agents. Consistent with previous studies[19,21,22], our study showed that ADC values increased on day 3 after treatment, while tumor volumes in the treated groups remained stable during the entire treatment period, which indicated that microstructural tumor tissue changes precede changes in tumor size. However, the complex interplay of decreased cell proliferation, increased apoptosis and necrosis, and the destruction of tumor microvasculature may contribute to the variations in ADC after treatment[4,21]. As shown in our study, considerable overlap in ADC was observed between the treated and control groups at each time point after treatment. In comparison, no significant overlap in f_p and D* measurements were observed between the control and treated groups, which provided a more reliable assessment of tumor tissue changes.

At the end of the treatment cycle (day 21), all measurements were similar between the treated and control groups. H&E staining showed that viable tumor tissues were mostly replaced by tumor necrosis, which was induced by chemotherapy in the treated groups or due to progressive growth in the control group. In addition, CD31 staining only identified a few endothelial cells in tumor microvasculature in both the control and treated groups on day 21, indicating the destruction of microvasculature.

The slow diffusion coefficient (D) is generally considered the pure diffusion coefficient describing extracellular and extravascular tissue water mobility[1,3,8]. We observed significant decreases in D (most D values were very close to 0) from day 7 to day 21 after treatment, which did not match the findings of previous studies[14,15]. For this reason, we did not calculate the percentage changes in D values at each time point further as we did with the other IVIM parameters shown in the results. Our explanation for this finding is that D in the two-compartment model may not represent real physiological changes, but may be a covariate parameter in the process of iterative converges of the nonlinear bi-exponential signal fitting. As discussed above, destruction of the tumor microenvironment after chemotherapy leads to a mixture of perfusion and diffusion components and thus can cause a deviation from the assumption of bi-exponential signal decay. Therefore, the term $V_{slow} \times e^{-bD}$ may become a constant term, meaning that D will need to decrease to a very small number so that the signal decay begins to follow a mono-exponential model. In this situation, the resultant D values may not reflect true physiological changes when it reaches a number close to zero. In order to measure more robust slow diffusion coefficients, we performed linear fitting of the logarithm DW signal decay with b-value = 200 and 1500 s/mm^2, where the perfusion effect can be ignored. We found that the slow diffusion coefficient derived from linear fitting of the high b-value DW signals increased after treatment (data not shown), similar to the ADC changes.

This study had some limitations. First, the sample size in each treated group was relatively small. Second, the bi-exponential IVIM-DWI model was the only model used to characterize the complicated tissue water mobility. There are several sophisticated diffusion models that have been investigated for multi-b-value signal fitting, such as the stretched exponential model, the diffusion kurtosis model, and the fractional order calculus (FROC) model[16,17]. The stretched model yields the intravoxel diffusion heterogeneity index and distributed diffusion coefficient. Similarly, the FROC model describes the anomalous diffusion process and yields a new set of parameters including the fractional order derivative in space (β) and a spatial parameter (μ). The value of β is mathematically equivalent to the heterogeneity index generated by the stretched model. We also used the FROC model to fit the diffusion decay; however, the changes in both β and μ were not stable and did not show differences between the treated and control groups during the course of the study (data not shown).

In addition, a recent study[23] suggested that although these sophisticated models may provide more information on tissue water diffusion, their biologic interpretation requires further refinement and they are not directly correlated with pathological characteristics. In contrast, our calculations based on the bi-exponential model were based on the theory that it might allow separation of water molecular diffusion from the microcirculation. Our results demonstrated that the fast diffusion coefficient and fractional volume provided valuable information reflective of tissue microvasculature changes secondary to chemotherapy and correlated well with each specific pathological finding. Interestingly, a recent study[24] compared a DW signal fitting plot using all these diffusion models, which clearly demonstrated that the bi-exponential model had the best fit of the signal decay with b < 1500 s/mm^2. Finally, the bi-exponential model was considered to be the most appropriate model for this study. Nonetheless, all of these diffusion models should be investigated further to compare their respective accuracies regarding tumor characterization and treatment response in future studies.

The present study demonstrated that the IVIM-derived tissue perfusion related diffusion coefficient and fraction parameters provided valuable information reflective of tissue microstructural and microvasculature

changes secondary to chemotherapy. The fast diffusion coefficient (D*) decreased, whereas the fast diffusion fractional volume (f_p) increased immediately after treatment and throughout the treatment course. ADC increased in both the treated and control groups with greater increases in the treated groups. No significant overlaps were observed in D* and f_p measurements between the treated and control groups, whereas ADC showed more overlap.

In conclusion, IVIM perfusion measurements offer a potentially accurate evaluation of chemotherapy efficacy and may facilitate individualized treatment planning and prompt treatment adjustment in gastric cancer patients.

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COMMENTS

Background

Early detection and accurate assessment of tumor response to chemotherapy is essential for individualized treatment planning in patients with gastric cancer.

Research frontiers

In a mouse gastric cancer model, the authors demonstrated that the intravoxel incoherent motion (IVIM)-derived tissue perfusion coefficient (D*) decreased, whereas the perfusion fraction (PF) increased immediately after chemotherapy and throughout the treatment course.

Innovations and breakthroughs

No significant overlaps were observed in D* and PF measurements between the treated and control groups; such overlaps were observed using the conventional apparent diffusion coefficient measurements.

Applications

IVIM-derived perfusion measurements offer the potential of accurate evaluation of chemotherapeutic efficacy. This imaging study is ready to be translated into a clinical study that may facilitate individualized treatment planning and prompt treatment adjustment in gastric cancer patients.

Terminology

The IVIM model developed by Le Bihan suggests that at the macroscopic level, the capillary network is distributed in space in a pseudorandom manner, and the overall movement of the blood's water molecules within capillaries (*i.e.*, perfusion) mimics the diffusion model. Consequently, IVIM diffusion-weighted magnetic resonance imaging (DW-MRI) allows the following to be calculated noninvasively without contrast agent administration: the "pure" (slow) diffusion coefficient (D), the pseudo-perfusion (fast) diffusion coefficient (D*), and the fractional volume (f_p).

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DW-MRI has emerged as a noninvasive imaging method, which exploits tissue water mobility reflective of the microstructural properties of tumor tissue and detects tissue changes after treatment. It was the aim of the authors to exploit IVIM-DW-MRI with a biexponential model for chemotherapy response evaluation in a gastric cancer mouse model. The authors demonstrated that in a mouse gastric cancer model the IVIM-derived tissue perfusion coefficient (D*) decreased whereas the PF increased immediately after chemotherapy and throughout the treatment course as well.

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ORIGINAL ARTICLE

Basic Study

Antioxidant axis Nrf2-keap1-ARE in inhibition of alcoholic liver fibrosis by IL-22

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Abstract

AIM

To explore the effect of interleukin (IL)-22 on *in vitro* model of alcoholic liver fibrosis hepatic stellate cells (HSCs), and whether this is related to regulation of Nrf2-keap1-ARE.

METHODS

HSC-T6 cells were incubated with 25, 50, 100, 200 and 400 μmol/L acetaldehyde. After 24 and 48 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to detect proliferation of HSCs to choose the best concentration and action time. We used the optimal concentration of acetaldehyde (200 μmol/L) to stimulate HSCs for 24 h, and treated the cells with a final concentration of 10, 20 or 50 ng/mL IL-22. The cell proliferation rate was detected by MTT assay. The cell cycle was analyzed by flow cytometry. The expression of nuclear factor-related factor (Nrf)2 and α-smooth muscle antigen was detected by western blotting and immunocytochemistry. The levels of malondialdehyde (MDA) and glutathione (GSH) were measured by spectrophotometry.

RESULTS

In the MTT assay, when HSCs were incubated with acetaldehyde, activity and proliferation were higher than in the control group, and were most obvious after 48 h treatment with 200 μmol/L acetaldehyde. The number of cells in G0/G1 phases was decreased and the number in S phase was increased in comparison with the control group. When treated with different concentrations of IL-22, HSC-T6 cell activity and proliferation rate were markedly decreased in a dose-dependent manner, and cell cycle progression was arrested from G1 to S phase. Western blotting and immunocytochemistry demonstrated that expression of Nrf2 total protein was not significantly affected. Expression of Nrf2 nuclear protein was low in the

control group, increased slightly in the model group (or acetaldehyde-stimulated group), and increased more obviously in the IL-22 intervention groups. The levels of MDA and GSH in the model group were significantly enhanced in comparison with those in the control group. In cells treated with IL-22, the MDA level was attenuated but the GSH level was further increased. These changes were dose-dependent.

CONCLUSION

IL-22 inhibits acetaldehyde-induced HSC activation and proliferation, which may be related to nuclear translocation of Nrf2 and increased activity of the antioxidant axis Nrf2-keap1-ARE.

Key words: Interleukin-22; Alcoholic liver fibrosis; Hepatic stellate cells; Nrf2; Oxidative stress

Core tip: We successfully established an *in vitro* cell model of alcoholic liver fibrosis (ALF). We investigated the influence of interleukin (IL)-22 on ALF and the possible mechanism involved. To our knowledge, this is the first study to confirm the inhibitory effect of IL-22 on ALF at the cellular level. We found that the effect was at least partly related to promotion of nuclear translocation of nuclear factor-related factor (Nrf)2 and increased activity of the antioxidant axis Nrf2-keap1-ARE. We aimed to provide a new target for research on ALF and new drug development.

Ni YH, Huo LJ, Li TT. Antioxidant axis Nrf2-keap1-ARE in inhibition of alcoholic liver fibrosis by IL-22. *World J Gastroenterol* 2017; 23(11): 2002-2011 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i11/2002.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i11.2002

INTRODUCTION

Alcoholic liver fibrosis (ALF) is a wound-healing response to prolonged alcoholic liver injury, which is characterized by excessive extracellular matrix accumulation. ALF is a turning point in the development of alcoholic liver disease (ALD), so treatment of ALF has become the focus of clinical research on ALD[1,2]. As well as other causes of liver fibrosis, activation and proliferation of hepatic stellate cells (HSCs) are key factors in fibrogenesis. Previous studies have found that acetaldehyde, the most harmful metabolite of alcohol, can trigger HSC activation and proliferation in alcoholic liver injury *via* inducing oxidative stress[3,4], whereas inhibiting oxidative stress or enhancing antioxidant capacity can reverse the activation and proliferation of HSCs induced by acetaldehyde.

Nuclear factor-related factor (Nrf)2 has a molecular weight of 66 kDa and was discovered by Moi *et al*[5] in 1994. Under normal conditions, cytoplasmic Nrf2 mostly combines with its binding protein Kelch-like ECH-associated protein (keap1) and is in an inactive state. Oxidative stress or stimulation of nucleophilic substances may trigger dissociation of Nrf2 from keap1 and release free Nrf2. Following dissociation, Nrf2 is rapidly translocated to the nucleus and transactivates the antioxidant response element (ARE) in the promoter region of many antioxidant genes, triggering transcription of downstream target genes (*e.g.*, *GSH* and *HO-1*). Thus, Nrf2 is the key regulatory factor in oxidative stress[6,7]. A significant body of evidence suggests that upregulation of Nrf2 or promotion of Nrf2 nuclear translocation can delay the progression of ALF[8,9]. Nrf2 promises to be one of the most important areas of research investigating the formation of ALF.

Interleukin (IL)-22 (also known as IL-10-related T-cell-inducible factor) is a member of the IL-10 cytokine family. It is secreted by T helper (Th)1, Th17 and Th22 cells and natural killer (NK)/NKT cells. By binding to a heterodimeric receptor complex IL-22R1/IL-10R2, IL-22 initiates the JAK/STAT3 signaling pathway[10]. IL-22 is one of the major inflammatory mediators associating with organ fibrosis, especially in the lungs and kidneys[10,11]. Previous animal and clinical research has shown that IL-22 and IL-22R1 expression is significantly increased in ALF, suggesting that IL-22 is involved in the process of ALF. This effect could be related to the promotion of liver progenitor cell/hepatocyte proliferation, inhibition of hepatocyte apoptosis, upregulation of metallothionein and glutathione (GSH) expression[12-14]. However, the activity of the antioxidant axis Nrf2-keap1-ARE in HSCs has not been reported to date.

Therefore, in the present study we used acetaldehyde as a stimulator of HSC-T6 cells to establish a model of ALF *in vitro*. Different concentrations of IL-22 were added to the culture, and the proliferation rate and activity of HSCs were detected, as well as nuclear translocation of Nrf2.

MATERIALS AND METHODS

Materials

HSC-T6 cells were purchased from the cell bank of the Central South University (Hunan, China). Acetaldehyde (40%) was purchased from Tianjin DaMao Chemical Reagents (Tianjin, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from Sijiqing (Hangzhou, China). Fetal calf serum (10%) was purchased from Hyclone (Logan, UT, United States). Recombinant mouse IL-22 was purchased from R&D Systems (Minneapolis, MN, United States). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit and nuclear-cytoplasmic protein extraction kit were purchased from Boster Bioengineering Company (Wuhan, China). Malondialdehyde (MDA) and GSH kits were purchased from Jiancheng Company (Nanjing, China). α-smooth muscle actin (SMA) and Nrf2 polyclonal antibody were

purchased from Abcam (Cambridge, MA, United States). Horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies were purchased from Boster Bioengineering.

Cell culture and grouping

Passaged and activated HSCs were seeded into 25-cm^2 sealable flasks and grown until the monolayers were 75%-80% confluent. HSCs were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 ℃ in a humidified incubator with 5% CO_2, until the cells showed adherent growth.

There were five groups of cells: normal control group; model group; and high-, medium- and low-dose IL-22 intervention groups. The normal control group was cultured in conventional DMEM for 48 h; for the model group, 200 μmol/L acetaldehyde was added to the DMEM for 48 h; and the IL-22 intervention groups were co-incubated with 200 μmol/L acetaldehyde and different concentrations (10, 20 or 50 ng/mL) of IL-22 for 24 h after pretreatment with 200 μmol/L acetaldehyde for 24 h.

Proliferation of HSCs was detected by MTT assay

HSC-T6 cells were seeded at 5 × 10^4/mL in a 96-well plate and incubated in 100 μL culture medium overnight. Six wells were used for each group. The outer wells were filled with sterile PBS. When the monolayers of HSC-T6 cells were 70%-80% confluent, DMEM with 10% fetal bovine serum was replaced by serum-deprived DMEM to synchronize the cells. We treated HSC-T6 cells with acetaldehyde at 25, 50, 100, 200 or 400 μmol/L for 24 or 48 h to establish an *in vitro* model of ALF. After treatment with acetaldehyde, 10 μL of 5 mg/mL MTT solution was added to form purple formazan. Subsequently, 100 μL formazan dissolving liquid was added to dissolve the formazan crystals. Results were measured using a microplate reader at an absorbance of 570 nm, and the 50% effective concentration (EC_{50}) value was obtained from the MTT viability growth curve. The HSC proliferation rate was calculated as follows: (OD of treated wells/OD of control wells) × 100%. Experiments were repeated three times and in triplicate.

We used the same method to test the effect of IL-22 on acetaldehyde-stimulated HSC-T6 cells. The cells were co-cultured with IL-22 at a final concentration of 10, 20 or 50 ng/mL after 24 h pretreatment with 200 μmol/L acetaldehyde. The OD was measured at 570 nm. The inhibitory rate was calculated as follows: [1 - (OD of treated wells/OD of model wells)] × 100%. Experiments were repeated three times and in triplicate.

Flow cytometric analysis of cell cycle distribution

Logarithmic growth phase HSC-T6 cells were inoculated in 6-well plates. When the monolayers were 70%-80% confluent, DMEM with 10% fetal bovine serum was replaced by serum-deprived DMEM to synchronize the cells, and different interventional treatments were added to the culture medium according to the experimental group. After treatment, HSC-T6 cells were trypsinized and resuspended in their original culture medium. The cells were harvested, washed and suspended in PBS twice, fixed in 70% ethanol at -20 ℃ overnight, and stained in 500 μL PBS containing propidium iodide (PI) (200 mg/mL RNase A + 50 μg/mL PI) at 37 ℃ for 30 min in the dark. Analysis was performed on a Cytomics FC500 flow cytometer (Beckman Coulter Inc., Brea, CA, United States).

Western blotting of expression of α-SMA and Nrf2 protein

After treatment, HSC-T6 cells were harvested by scraping the cells from the culture dishes. Cell lysates were prepared using nuclear and cytoplasmic extraction kits for the detection of Nrf2 and α-SMA proteins. Protein concentrations were determined by the BCA Protein Assay Kit (Boster Bioengineering Company). After separation by 10% SDS-PAGE (140 V for 55 min), the proteins (60 μg) were transferred onto a PVDF membrane. The blots were incubated overnight at 4 ℃ with primary antibodies diluted with TBS solution containing 0.05% Tween 20 (TBST) after 5% nonfat milk blocking for 3 h at room temperature. The α-SMA antibody was diluted to 1:500, Nrf2 antibody to 1:1000, and β-actin antibody to 1:6000. On the next day, the membranes were washed with TBST three times and probed for 1 h with HRP-conjugated goat anti-rabbit IgG antibody (1:3000). TBST washing was repeated, and the immunoreactive band intensities were measured by grey intensity analysis using ImageJ software, and the gray values of β-actin protein bands were used to normalize the gray values of each target protein. All experiments were performed at least three times.

Protein localization of α-SMA and Nrf2 evaluated by immunocytochemistry

HSC-T6 cells were seeded at 2 × 10^4/mL, with 0.5 mL/well on coverslips in 24-well plates. Six wells were used for each group. Different interventional treatments were added to the culture medium according to the experimental group. After treatment, coverslips were removed, washed with PBS, fixed in 4% polyformaldehyde, and membranes were disrupted with 0.1% Triton-X100. Then, endogenous peroxidase was blocked by 3% H_2O_2 and target proteins nonspecific binding by 5% goat serum (every two steps included washing with PBS three times for 2 min each), primary rabbit polyclonal anti-α-SMA (1:100) and anti-Nrf2 (1:200) were applied and incubated in a humidified chamber overnight at 4 ℃, followed by biotinylated goat anti-rabbit secondary antibody

Table 1 Effects of acetaldehyde on hepatic stellate cells-T6 cell proliferation for 24 h and 48 h, and maximum OD value after exposure to 200 μmol/L acetaldehyde

Concentration of acetaldehyde (μmol/L)	24 h		48 h	
	OD	Proliferation rate	OD	Proliferation rate
0	0.2743 ± 0.0134		0.4154 ± 0.0130	
25	0.3409 ± 0.0118[a]	124%	0.4983 ± 0.0258[a]	120%
50	0.3732 ± 0.0152[a]	136%	0.5733 ± 0.0208[a]	138%
100	0.3807 ± 0.0157[a]	139%	0.6181 ± 0.0351[a]	149%
200	0.5019 ± 0.0098[a]	183%	0.7649 ± 0.0333[a]	184%
400	0.4524 ± 0.0104[a]	165%	0.7069 ± 0.0265[a]	170%

Data are expressed as mean ± SD. [a]$P < 0.05$ *vs* the control group.

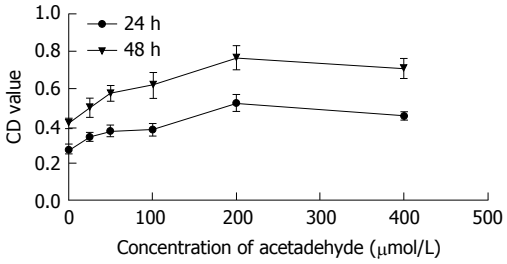

Figure 1 Effects of acetaldehyde for 24 or 48 h on HSC-T6 cell proliferation.

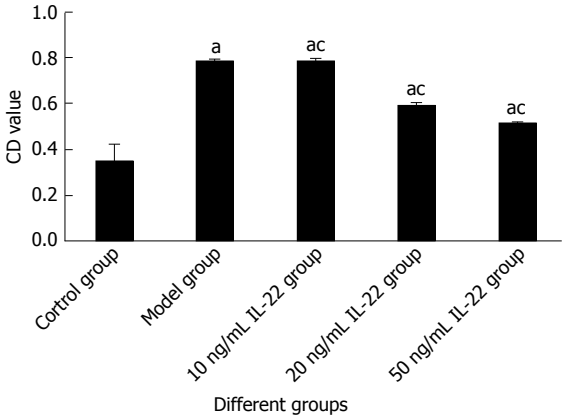

Figure 2 Effects of interleukin-22 on HSC-T6 cells proliferation. [a]$P < 0.05$ *vs* the control group; [c]$P < 0.05$ *vs* the model group. HSCs: Hepatic stellate cells; IL: Interleukin.

for 1 h at room temperature. After rinsing with PBS, the coverslips were counterstained by hematoxylin and dehydrated. The signal was visualized by light microscopy and analyzed by measuring the OD of positive staining using the Scanscope Digital Pathology Scanning System (Aperio; Leica Biosystems, Wetzlar, Germany).

Spectrophotometry of MDA and GSH

Logarithmic growth phase HSC-T6 cells were inoculated in 6-well plates, and different interventional treatments were added to the culture medium according to the experimental group, with each group repeated in three wells. Cell supernatants were collected and centrifuged at 3000 × *g* for 10 min. Then blank pipes, standard pipes and experiment pipes were set up according to MDA, GSH kit instruction. The OD value was measured at 532 and 420 nm respectively, and the content of MDA (nmol/L) and GSH (NU/L) were calculated.

Statistical analysis

SPSS version 17.0 was used for all statistical analysis and data were expressed as mean ± SD. If the data showed a normal distribution and homogeneity of variance, one-way ANOVA was used to assess the significance of differences between the mean values. Multiple pair-wise comparisons were conducted using the least significant difference (LSD). Otherwise, Welch's test was used to assess the significance of differences between the mean values. Multiple pairwise comparisons were conducted using Dunnett's T3 test. The difference was statistically significant at $P < 0.05$.

RESULTS

Effects of IL-22 on HSC proliferation

Compared with the control group, OD was significantly increased after stimulation with the graded concentrations of acetaldehyde. According to the growth curve, EC_{50} = 100 μmol/L was obtained. At this concentration, we found that OD reached a maximum at 48 h after exposure to 200 μmol/L acetaldehyde. Then, we determined the concentration and incubation time that best mimicked HSC activation seen in an *in vivo* model of ALF (Table 1 and Figure 1). In the experiment group, intervention with different concentrations of IL-22 significantly reduced OD. There was a negative correlation between OD and dose of IL-22 (Table 2 and Figure 2).

Cell cycle distribution of HSCs

After stimulation with acetaldehyde at the optimal concentration and duration, HSCs were more abundant in S phase (42.94%) and less abundant in G1 phase (51.41%) in comparison with the control group (40.25% and 52.01%, respectively), indicating that HSCs were activated and proliferated. The percentage of HSCs in S phase gradually decreased with increased concentrations of IL-22, while the percentage in G1

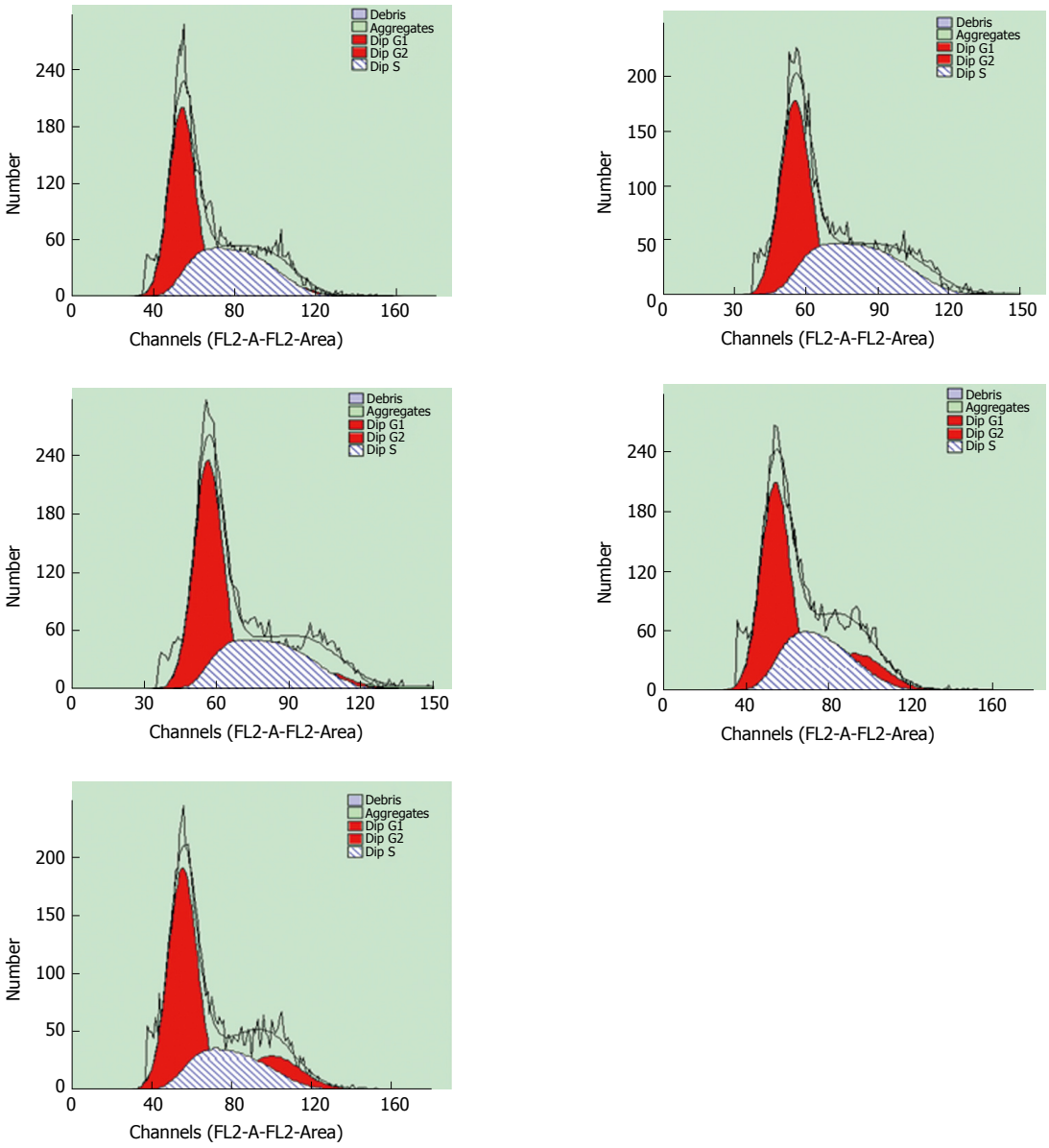

Figure 3 Effects of interleukin-22 on the cell cycle distribution of HSC-T6 cells. HSCs: Hepatic stellate cells; IL: Interleukin.

phase was gradually increased in a dose-dependent manner when compared with the model group (Figures 3 and 4).

Effects of IL-22 on expression of α-SMA and Nrf2 protein

In the control group, there was basic expression of α-SMA. In comparison, the expression of α-SMA was significantly increased in the model group. However, α-SMA expression was gradually reduced as IL-22 concentration increased, and the difference was significant in comparison with the model group.

Expression of Nrf2 total protein showed no significant difference between all the groups. Compared with the control group, expression of Nrf2 nuclear protein was increased slightly in the model group, and increased significantly in the IL-22 intervention groups, in a dose-dependent manner (Figures 5 and 6).

Expression of α-SMA and Nrf2 protein detected by immunocytochemistry

HSC-T6 cells with α-SMA-positive expression had brown granules in the cytoplasm, although the degree of staining was varied. In the control group, the staining of the cytoplasm was light brown, whereas it was obviously deepened in the model group. Treatment with different concentrations of IL-22 gradually reduced the depth of brown staining in the cytoplasm in a dose-dependent manner. The difference was significant (Figure 7).

For expression of Nrf2 nuclear protein, immunocytochemistry showed that in the IL-22 intervention groups, the positive rate of nuclear staining was higher than in the model group (27.8%, 62.5% and 84.6% in the low-, middle- and high-dose of IL-22 intervention groups and 16.7% in the model group), whereas there was rarely positive nuclear staining in the control

Table 2 Effects of interleukin-22 on the acetaldehyde-induced HSC-T6 cells proliferation

Group	OD	Proliferation inhibition rate
Control group	0.3854 ± 0.0366	
Model group	0.7860 ± 0.0129[a]	-
10 ng/mL IL-22 group	0.6761 ± 0.0129[ac]	14%
20 ng/mL IL-22 group	0.5903 ± 0.0164[ac]	25%
50 ng/mL IL-22 group	0.5095 ± 0.0101[ac]	35%

OD was significantly decreased along with increased IL-22 dose, indicating that IL-22 dose-dependently inhibited proliferation of HSCs. Data are expressed as mean ± SD. [a]$P < 0.05$ *vs* the control group; [c]$P < 0.05$ *vs* the model group. HSCs: Hepatic stellate cells; IL: Interleukin.

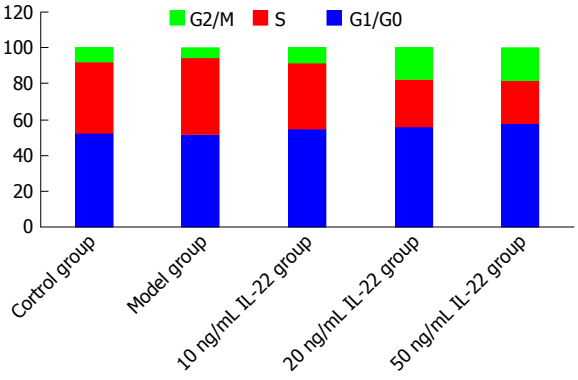

Figure 4 Cell cycle distribution of HSC-T6 cells in different groups. HSCs: Hepatic stellate cells.

group (7.6%) (Figures 8 and 9).

MDA and GSH in the supernatant determined by spectrophotometry

Compared with the control group, the content of MDA and GSH was increased in the model group. When cells were treated with different concentrations of IL-22, the MDA level was attenuated and the content of GSH was further elevated, both in a dose-dependent manner. The differences were significant (Table 3, Figures 10 and 11).

DISCUSSION

ALD is a chronic liver injury caused by long-term heavy alcohol drinking. Its pathological stages comprise steatosis (alcoholic fatty liver), steatohepatitis (alcoholic hepatitis) and liver fibrosis/cirrhosis[15-17]. ALF is regarded as a turning point in the development and progression of ALD, so timely treatment of ALF can reverse ALD[1,2]. Previous research has shown that, as the principal metabolite of ethanol, acetaldehyde triggers activation and proliferation of HSCs, which is the central feature of ALF[3,4]. α-SMA is the most important activation marker of HSCs; it is mostly expressed in activated HSCs and can be used to assess the extent of liver fibrosis[18-20]. As our results demonstrated, after stimulation by different concentrations of acetaldehyde for different times, the

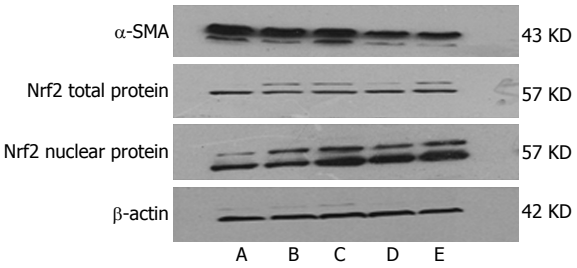

Figure 5 Effects of interleukin-22 on expression of α-smooth muscle antigen and nuclear-factor-related factor 2 protein in acetaldehyde-induced HSC-T6 cells. A: Control group; B: Model group; C: 10 ng/mL IL-22 group; D: 20 ng/mL IL-22 group; E: 50 ng/mL IL-22 group. IL: Interleukin; SMA: Smooth muscle antigen; Nrf2: Nuclear-factor-related factor 2.

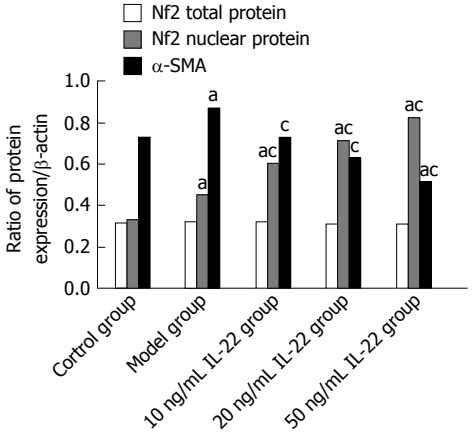

Figure 6 Effects of interleukin-22 on the expression of target protein. [a]$P < 0.05$ *vs* the control group; [c]$P < 0.05$ *vs* the model group. IL: Interleukin; Nrf2: Nuclear-factor-related factor 2; α-SMA: α-smooth muscle antigen.

proliferation rate of HSCs was significantly increased. It was most obvious when 200 μmol/L acetaldehyde was added for 48 h, which agreed with previous studies[21-23] and these conditions were used for later model establishment. We demonstrated by flow cytometry that acetaldehyde markedly decreased HSCs in G0/G1 phases but increased the HSCs in S phase. Western blotting and immunocytochemistry also showed that, in response to acetaldehyde-induced stimulation, expression of α-SMA was obviously increased, adding to a growing body of evidence about the successful establishment of an *in vitro* model of ALF.

Oxidative stress is one of the clearly important mechanisms of ALF, which is characterized by accumulation of lipid peroxidation product MDA and depletion of endogenous antioxidant GSH[3,4]. A large body of evidence[24,25] has demonstrated that acetaldehyde serves as one of the most important stimulants of ALF by triggering oxidative stress. However, reducing oxidative stress blocks HSC activation and proliferation, as well as prevents ALF. Our results also showed that compared with the control group, the contents of MDA and GSH were significantly increased in the model group. This shows that lipid peroxidation and antioxidant capacity are synchronously activated in the early stage

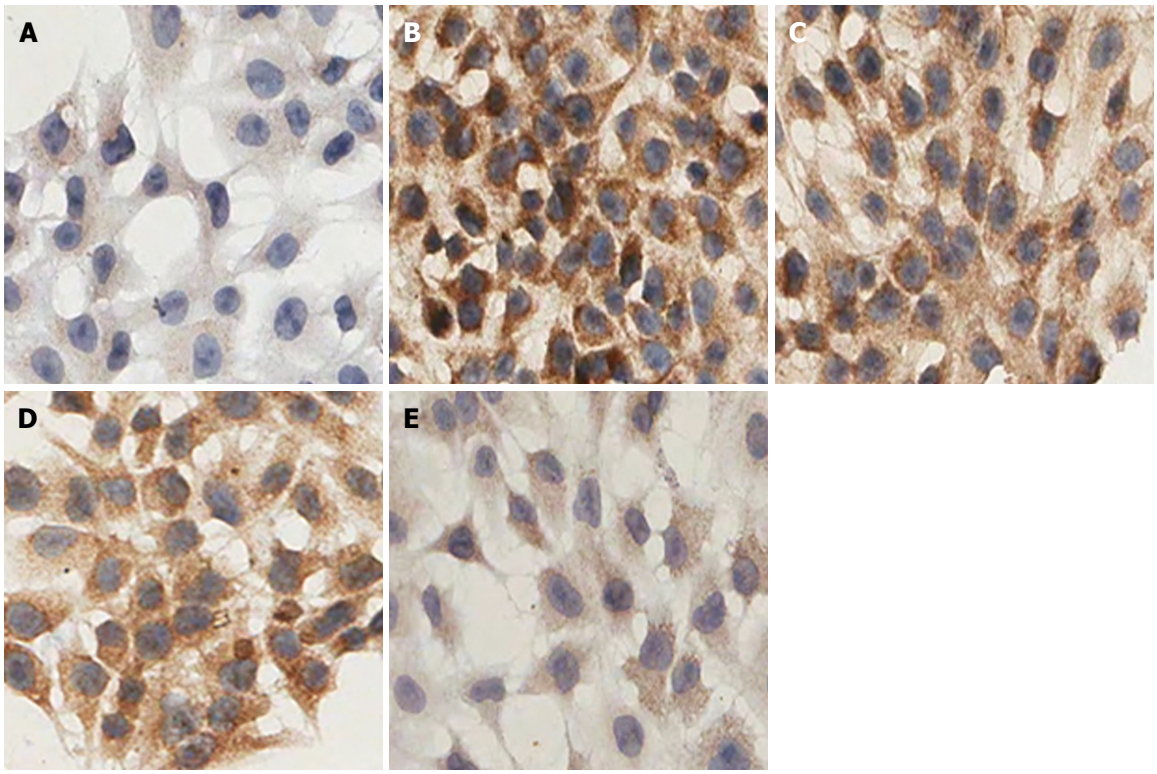

Figure 7 Effects of interleukin-22 on expression of α-smooth muscle antigen (magnification × 400). A: Control group; B: Model group; C: 10 ng/mL IL-22 group; D: 20 ng/mL IL-22 group; E: 50 ng/mL IL-22 group. All HSC-T6 cells with α-SMA positive expression had brown granules in the cytoplasm, however, the degree of staining varied. In the control group, the cytoplasm was stained light brown, whereas it was obviously deepened in the model group. When treated with different concentrations of IL-22, the staining in the cytoplasm was gradually reduced in a dose-dependent manner. IL: Interleukin; SMA: Smooth muscle antigen.

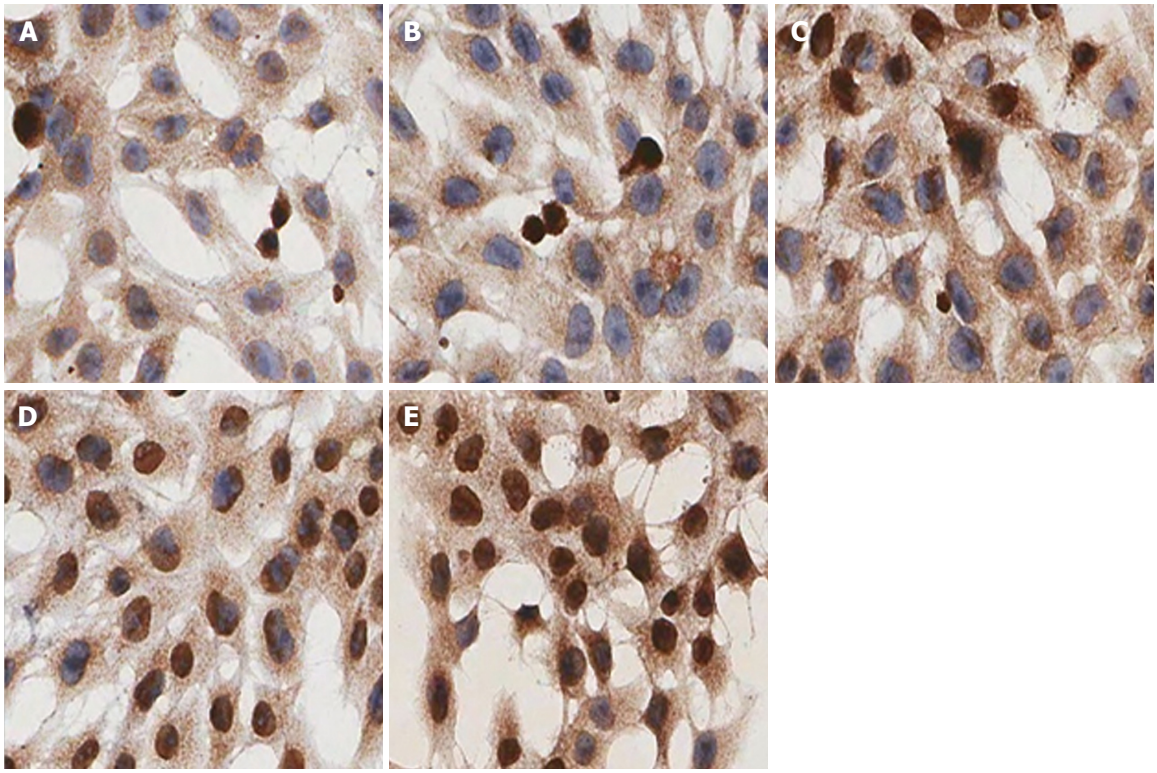

Figure 8 Effects of interleukin-22 on nuclear translocation of nuclear-factor-related factor 2. A: Control group; B: Model group; C: 10 ng/mL IL-22 group; D: 20 ng/mL IL-22 group; E: 50 ng/mL IL-22 group. Magnification: × 400. IL: Interleukin; SMA: Smooth muscle antigen.

Table 3 Content of malondialdehyde and glutathione in the supernatant

Group	Acetaldehyde (μmol/L)	IL-22 (ng/mL)	MDA (nmol/mL)	GSH (NU/L)
Control group	0	0	1.3654 ± 0.1022	12.8569 ± 0.3699
Model group	200	0	7.5073 ± 0.6126[a]	24.6972 ± 0.5043[a]
IL-22 groups				
Low	200	10	5.1256 ± 0.3835[ac]	33.8866 ± 2.6506[ac]
Medium	200	20	3.3195 ± 0.2963[ac]	44.5935 ± 4.1386[ac]
High	200	50	2.8141 ± 0.0720[ac]	55.6834 ± 1.1671[ac]

Data are expressed as mean ± SD. [a]$P < 0.05$ *vs* the control group; [c]$P < 0.05$ *vs* the model group. GSH: Glutathione; MDA: Malondialdehyde; IL: Interleukin.

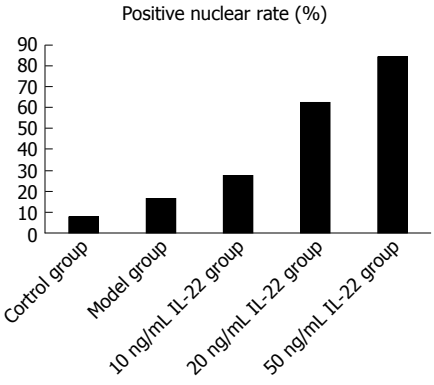

Figure 9 Effects of interleukin-22 on positive rate of nuclear-factor-related factor 2 nuclear staining. IL: Interleukin.

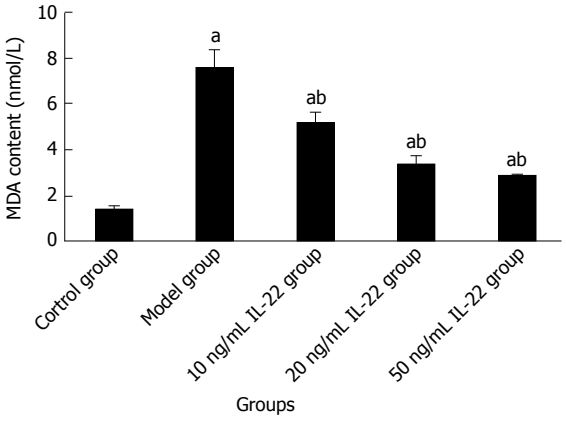

Figure 10 Content of malondialdehyde in different groups. [a]$P < 0.05$ *vs* the control group; [b]$P < 0.05$ *vs* the model group. MDA: Malondialdehyde; IL: Interleukin.

of ALF. As the disease progresses, the antioxidant system is exhausted, which leads to the persistence of oxidative stress and formation of liver fibrosis. So, focusing on the relationship between oxidative stress and fibrogenesis is important to ALF treatment.

Nrf2 is an important transcription factor in the process of antioxidative stress response[6,7]. Under basal conditions, Nrf2 is constitutively sequestered in the cytoplasm by its binding protein keap1, which causes proteasomal degradation of Nrf2. However, under conditions of oxidative stress, the ubiquitination degradation pathway of Nrf2 is inhibited, resulting in cytoplasmic Nrf2 deposition. Free Nrf2, dissociated

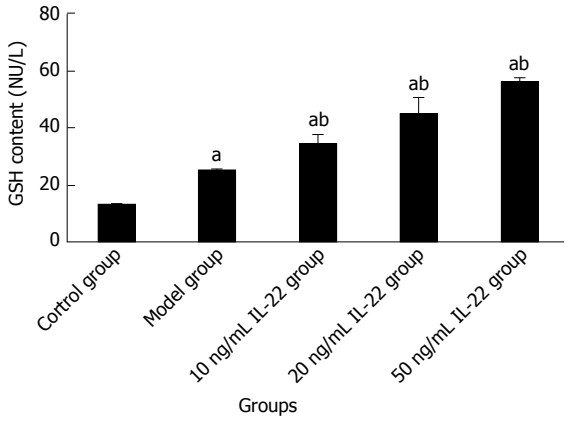

Figure 11 Content of glutathione in different groups. [a]$P < 0.05$ *vs* the control group; [b]$P < 0.05$ *vs* the model group. GSH: Glutathione; IL: Interleukin.

from keap1, moves into the nucleus, combines with ARE, and serves as a transcriptional factor to regulate the expression of corresponding downstream genes (*e.g.*, *GSH* and *HO-1*). This comes with enhanced oxidation resistance and reduced oxidative stress[6,7,26]. So, the means by which to increase the expression of Nrf2 or promote Nrf2 nuclear translocation could be an important target for the treatment and prevention of liver fibrosis.

IL-22 belongs to the IL-10 cytokine superfamily and is secreted by Th1, Th17, Th22 and NK/NKT cells. It mediates various types of biological behavior *via* a heterodimeric receptor complex IL-22R1/IL-10R2 that is exclusively expressed in epithelial cells and some fibroblasts. Binding of IL-22 to its receptor results in activation of signaling cascades, including JAK/STAT, ERK and JNK. In the liver, the receptor complex is selectively expressed in hepatocytes, liver progenitor cells and HSCs[10,11]. Ki *et al*[12] demonstrated an antioxidant role of IL-22 in ALF, indicated by the upregulation of antioxidant proteins metallothionein I/II. Xing *et al*[13] established an animal model of chronic alcoholic hepatitis using high-fat diet, carbonyl and Fe(III) complex factors. Based on this model, they found that IL-22 reduced liver lipid peroxidation, restored the level of GSH, and inhibited oxidative stress. In their later research about acute liver injury induced by D-galactosamine/lipopolysaccharide, they confirmed the inducing effect of IL-22 on expression of Bcl-x, heme oxygenase-1 and redox factor-1, also

indicating that IL-22 has liver-protective effects of anti-inflammation, anti-apoptosis and anti-oxidation. And, based on its cell-targeted characteristic so that it would not trigger excessive immune reactions, the clinical toxicity was very slight[12-14,27]. We also showed that IL-22 inhibited activation and proliferation of HSCs in a dose-dependent manner; the population of cells in G0/G1 phases was increased dramatically, while that in S phase was decreased markedly when compared with the number of HSCs maintained in the acetaldehyde control group. This indicates that IL-22 provoked G1/S phase arrest of acetaldehyde-induced HSCs. We used western blotting and immunocytochemistry to explore the possible mechanism. We showed that expression of Nrf2 total protein did not differ among the groups, whereas IL-22 treatment resulted in a dose-dependent increase in Nrf2 nuclear protein. This indicates that IL-22 promotes Nrf2 nuclear translocation, activates the Nrf2-keap1-ARE signaling pathways, increases expression of its downstream target protein GSH, and inhibits oxidative stress and progression of ALF.

The limit to our study is that we only investigated the inhibitory effect of IL-22 on ALF at the cellular level, while animal experiments and clinical research about IL-22 and ALF were not sufficiently performed. Further investigation of the relevant mechanisms is needed.

In summary, we established an *in vitro* model of acetaldehyde-induced ALF. We further demonstrated that IL-22 effectively inhibited activation and proliferation of HSCs, followed by delayed disease progression of ALF. This may partly be related to promoting Nrf2 nuclear translocation and enhancing the activity of the antioxidant axis Nrf2-keap1-ARE. These results could provide an experimental and theoretical basis for new drug development for treatment of ALF.

COMMENTS

Background

Alcoholic liver fibrosis (ALF) is the turning point and research focus of alcoholic liver disease. Oxidative stress-induced hepatic stellate cell (HSC) activation and proliferation have been shown to be the key factors in ALF. In the process, acetaldehyde is the most important effector. Nuclear factor-related factor (Nrf)2 is the key regulatory factor of antioxidant responses, and upregulation of Nrf2 or promotion of Nrf2 nuclear translocation delays the progression of ALF. Interleukin (IL)-22 belongs to the IL-10 cytokine superfamily and has liver-protective effects of anti-inflammation, anti-apoptosis, anti-oxidation and anti-fibrosis. However, whether it regulates the activity of the antioxidant axis Nrf2-keap1-ARE in HSCs has not yet been elucidated.

Research frontiers

Upregulation of Nrf2 or promotion of Nrf2 nuclear translocation delays the progression of ALF. IL-22 is a novel organ fibrosis-related cytokine. It delays the progression of liver fibrosis *via* promotion of liver progenitor cell/hepatocyte proliferation, inhibition of hepatocyte apoptosis, and upregulation of metallothionein (MTI/II) and glutathione (GSH) expression.

Innovations and breakthroughs

This is the first study to confirm the inhibitory effect of IL-22 on ALF at the cellular level. More important, we found that the effect was at least partly related to the promoted nuclear translocation of Nrf2 and the increased activity of antioxidant axis Nrf2-keap1-ARE.

Applications

Acetaldehyde (200 μmol/L for 48 h) mimics *in vivo* HSC activation and proliferation of ALF, indicating the successful establishment of an *in vitro* model of ALF. Based on the model, IL-22 dose-dependently inhibited the activation and proliferation of HSCs and progression of ALF. This was partly related to enhancing the activity of antioxidant axis Nrf2-keap1-ARE in HSCs. These results could provide an experimental and theoretical target and basis for new drug development for treatment of ALF.

Terminology

The Nrf2-keap1-ARE axis is an important internal antioxidative stress reaction system. Under basal conditions, cytoplasmic Nrf2 mostly combines with its binding protein keap1 and is in an inactive state. Oxidative stress may trigger dissociation of Nrf2 from keap1 and release free Nrf2. Nrf2 rapidly translocates into the nucleus and transactivates ARE in the promoter region of many antioxidant genes, triggering transcription of downstream antioxidant proteins (*e.g.*, GSH and heme oxygenase-1).

Peer-review

This study investigated proliferation and response to acetaldehyde, a metabolite of alcohol, as an *in vitro* model of ALF. The authors found that acetaldehyde increases HSCs' proliferation, decreases G0/G1 phases, and increases S phase. IL-22 can return the acetaldehyde effect on the cell proliferation which can be mediated through Nrf2-keap1-ARE.

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ORIGINAL ARTICLE

Basic Study

Expression of CRM1 and CDK5 shows high prognostic accuracy for gastric cancer

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Data sharing statement: All available data can be obtained by contacting the corresponding author.

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Abstract

AIM

To evaluate the predictive value of the expression of chromosomal maintenance (CRM)1 and cyclin-dependent kinase (CDK)5 in gastric cancer (GC) patients after gastrectomy.

METHODS

A total of 240 GC patients who received standard gastrectomy were enrolled in the study. The expression level of CRM1 and CDK5 was detected by immunohistochemistry. The correlations between CRM1 and CDK5 expression and clinicopathological factors were explored. Univariate and multivariate survival analyses were used to identify prognostic factors for GC. Receiver operating characteristic analysis was used to compare the accuracy of the prediction of clinical outcome by the parameters.

RESULTS

The expression of CRM1 was significantly related to size of primary tumor (P = 0.005), Borrmann type (P = 0.006), degree of differentiation (P = 0.004), depth of invasion (P = 0.008), lymph node metastasis (P = 0.013), TNM stage (P = 0.002) and distant metastasis

(P = 0.015). The expression of CDK5 was significantly related to sex (P = 0.048) and Lauren's classification (P = 0.011). Multivariate Cox regression analysis identified that CRM1 and CDK5 co-expression status was an independent prognostic factor for overall survival (OS) of patients with GC. Integration of CRM1 and CDK5 expression could provide additional prognostic value for OS compared with CRM1 or CDK5 expression alone (P = 0.001).

CONCLUSION

CRM1 and CDK5 co-expression was an independent prognostic factors for GC. Combined CRM1 and CDK5 expression could provide a prognostic model for OS of GC.

Key words: Gastric cancer; CRM1; CDK5; Prognosis

Core tip: Our study shows that low expression of chromosomal maintenance (CRM)1 and cyclin-dependent kinase (CDK)5 was associated with poor prognosis of gastric cancer patients. The expression of CRM1 or CDK5 influenced the prognostic value of each other. Combined CRM1 and CDK5 expression had better prognostic power than their individual expression had.

Sun YQ, Xie JW, Xie HT, Chen PC, Zhang XL, Zheng CH, Li P, Wang JB, Lin JX, Cao LL, Huang CM, Lin Y. Expression of CRM1 and CDK5 shows high prognostic accuracy for gastric cancer. *World J Gastroenterol* 2017; 23(11): 2012-2022 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i11/2012.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i11.2012

INTRODUCTION

Gastric cancer (GC) is the third leading cause of cancer-related death worldwide, although its incidence and mortality have decreased dramatically over the last 50 years[1]. In 2011 there were about 420000 new cases diagnosed (70% men and 30% women) and 300 000 deaths due to this disease in China[2-4]. Clinically, the prognostic classification model for outcomes of GC patients is mainly the TNM staging system based on the histopathological score[5], whereas the underlying molecular and cellular processes during carcinogenesis of GC are ignored. Patients with the same TNM stage may have wide variations in survival owing to different genetic mutation status[6]. Therefore, a better understanding of the molecular pathology might provide better prognostic biomarkers and guidance for more precise treatment for GC patients.

The human nuclear export protein chromosomal maintenance (CRM)1 (also known as exportin 1) has been reported to control multiple processes during cellular mitosis and is important in mediating nuclear export of cargo proteins that contain specific leucine-rich nuclear export signal (NES) consensus sequences[7,8]. Previous studies have demonstrated that CRM1 is important for the functions of proteins such as epidermal growth factor receptor, p53, p27, cyclin-dependent kinase (CDK)5 and Akt1[9-13]. The prognostic value of CRM1 expression has been reported in many types of cancer including ovarian cancer[14], osteosarcoma[15], glioma[16], pancreatic cancer[17] and esophageal squamous cell carcinoma[18]. However, whether CRM1 expression contributes to the development or progression of GC is not known.

CDK5 is a proline-directed serine/threonine kinase and participates in a variety of pathological and physiological functions[19,20]. Increasing evidence suggests a role for CDK5 in cancer tumorigenesis and progression[21,22]. Our previous work has demonstrated that in GC, CDK5 downregulation is an independent prognostic factor and the nuclear localization of CDK5 is critical for its tumor-suppressor function[23]. Given that CRM1 regulates CDK5 cytoplasm localization in neurons[12], we hypothesized that the functional correlation between CRM1 and CDK5 may affect the prognostic power of each molecule. In the present study, we examined the expression of CRM1 and CDK5 in 240 gastric tumor tissues and analyzed their correlation with patient clinicopathological features.

MATERIALS AND METHODS

Patients and specimens

The study cohort was composed of samples from 240 patients (178 men and 62 women, mean age: 59.5 years) with gastric adenocarcinoma, who had undergone gastrectomy at the Department of Gastric Surgery, Fujian Medical University Union Hospital, between January 2009 and December 2009. Following surgery, routine chemotherapy was given to patients with advanced disease and no radiation treatment was administered to any of the patients. Eligibility criteria for patients included in this study were: (1) histologically proven adenocarcinoma; (2) no other gastric tumors such as gastric stromal tumor; (3) no history of gastrectomy or other malignancy; (4) no prior neoadjuvant chemotherapy; and (5) availability of complete clinicopathological and survival data (Figure 1). The study was performed with the approval of the Ethics Committee of Fujian Medical Union Hospital. Written consent was given by the patients for their information and specimens to be stored in the hospital database and used for research.

Clinicopathological and survival data

The clinical and pathological data were recorded prospectively for the retrospective analysis. The clinicopathological data for the 240 GC patients included age, sex, size of primary tumor, location of primary tumor, degree of differentiation, histological

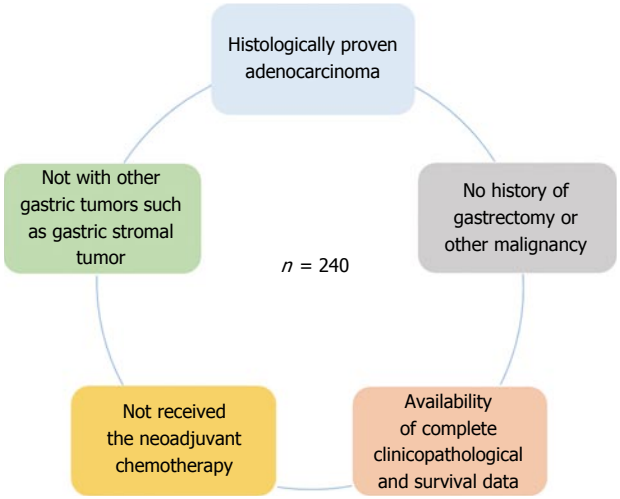


Figure 1 Eligibility criteria for patient inclusion.

type, Lauren's classification, Borrmann type, depth of invasion, lymph node metastasis, TNM stage, vessel invasion and distant metastasis. The pathological stage of the tumor was reassessed according to the 2010 International Union Against Cancer on GC TNM Classification (seventh edition)[5]. Overall survival (OS) was defined as the time from curative surgery to death or the last clinical follow-up. After surgery, all patients were followed by outpatient visits, telephone calls and letters every 3 mo in the first 2 years, every 6 mo in the next 3 years, and every year afterwards or until death. The deadline for follow-up was October 2015. All patients had follow-up records for > 5 years.

Immunohistochemistry

Paraffin blocks that contained sufficient formalin-fixed tumor specimens were serial sectioned at 4 μm and mounted on silane-coated slides for immunohistochemistry analysis. The sections were deparaffinized with dimethylbenzene and rehydrated through 100, 100, 95, 85, and 75% ethanol. Antigen retrieval treatment was done in 0.01 mol/L sodium citrate buffer (autoclaved at 121 ℃ for 2 min, pH 6.0) and endogenous peroxidase was blocked by incubation in 3% H_2O_2 for 10 min at room temperature. The sections were then washed in phosphate-buffered saline (PBS) and blocked with 10% goat serum (ZhongShan Biotechnology, China) for 30 min and incubated with rabbit anti-human CRM1 (ab24189, 1:200 dilution; Abcam, Cambridge, MA, United States) or CDK5 (sc-173, 1:150 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, United States) antibody in a humidified chamber at 4 ℃ overnight. Following three additional washes in PBS, the sections were incubated with horseradish-peroxidase-conjugated secondary antibody for 30 min at room temperature. The visualization signal was developed with diaminobenzidine solution and all slides were counterstained with 20% hematoxylin. Finally, all slides were dehydrated and mounted on coverslips. For negative controls, the primary antibody diluent was used to replace primary antibody.

Evaluation of immunostaining intensity

The stained tissue sections were reviewed under a microscope by two pathologists who were blinded to the clinical parameters, and scored independently according to the intensity of cellular staining and the proportion of stained tumor cells[6]. The CRM1 and CDK5 proteins were immunohistochemically stained yellowish to brown in the cytoplasm and/or nuclei of cancer cells. The expression pattern of CRM1 and CDK5 was all or none in tumor tissues, suggesting the score for the proportion of stained tumor cells was unavailable. The staining intensity was scored as 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellow brown), and 3 (strong staining, brown) (Figure 2). The CRM1 and CDK5 protein expression was considered low if the score was $\leqslant 1$ and high if it was $\geqslant 2$.

Statistical analysis

IBM SPSS version 19.0 (SPSS, Chicago, IL, United States) was used for all statistical analyses. χ^2 and Fisher's exact tests were used to analyze categorical data. Univariate survival analysis was performed using the Kaplan-Meier method, and the significance of difference between groups was analyzed using the log-rank test. The stepwise Cox proportional hazards regression model was used for multivariate survival analysis, with adjustments for variables that may have been significant prognostic factors according to the univariate analysis. Receiver operating characteristic (ROC) analysis was used to compare the accuracy of the prediction of clinical outcome by the parameters. All *P* values were two-sided and statistical significance was determined at $P < 0.05$.

RESULTS

Expression status of CRM1 and CDK5 in GC

We examined CRM1 and CDK5 protein expression in tumor tissues from 240 GC patients using immunohistochemistry. The expression of CRM1 and CDK5 proteins were scored as low in 149 (62.08%) and 91 (37.92%) samples, and high in 91 (37.92%) and 149 (62.08%) samples, respectively. Based on the combined expression of CRM1 and CDK5, we classified the patients into three subtypes: CRM1 and CDK5 high ($n = 63$), CRM1 or CDK5 low ($n = 114$) and CRM1 and CDK5 low ($n = 63$).

Correlation between CRM1 and CDK5 expression and clinicopathological parameters in GC patients

The correlation between expression of CRM1 and CDK5 and the clinicopathological features were analyzed (Table 1). CRM1 expression was significantly related to size of primary tumor ($P = 0.005$), Borrmann type (*P*

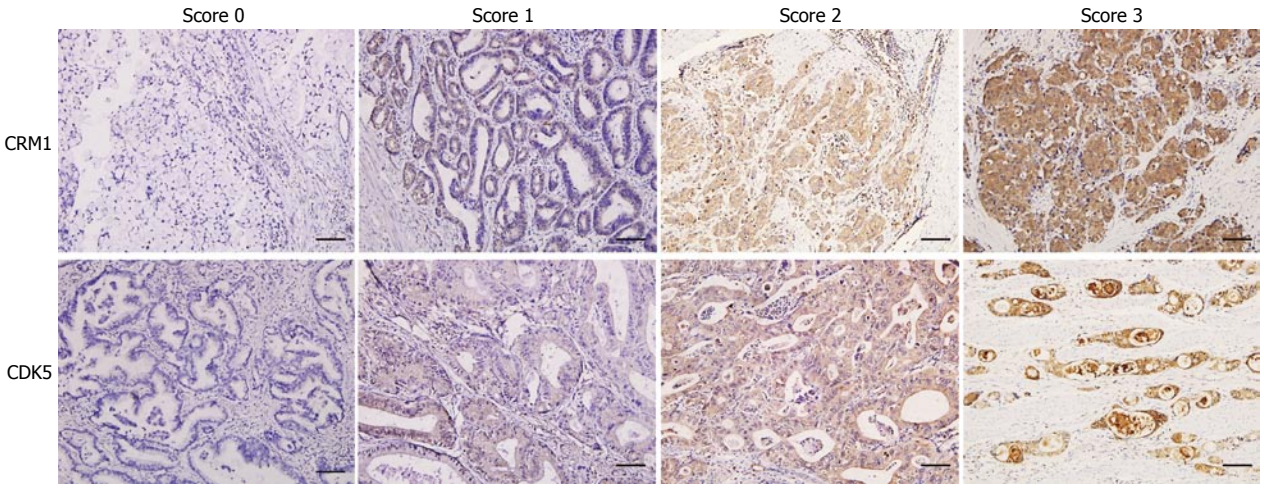


Figure 2 Immunohistochemical staining of CRM1 and CDK5 expression in gastric cancer tissue and the criteria for immunohistochemistry scoring. Score 0: no staining, Score 1: weak staining, Score 2: moderate staining, Score 3: strong staining. The protein expression was considered low if the score was ≤ 1 and high if it was ≥ 2. Scale bar = 100 μm.

= 0.006), degree of differentiation (P = 0.004), depth of invasion (P = 0.008), lymph node metastasis (P = 0.013), TNM stage (P = 0.002) and distant metastasis (P = 0.015). The expression of CDK5 was significantly related to sex (P = 0.048) and Lauren's classification (P = 0.011). The correlation between combined CRM1 and CDK5 expression and the clinicopathological features was also analyzed. The combined CRM1 and CDK5 expression was significantly related to size of primary tumor (P = 0.026), degree of differentiation (P = 0.007), Lauren's classification (P = 0.019), lymph node metastasis (P = 0.015), TNM stage (P = 0.035) and vessel invasion (P = 0.021) (Table 2).

Prognostic value of CRM1 and CDK5 expression

To elucidate the prognostic value of CRM1 and CDK5 expression, univariate Kaplan-Meier and multivariate Cox regression analyses were used. Univariate analysis revealed that OS was significantly associated with size and location of primary tumor, Borrmann type, degree of differentiation, depth of invasion, lymph node metastasis, TNM stage, vessel invasion, distant metastasis, CRM1 and CDK5 expression, but not with sex, age at surgery, histological type, and Lauren's classification (Table 3). The hazard ratio and 95%CI for OS were compared among the subgroups. OS was shorter in patients with low expression of CRM1 or CDK5 in comparison to the corresponding patients with high CRM1 or CDK5 expression (Figure 3).

The 3- and 5-year cumulative survival rates were 54.1% and 39.7% for patients with low CRM1 expression, and 67.0% and 61.5% for those with high CRM1 expression. The mean survival time for patients with low and high expression of CRM1 was 44.6 and 56.5 mo, respectively. Clearly, GC patients with low expression of CRM1 had a poorer prognosis than those with high CRM1 expression (P < 0.05) (Figure 4A). The 3- and 5-year cumulative survival rates were 49.5% and 39.3% for GC patients with low expression of CDK5, and 63.6% and 53.4% for those with high CDK5 expression. The mean survival time for GC patients with low and high expression of CDK5 was 43.4 and 53.1 mo, respectively, suggesting a shorter OS for GC patients with low expression of CDK5 (P < 0.05) (Figure 4B).

We evaluated the prognostic value of the combined CRM1 and CDK5 expression. The patients with simultaneous high expression of CRM1 and CDK5 displayed better survival in comparison with the rest of the patients in Kaplan-Meier analysis (Figure 4C). The 3- and 5-year cumulative survival rates were 47.6% and 34.3% for the simultaneous low CRM1 and CDK5 expression patient group, 55.9% and 45.2% for the CRM1 or CDK5 low expression patient group, and 73.0% and 66.7% for the simultaneous high CRM1 and CDK5 expression patient group, respectively. The mean survival time was 41.5 mo for patients with CRM1 and CDK5 low expression; 46.9 mo for those with CRM1 or CDK5 low expression; and 61.1 mo for those with CRM1 and CDK5 high expression (Table 3).

The clinicopathological parameters that were correlated with patient survival in univariate analysis were included in multivariate analysis. CRM1 and CDK5 coexpression status, tumor size, tumor location, and TNM stage were independent prognostic factors for patients with GC, whereas vessel invasion and Borrmann type were not (Table 4).

Improvement of CDK5 prognostic model with CRM1 expression

In our previous work, we demonstrated that down-regulation of CDK5 in GC was an independent prognostic factor. To improve the prognostic accuracy of OS in GC patients, we combined CRM1 and CDK5 expression to generate a predictive model. ROC analysis was applied to compare the prognostic accuracy between

Table 1 Relationships between CRM1 and CDK5 protein expression (immunohistochemical staining) in gastric cancer tissues and various clinicopathological variables

Variables	Total	CRM1 expression				CDK5 expression			
		Low (n = 149)	High (n = 91)	χ^2	P value	Low (n = 91)	High (n = 149)	χ^2	P value
Gender									
Male	178	110	68	0.024	0.877	61	117	3.893	0.048[1]
Female	62	39	23			30	32		
Age at surgery (yr)									
≤ 60	120	78	42	0.867	0.352	46	74	0.018	0.894
> 60	120	71	49			45	75		
Size of primary tumor (cm)									
≤ 5	99	51	48	7.995	0.005[1]	35	64	0.470	0.493
> 5	141	98	43			56	85		
Location of primary tumor									
Upper 1/3	56	33	23	5.290	0.152	22	34	1.718	0.633
Middle 1/3	59	39	20			21	38		
Lower 1/3	103	59	44			37	66		
More than 1/3	22	18	4			11	11		
Borrmann type									
Early stage	10	4	6	10.118	0.006[1]	5	5	0.774	0.679
Ⅰ + Ⅱ type	89	46	43			32	57		
Ⅲ + Ⅳ type	141	99	42			54	87		
Degree of differentiation									
Well/moderate	96	49	47	8.287	0.004[1]	30	66	3.021	0.082
Poor and not	144	100	44			61	83		
Lauren's classification									
Intestinal type	46	33	13	2.254	0.176	25	21	6.527	0.011[1]
Diffuse type	294	116	78			66	128		
Histological type									
Papillary	7	4	3	2.958	0.398	3	4	7.052	0.070
Tubular	187	112	75			63	124		
Mucinous	20	13	7			10	10		
Signet-ring cell	26	20	6			15	11		
Depth of invasion									
T1	40	18	22	11.908	0.008[1]	15	25	2.145	0.543
T2	27	13	14			8	19		
T3	62	38	24			21	41		
T4	111	80	31			47	64		
Lymph node metastasis									
N0	63	29	34	10.781	0.013[1]	23	40	4.868	0.182
N1	40	29	11			11	29		
N2	43	26	17			14	29		
N3	94	65	29			43	51		
TNM stage									
Ⅰ	44	18	26	15.074	0.002[1]	15	29	1.058	0.787
Ⅱ	55	33	22			19	36		
Ⅲ	123	82	41			49	74		
Ⅳ	18	16	2			8	10		
Vessel invasion									
Negative	230	141	89	1.423	0.233	88	142	0.278	0.598
Positive	10	8	2			3	7		
Distant metastasis									
Negative	222	133	89	5.940	0.015[1]	83	139	0.352	0.553
Positive	18	16	2			8	10		

[1]P < 0.05, statistical significance. CRM: Chromosomal maintenance; CDK: Cyclin-dependent kinase.

combined CRM1 and CDK5 expression and CRM1 or CDK5 expression alone. Combination of CRM1 and CDK5 expression showed significantly higher prognostic accuracy [area under the curve (AUC): 0.622, 95%CI: 0.551-0.694, P = 0.001] than CRM1 expression alone (AUC: 0.585, 95%CI: 0.512-0.657, P = 0.024) or CDK5 expression alone (AUC: 0.575, 95%CI: 0.503-0.648, P = 0.045) (Figure 5). All these results indicated that the combined CRM1 and CDK5 expression provided better prognostic power for GC patient OS.

DISCUSSION

Increasing evidence has demonstrated that the karyoplasm localization of CDK5 is important for its multiple pathological and physiological functions, including neuronal migration during brain development, neuronal cell survival and tumor development and

Table 2 Relationships between different CRM1 and CDK5 protein expression status in gastric cancer tissues and various clinicopathological variables

Variables	Total	CRM1 and CDK5 High expression	CRM1 or CDK5 Low expression	CRM1 and CDK5 Low expression	χ^2	*P* value
Gender						
Male	178	42	87	49	2.553	0.279
Female	62	21	27	14		
Age at surgery(yr)						
≤ 60	120	35	54	31	1.109	0.574
> 60	120	28	60	32		
Size of primary tumor (cm)						
≤ 5	99	22	42	35	7.275	0.026[1]
> 5	141	41	72	28		
Location of primary tumor						
Lower 1/3	56	18	19	19	10.848	0.093
Middle 1/3	59	14	32	13		
Upper 1/3	103	22	52	29		
More than 1/3	22	9	11	2		
Borrmann type						
Early stage	10	2	5	3	6.035	0.197
Ⅰ + Ⅱ type	89	20	38	31		
Ⅲ + Ⅳ type	141	41	71	29		
Degree of differentiation						
Well/moderate	96	18	43	35	10.027	0.007[1]
Poor and not	144	45	71	28		
Lauren's classification						
Intestinal type	46	17	24	5	7.875	0.019[1]
Diffuse type	194	46	90	58		
Histological type						
Papillary	7	2	3	2	11.127	0.850
Tubular	187	44	87	56		
Mucinous	20	5	13	2		
Signet-ring cell	26	12	11	3		
Depth of invasion						
T1	40	8	17	15	10.996	0.088
T2	27	4	13	10		
T3	62	16	27	19		
T4	111	35	57	19		
Lymph node metastasis						
N0	63	15	22	26	15.845	0.015[1]
N1	40	9	22	9		
N2	43	7	26	10		
N3	94	32	44	18		
TNM stage						
Ⅰ	44	8	17	19	13.543	0.035[1]
Ⅱ	55	14	24	17		
Ⅲ	123	33	65	25		
Ⅳ	18	8	8	2		
Vessel invasion						
Negative	230	62	105	63	7.757	0.021[1]
Positive	10	1	9	0		
Distant metastasis						
Negative	222	55	106	61	4.191	0.123
Positive	18	8	8	2		

[1]$P < 0.05$, statistical significance. CRM: Chromosomal maintenance; CDK: Cyclin-dependent kinase.

progression[23-27]. CDK5 has no intrinsic nuclear localization signal and its nuclear localization relies on p27[12]. In the absence of p27, two weak NESs on CDK5 bind to CRM1, leading to the cytoplasmic shuttle of CDK5[12]. In this study, low CDK5 expression was associated with poorer prognosis (Figure 4B), which was consistent with our previous discovery that CDK5 acted as a tumor suppressor in GC[23]. However, CRM1 is usually considered as an oncogene and involved in the nuclear export of a number of proteins including p53, p21, c-ABL and FOXOs[28-30]. Forgues *et al*[31] found that cytoplasmic sequestration of CRM1 is frequently associated with hepatocellular carcinoma. In this work, high CRM1 expression was associated with longer GC patient survival (Figure 4A), suggesting that CRM1 exerts a tumor suppressive role in GC. Considering the oncogenic role of CDK5 in many other types of cancer such as hepatocellular carcinoma[24], breast cancer[32]

Table 3 Univariate analysis of the correlation between clinicopathological parameters and survival of patients with gastric cancer

Clinicopathological parameters	Cumulative survival rates (%)		Mean survival time (mo)	Log-rank test	*P* value
	3 yr	5 yr			
Gender					
Male	66.1	48.3	49.022	0.092	0.762
Female	56.6	48.0	49.324		
Age at surgery (yr)					
≤ 60	60.8	48.1	49.510	0.022	0.882
> 60	57.2	47.9	49.285		
Size of primary tumor (cm)					
≤ 5	84.8	73.4	66.451	44.251	0.000[1]
> 5	41.1	30.4	37.516		
Location of primary tumor					
Upper 1/3	51.8	38.7	44.354	28.888	0.000[1]
Middle 1/3	42.4	33.9	39.508		
Lower 1/3	76.5	66.7	61.597		
More than 1/3	31.8	22.7	30.500		
Borrmann type					
Early stage	90.0	90.0	72.186	41.770	0.000[1]
Ⅰ + Ⅱ type	81.9	71.5	64.835		
Ⅲ + Ⅳ type	42.6	30.4	38.102		
Degree of differentiation					
Well/moderate	70.8	60.3	57.397	8.644	0.003[1]
Poor and not	49.8	39.9	44.056		
Lauren's classification					
Intestinal type	66.8	50.7	53.287	0.649	0.420
Diffuse type	56.2	47.4	48.471		
Histological type					
Papillary	57.1	57.1	50.857	1.026	0.752
Tubular	57.2	47.0	48.339		
Mucinous	75.0	53.6	53.850		
Signet-ring cell	60.2	48.2	51.110		
Depth of invasion					
T1	97.5	94.9	78.311	64.970	0.000[1]
T2	88.9	74.1	67.889		
T3	59.2	46.0	48.764		
T4	37.8	25.2	34.461		
Lymph node metastasis					
N0	88.9	80.8	70.120	59.862	0.000[1]
N1	69.5	69.5	61.079		
N2	58.1	34.9	43.674		
N3	33.0	23.3	32.911		
TNM stage					
Ⅰ	97.7	95.4	78.211	71.616	0.000[1]
Ⅱ	76.1	61.3	60.241		
Ⅲ	40.7	29.2	38.186		
Ⅳ	27.8	16.7	22.518		
Vessel invasion					
Negative	60.8	49.3	50.492	8.264	0.004[1]
Positive	20.0	20.0	23.400		
Distant metastasis					
Negative	60.7	50.6	51.544	20.223	0.000[1]
Positive	16.7	16.7	22.518		
CRM1 expression					
Low	54.1	39.7	44.590	7.707	0.005[1]
High	67.0	61.5	56.540		
CDK5 expression					
Low	49.5	39.3	53.058	6.234	0.013[1]
High	63.6	53.4	43.438		
CRM1/CDK5 expression					
CRM1 and CDK5 Low	47.6	34.3	41.487	13.683	0.001[1]
CRM1 or CDK5 Low	55.9	45.2	46.873		
CRM1 and CDK5 High	73.0	66.7	61.069		

[1]$P < 0.05$, statistical significance. CRM: Chromosomal maintenance; CDK: Cyclin-dependent kinase.

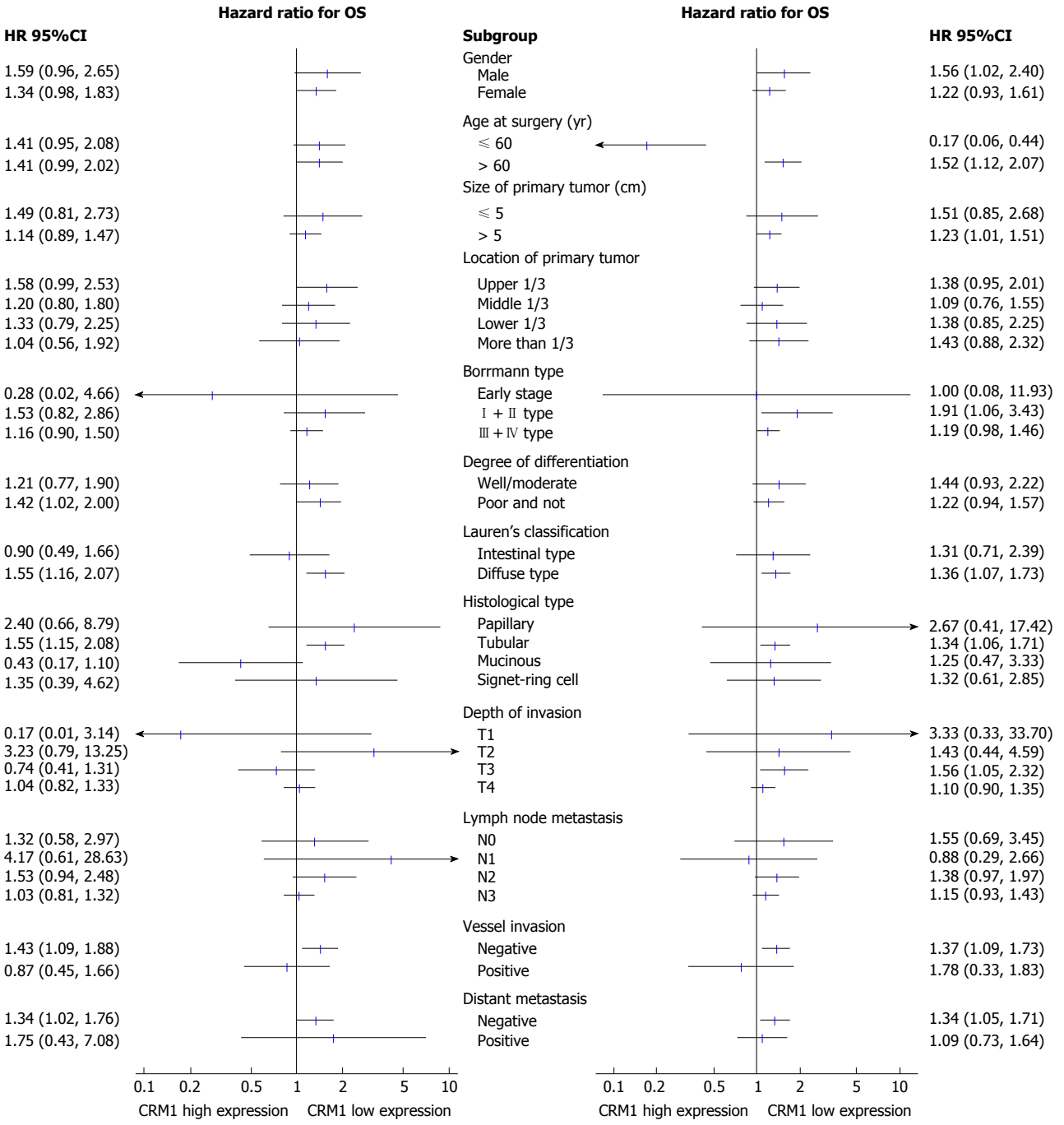


Figure 3 Forest plot showing hazard ratios (oblongs) and 95%CI (bars) for overall survival of subgroups from the 240 gastric cancer patients with different CRM1 (left) and CDK5 (right) expression status. HR: Hazard ratio; OS: Overall survival; CRM: Chromosomal maintenance; CDK: Cyclin-dependent kinase.

Table 4 Multivariate analysis of the correlation between clinicopathological parameters and survival time of patients with gastric cancer

Covariates	Coefficient	Standard error	HR	95% CI for HR	*P* value
Tumor location (cardia *vs* others)	0.451	0.202	1.570	1.057-2.333	0.026[1]
Tumor size (≥ 5 *vs* < 5 cm)	0.723	0.232	2.060	1.309-3.243	0.002[1]
Vessel invasion (positive *vs* negative)	NA	NA	NA	NA	NA
TNM stage (stage Ⅲ and Ⅳ *vs* Ⅰ and Ⅱ)	1.086	0.243	1.961	1.839-4.768	0.000[1]
CDK5 and CRM1 expression					
(low/high *vs* high/high)	0.568	0.254	1.765	1.074-2.903	0.025[1]
(low/low *vs* high/high)	0.769	0.269	2.158	1.274-3.657	0.004[1]
Borrmann type (type early, Ⅰ, Ⅱ *vs* Ⅲ, Ⅳ)	NA	NA	NA	NA	NA

[1]$P < 0.05$, statistical significance. NA: Not available.

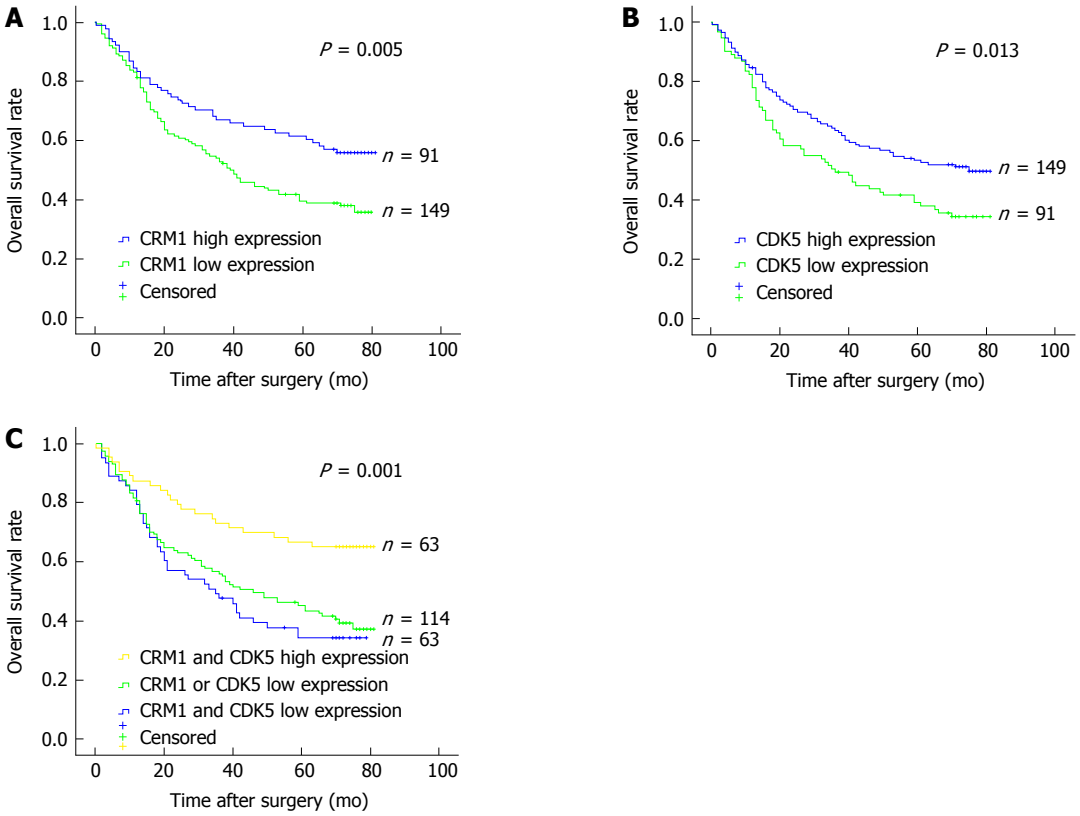
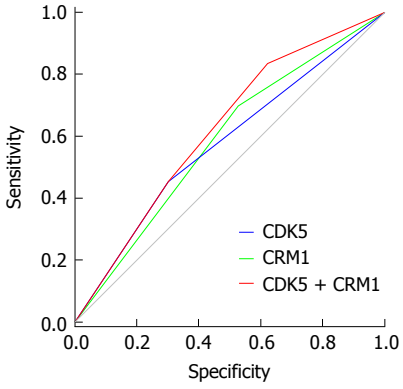


Figure 4 Kaplan-Meier analysis of the correlation between expression of CRM1 (A), CDK5 (B) and combined CRM1 and CDK5 expression (C) and the overall survival of gastric cancer patients. CRM: Chromosomal maintenance; CDK: Cyclin-dependent kinase.



	AUC	95%CI	*P*
CDK5	0.575	0.503-0.648	0.045
CRM1	0.585	0.512-0.657	0.024
CDK5 + CRM1	0.622	0.551-0.694	0.001

Figure 5 Receiver operating characteristic analysis of the sensitivity and specificity of the predictive value of the combined CRM1 and CDK5 expression model, CRM1 expression model and CDK5 expression model. CRM: Chromosomal maintenance; CDK: Cyclin-dependent kinase.

and neuroendocrine thyroid cancer[25], it is possible that the shift of CDK5 function in GC affects the function of CRM1. In addition, we recently found that CDK5RAP3, a binding protein of the CDK5 activator p35, negatively regulates the β-catenin signaling pathway by repressing glycogen synthase kinase-3β phosphorylation and acts as a tumor suppressor in GC[33]. The differential expression or activities of other CDK5-binding partners such as CDK5RAP3 may also affect the functions of CDK5 and CRM1 among different cancer types.

The fact that either CDK5 or CRM1 expression could influence the prognostic power of the other (Figure 4C) seemed to support this hypothesis. Further analysis with ROC revealed that combination of CRM1 and CDK5 expression showed significantly higher prognostic accuracy than CRM1 or CDK5 expression alone (P = 0.001) (Figure 5), indicating that combined CRM1 and CDK5 expression show more prognostic power for OS of patients with GC. Taken together, our present study suggested that CRM1 and CDK5 should receive considerable attention as effective markers for predicting therapeutic outcomes, but the profound molecular roles of CRM1 and CDK5 in GC remain far from being fully elucidated and need further research.

In addition, we found that low CRM1 expression was associated with lymph node metastasis in GC (Table 1). This suggested that the identification of CRM1 expression in preoperative mucosal biopsies from GC patients may indicate the necessity for a more aggressive lymphadenectomy, although further studies in a larger cohort of patients are needed.

In conclusion, our results suggested that combined CRM1 and CDK5 expression was an independent prognostic factor for OS and showed more prognostic power in GC patients. Considering the inferior prognosis of the CRM1 and/or CDK5 low patients, more frequent

follow-up is probably needed for these patients after surgery.

COMMENTS

Background

To evaluate the prognostic value of the expression of chromosomal maintenance (CRM)1 and cyclin-dependent kinase (CDK)5 for gastric cancer (GC) patients after gastrectomy.

Research frontiers

CDK5 downregulation was an independent prognostic factor and the nuclear localization of CDK5 was critical for its tumor suppressor function in GC. Given that CRM1 regulates CDK5 karyoplasm localization in neurons, we hypothesized that the functional correlation between CRM1 and CDK5 may affect the prognostic power of each molecule. In the present study, we examined the expression of CRM1 and CDK5 in 240 gastric tumor tissues and analyzed their correlation with patient clinicopathological features.

Innovations and breakthroughs

CRM1 and CDK5 coexpression was an independent prognostic factor for patients with GC. The present results suggested that combined CRM1 and CDK5 expression could provide a better prognostic model for overall survival (OS) of GC patients.

Applications

The presented results suggested that combined CRM1 and CDK5 expression was an independent prognostic factor for OS of GC patients and showed more prognostic power than individual factors alone. Considering the inferior prognosis of the CRM1 and/or CDK5 low patients, more frequent follow-up is probably needed for these patients after surgery.

Peer-review

The authors investigate whether combined expression of CDK5 and CRM1 correlates with clinic-pathological parameters in GC. The manuscript is sound and the experiments/correlations are well-performed.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Colonic ulcerations may predict steroid-refractory course in patients with ipilimumab-mediated enterocolitis

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Author contributions: Jain A, Lipson EJ and Lazarev MG conceived the study and initial study design; Sharfman W contributed to study design; Lipson EJ and Sharfman WH contributed the patient cohort; Brant SR assisted with data analysis and interpretation; Jain A wrote the primary draft of the manuscript; all authors contributed to data analysis/interpretation, and drafting/revising the manuscript, and have approved the final version of this manuscript, including the authorship list.

Institutional review board statement: The study was approved by the Institutional Review Board of the Johns Hopkins University.

Informed consent statement: Informed consent was not obtained from patients given that this was a retrospective cohort study with low likelihood of identifiable patient information - only anonymized data are presented. The study was approved by our Institutional Review Board as being exempt from informed consent requirements based on the nature and design of the study.

Conflict-of-interest statement: Jain A and Lazarev MG have no declarations. Lipson EJ has served as a consultant for Bristol-Myers Squibb, EMD, Serono, Merck and Novartis; He has received research funding from Genentech and AstraZeneca. Sharfman WH has served as a consultant for Merck, Genentech, and Castle Biosciences, and he has received research funding from Bristol-Myers Squibb, Glaxo Smith Kline, and Novartis. Brant SR has served as an advisory board member for Asana Medical Inc., and he has received research funding from Johnson and Johnson.

Data sharing statement: The original anonymous dataset is available on request from the corresponding author at AnimeshJ@med.unc.edu.

Manuscript source: Invited manuscript

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Abstract

AIM

To investigate management of patients who develop ipilimumab-mediated enterocolitis, including association of endoscopic findings with steroid-refractory symptoms and utility of infliximab as second-line therapy.

METHODS

We retrospectively reviewed all patients at our center

with metastatic melanoma who were treated with ipilimumab between March 2011 and May 2014. All patients received a standard regimen of intravenous ipilimumab 3 mg/kg every 3 wk for four doses or until therapy was stopped due to toxicity or disease progression. Basic demographic and clinical data were collected on all patients. For patients who developed grade 2 or worse diarrhea (increase of 4 bowel movements per day), additional data were collected regarding details of gastrointestinal symptoms, endoscopic findings and treatment course. Descriptive statistics were used.

RESULTS

A total of 114 patients were treated with ipilimumab during the study period and all were included. Sixteen patients (14%) developed ⩾ grade 2 diarrhea. All patients were treated with high-dose corticosteroids (1-2 mg/kg prednisone daily or equivalent). Nine of 16 patients (56%) had ongoing diarrhea despite high-dose steroids. Steroid-refractory patients received one dose of intravenous infliximab at 5 mg/kg, and all but one had brisk resolution of diarrhea. Fourteen of the patients underwent either colonoscopy or sigmoidoscopy with variable endoscopic findings, ranging from mild erythema to colonic ulcers. Among 8 patients with ulcers demonstrated by sigmoidoscopy or colonoscopy, 7 patients (88%) developed steroid-refractory symptoms requiring infliximab. With a median follow-up of 264 d, no major adverse events associated with prednisone or infliximab were reported.

CONCLUSION

In patients with ipilimumab-mediated enterocolitis, the presence of colonic ulcers on endoscopy was associated with a steroid-refractory course.

Key words: Ipilimumab; Melanoma; Enterocolitis; Colitis; Infliximab; Corticosteroid; Colonic ulcer

Core tip: Immune-mediated enterocolitis is a common toxicity of ipilimumab therapy for melanoma. Infliximab is often needed as a second line therapy in steroid refractory cases. Our findings suggest that colonic ulcers seen on lower gastrointestinal endoscopy may predict a steroid refractory disease course. This would support a role for endoscopy in select cases, and suggest that early initiation of infliximab therapy may be appropriate in patients with colonic ulceration. These results require further exploration in larger patient cohorts.

Jain A, Lipson EJ, Sharfman WH, Brant SR, Lazarev MG. Colonic ulcerations may predict steroid-refractory course in patients with ipilimumab-mediated enterocolitis. *World J Gastroenterol* 2017; 23(11): 2023-2028 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i11/2023.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i11.2023

INTRODUCTION

Ipilimumab (Yervoy™, Bristol-Myers Squibb, Princeton, NJ, United States) is a first-in-class monoclonal antibody that blocks the immune regulatory molecule cytotoxic T-lymphocyte antigen-4 (CTLA-4), resulting in T-cell activation. It was approved by the United States Food and Drug Administration (FDA) in 2011 after two phase 3 studies demonstrated a survival benefit in patients with advanced melanoma[1-5]. It is now being tested in clinical trials in patients with a variety of tumor types.

Ipilimumab administration is associated with numerous immune-related adverse effects (irAE) including enterocolitis, hepatitis, hypophysitis, dermatitis, as well as several others[1,4,6]. Enterocolitis is among the most common toxicities, manifesting as diarrhea, abdominal pain, nausea or hematochezia. The overall incidence of any diarrhea (grade 1 or higher) has been reported as 31%-43% in various studies[1,3-5,7].

Immune-mediated diarrhea is typically treated based on severity. Anti-diarrheal medications such as loperamide are often used for patients with mild symptoms. Corticosteroids are generally administered in cases of moderate to severe diarrhea. In some steroid-refractory cases, the tumor necrosis factor alpha inhibitor infliximab has demonstrated efficacy in alleviating symptoms[8-10]. As more patients receive ipilimumab and similar immune therapies (such as anti-PD-1 therapies), gastroenterologists are increasingly being asked to assist in the management of patients immune-mediated enterocolitis. Questions remain regarding the role of endoscopy, and the optimal timing of infliximab administration.

To better understand the clinical features and treatment approaches to ipilimumab-mediated enterocolitis, we examined our single-center experience with post-FDA approval ipilimumab therapy in a large cohort of patients with metastatic melanoma.

MATERIALS AND METHODS

We conducted a retrospective study of all patients treated with ipilimumab for advanced melanoma from March 2011 to May 2014 at our institution. All patients were treated with a standard regimen of 3 mg/kg of intravenous ipilimumab administered every 3 wk for 4 total doses or until development of drug toxicity, disease progression or death. Diarrhea was graded based on the National Cancer Institute's (NCI's) Common Terminology Criteria for Adverse Events (CTCAE), version 4.0: (1) Grade 1 - increase of < 4 stools per day over baseline; (2) Grade 2 - increase of 4-6 stools per day; (3) Grade 3 - increase of > 6 stools per day, or incontinence or hospitalization; (4) Grade 4 - life-threatening consequences; and (5) Grade 5 - death.

Using the electronic medical record, demographic and clinical data were collected on all patients, including age, sex, peripheral white blood cell count, lymphocyte

Table 1 Baseline characteristics of patients

	Ipilimumab induced enterocolitis	Ipilimumab without enterocolitis
Demographics		
Total number of patients	16	98
Mean age in years	63	61
Female sex, *n* (%)	8 (50)	28 (29)
Laboratory characteristics prior to ipilimumab		
Mean white blood cell count, cells/cu. mm (range)	6720 (3510-17100)	7530 (1080-25800)
Reference range 4500-11000 cells/cu. mm		
Mean lymphocyte count, cells per cu. mm (range)	1570 (592-4610)	1440 (97-4420)
Reference range 1150-4800 cells/cu. mm		
Mean neutrophil count, cells per cu. mm (range)	4350 (2040-11610)	5200 (668-23500)
Reference range 1800-7000 cells/cu. mm		

Table 2 Clinical features and treatment of 16 patients with ipilimumab-mediated enterocolitis *n* (%)

Onset of diarrhea	
After 1 dose of ipilimumab	3 (19)
After 2 doses of ipilimumab	7 (43)
After 3 doses of ipilimumab	3 (19)
After 4 doses of ipilimumab	3 (19)
Diarrhea details	
Number of bowel movements/day, median (range)	6 (5-12)
Grade 2 diarrhea	9 (56)
Grade 3 diarrhea	7 (44)
Grade 4/5 diarrhea	0
Associated symptoms	
Abdominal pain	10 (63)
Nausea or vomiting	3 (19)
Fever	2 (13)
Anorexia	2 (13)
Endoscopic findings	
Mucosal erythema, edema, or erosions only	6 (43)
Ulcers	8 (57)
Treatment of diarrhea	
High dose corticosteroids	16 (100)
Infliximab	9 (56)

count, neutrophil count, platelet count, information on melanoma treatment response, and mortality. Charts for all patients were reviewed to determine which patients developed grade 2 or worse diarrhea. For patients who developed at least grade 2 diarrhea, additional data were collected, including details of diarrhea, other gastrointestinal symptoms, endoscopy results, pathology results, therapy for diarrhea, and response to therapy. Descriptive statistics were used to evaluate the data. Missing data variables were omitted from the analysis (pairwise deletion).

The study was approved by the Institutional Review Board of the Johns Hopkins University.

RESULTS

Patient characteristics

A total of 114 patients were treated with ipilimumab for metastatic melanoma during the study period; all were included in the study. Baseline demographic and laboratory characteristics were similar between patients who developed grade 2 (increase of 4 bowel movements per day) or worse diarrhea and those who did not (Table 1). A total of 16 patients (14%) developed grade 2 or worse diarrhea.

Out of 114 total patients, baseline CBC and differential data were missing for 2 patients. One patient moved to another city only 21 d after starting ipilimumab therapy and no additional follow-up data were available regarding her condition. All other data were available for analysis.

Clinical features

Sixteen patients developed ipilimumab-mediated enterocolitis. Clinical features and treatment outcomes are shown in Table 2. Onset of diarrhea occurred after a median of 2 doses of ipilimumab and after a median of 33 d from the first dose of ipilimumab (range 5-94 d). Patients had a median of 6 bowel movements per day with stool being described as watery and non-bloody in most patients; one patient reported trace amounts of blood in the stool initially. Most patients (63%) reported abdominal pain with a cramping character, while a minority of patients had fever, anorexia, or nausea.

Workup and endoscopic findings

Standard medical workup included polymerase chain reaction stool test for *Clostridium difficile* toxin and stool culture for routine enteric pathogens (including *Salmonella, Campylobacter, Shigella,* and *Escherichia coli*), which were negative in all patients. Testing for celiac disease was not routinely performed. All but two patients underwent endoscopic evaluation with either flexible sigmoidoscopy (4 patients) or full colonoscopy (10 patients). Endoscopic appearance was variable: some patients had only mild edema and erythema of the mucosa (6 patients), while others had ulcers in the colon (8 patients). All 10 patients who underwent a full colonoscopy had at least patches of abnormal mucosa in the right and left colon. Histologic analysis revealed crypt apoptosis, crypt abscesses, and/or cryptitis in 12 of 14 patients (86%).

Treatment of enterocolitis

Patients with grade 2 diarrhea were treated with high-

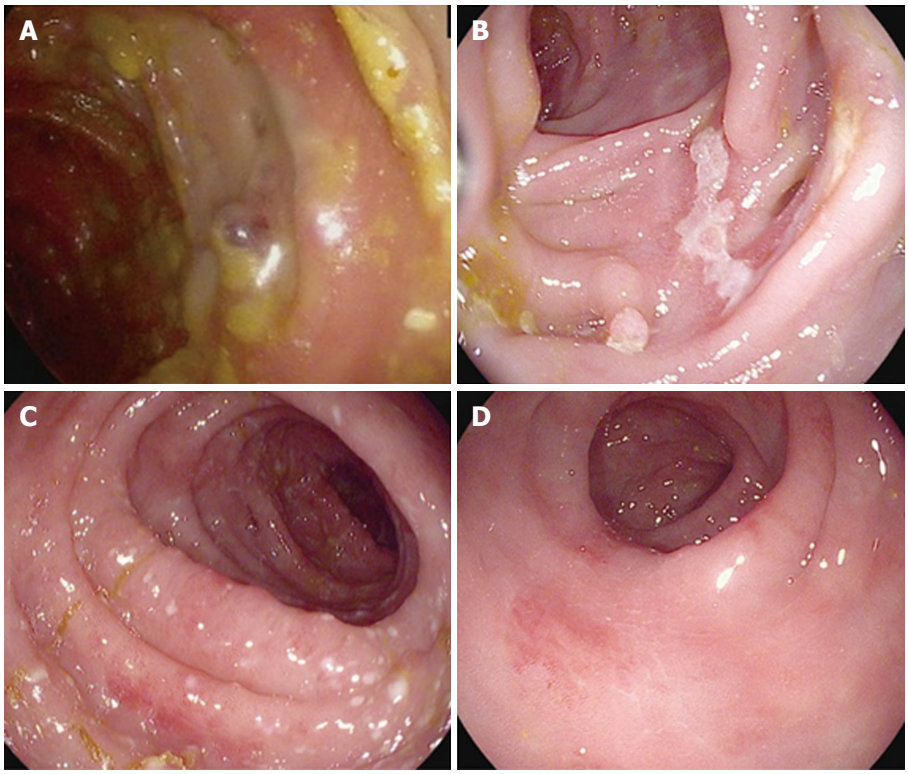

Figure 1 Sample endoscopic images from patients with ipilimumab-mediated enterocolitis. A: A large, punched-out ulcer in the center of the image; B: A linear colonic ulcer; C: A segment of mild, diffuse colitis with punctate areas of erythema and colitis; D: Patchy, mild erythema. Patients from A and B had a steroid refractory course and required infliximab, while patients from C and D had steroid responsive symptoms.

dose corticosteroids (1-2 mg/kg prednisone per day or equivalent). Most patients were also treated with loperamide at the onset of symptoms. Ipilimumab therapy was suspended at the onset of grade $\geqslant$ 2 diarrhea.

In 7 patients (44%), gastrointestinal symptoms resolved after administration of high-dose corticosteroids. Nine patients (56%) experienced ongoing diarrhea despite steroids and were treated with a single dose of 5 mg/kg of intravenous infliximab. Eight patients (89%) reported improvement of gastrointestinal symptoms within 1-2 wk of infliximab therapy. One patient had ongoing diarrheal symptoms after one dose of infliximab. Prednisone was weaned off, but he continued to have symptoms. He was treated with a second dose of infliximab 9 wk after the first dose with improvement in diarrhea.

Seven of 8 patients (88%) who had mucosal ulceration on sigmoidoscopy or colonoscopy developed steroid refractory diarrhea requiring infliximab, whereas only 2 of 6 patients without colonic ulcerations required infliximab (positive likelihood ratio = 3.89, 95%CI: 0.65-23.2; negative likelihood ratio = 0.28, 95%CI: 0.08-1.02). Also of note, the one patient who required 2 doses of infliximab had multiple long ulcers (approximately 1 centimeter ulcers) on colonoscopy. See Figure 1 for summary of endoscopic findings and treatment outcomes. Grade of diarrhea did not appear to correlate with steroid refractory symptoms.

A case of Ipilimumab-mediated enteritis

One patient developed severe diarrhea with 12 bowel movements per day only 8 d after her first dose of ipilimumab. She presented to the hospital with diarrhea and fevers 4 d after the onset of symptoms. A CT scan of the abdomen and pelvis revealed moderate diffuse thickening of the small bowel, which was not present on an abdominal CT scan 1 wk prior to receiving ipilimumab. Sigmoidoscopy performed the following day showed normal colonic mucosa and no evidence of colitis on histology. She was diagnosed with ipilimumab-mediated enteritis but did not improve with prednisone 80mg daily. After 2.5 wk of prednisone therapy, she was given a dose of infliximab with resolution of her diarrhea within 7 d.

Completion of Ipilimumab course

Three patients had already received all four doses of ipilimumab prior to developing enterocolitis. Four other patients were ultimately able to resume ipilimumab and complete the full 4 doses of therapy (2 patients who only received steroids, and 2 patients who received steroids and infliximab). Hence a total of 7 of 16 (44%) patients ultimately completed all 4 doses of ipilimumab therapy.

Follow-up and adverse effects

Follow-up was available on patients for a median of 264 d (range 17-1055) from the date of first ipilimumab

infusion to last oncology follow-up or death. One patient suffered labile mood while on high-dose prednisone and another patient reported hyperglycemia and vaginal candidiasis while on high-dose prednisone. No other adverse events, including other infections or bowel perforation, were reported through the follow-up period.

DISCUSSION

Diarrhea and enterocolitis are among the most common immune-related adverse events associated with ipilimumab therapy. With the increasing use of immune-based therapies for patients with various cancer types, gastroenterologists are likely to see a growing burden of immune-mediated gastrointestinal toxicities. In the present analysis, we report a single center experience of 114 patients with metastatic melanoma who presented with ipilimumab-mediated enterocolitis. Overall, 14% of patients developed grade $\geqslant$ 2 diarrhea. More than half of those patients had steroid-refractory symptoms requiring treatment with infliximab. Interestingly, 8 of 9 patients (88%) with ulcers found on colonoscopy/sigmoidoscopy experienced a steroid-refractory course requiring infliximab (Figure 1).

While the cohort size in our study is relatively small, this potential association deserves further investigation. If validated, this finding could have important implications in the management of some patients with ipilimumab-mediated enterocolitis. Prompt evaluation with colonoscopy/sigmoidoscopy may provide prognostic information, allowing clinicians to consider initiation of infliximab therapy in patients whose symptoms do not rapidly respond to corticosteroids and who are found to have mucosal ulceration.

Prior studies have estimated incidence of ipilimumab-mediated diarrhea (any grade) to be in the range of 14%-29%[1,3,10,11]. In our cohort, we observed $\geqslant$ grade 2 and grade 3 diarrhea in 14% and 6% of patients, respectively, which is consistent with previous reports[1,4,5,10,12].

Initial experience with ipilimumab in the clinical trial setting seemed to indicate that steroid-refractory colitis was rare. For example, in a landmark phase 3 trial, only 4 of 179 (2%) patients with diarrhea or colitis required treatment with infliximab[1]. An earlier phase 2 study by Beck and colleagues reported refractory enterocolitis requiring infliximab in only 4 of 41 (10%) patients with enterocolitis[11].

By contrast, more contemporary experience with ipilimumab in the post-FDA approval era has provided evidence that steroid-refractory enterocolitis may be more common than initially thought. A large, retrospective, single-center review recently reported steroid-refractory symptoms in approximately 30% of patients with a serious immune-related adverse event[10]. Another recent study reported steroid-refractory symptoms in 26% of patients with ipilimumab-induced colitis. In our study, 9 of 16 (56%) patients had steroid-refractory symptoms requiring infliximab rescue therapy.

Of those, 8 patients experienced symptom improvement after a single dose of infliximab. One patient experienced lingering symptoms requiring a second dose of infliximab. This is consistent with previously published series, which have reported efficacy rates of infliximab in treating steroid-refractory ipilimumab-induced diarrhea between 72%-100%[8-10]. Administration of steroids and infliximab does not seem to adversely affect the anti-tumor response of immune based therapy. A recent report from the Memorial Sloan Kettering group indicates that the use of immune suppressive medications for the treatment of immune-related adverse events did not adversely impact overall survival or time to change in oncologic therapy in patients with melanoma[10].

The mechanism of ipilimumab-mediated enterocolitis has not been precisely elucidated. Indeed, with the increase availability of novel biologic agents, there is a growing recognition of the spectrum biologic drug-mediated enterocolitis. Each of these drugs may affect different lymphocyte targets of the gut mucosal immune system leading to enterocolitis. Ipilimumab specifically targets the inhibitory T-cell surface protein CTLA-4. Prior investigation has shown that T-cell depletion does not appear to be a feature in the pathogenesis of ipilimumab-mediated enterocolitis[7]. Berman and colleagues posit that ipilimumab results in dysregulation of mucosal immunity, evidenced by alterations in antibody levels to various microbial antigens and elevated fecal calprotectin levels[13]. Further understanding of ipilimumab and other biologic-mediated enterocolitides may have broader implications for understanding other inflammatory gastrointestinal disease states.

In conclusion, although our study was limited by its retrospective design, small cohort size and the fact that all patients were treated at a single tertiary center, our findings raise the possibility that the presence of colonic ulcers in patients with ipilimumab-mediated enterocolitis may predict a steroid refractory course. These results should be explored in larger patient cohorts in order to elucidate the utility of endoscopic evaluation and early administration of infliximab.

COMMENTS

Background

Ipilimumab is a novel T-cell activating therapy used in the treatment of melanoma and potentially other malignancies. Immune-mediated enterocolitis is one of the well-known toxicities of this drug. Treatment paradigms for the management of ipiliumumab-mediated enterocolitis are evolving. In particular, the management of corticosteroid refractory cases is still being understood. As immune activating therapies are being increasingly used in the oncology realm, management of patients with immune-mediated enterocolitis will continue to be a clinical challenge.

Research frontiers

First line therapy for ipilimumab-mediated enterocolitis often includes

corticosteroids, with growing evidence to support the use of infliximab in refractory cases. There are no well-known predictors of steroid refractory disease course and the role of endoscopy in such cases is unclear. In this report, the authors report on a large cohort of patients treated with ipilimumab with particular focus on the role of endoscopic findings.

Innovations and breakthroughs

In the present manuscript, the authors present the novel finding that severity of endoscopic findings, namely colonic ulcers, could be a predictor of steroid refractory disease course in patients with ipilimumab-mediated enterocolitis.

Applications

This manuscript would support a role for colonoscopy or sigmoidoscopy in patients with ipilimumab-mediated enterocolitis. Patients with worse endoscopic findings, such as colonic ulcers, may be more likely to have refractory disease course and this may be a reason to offer earlier rescue therapy, such as infliximab, in such patients.

Terminology

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is an immune regulatory molecule found on the surface of T-lymphocytes. CTLA-4 is the target of ipilimumab and activation of CTLA-4 results in T-cell activation.

Peer-review

This is an interesting and well-written paper.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Predictive factors for compliance with transanal irrigation for the treatment of defecation disorders

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Author contributions: Leroi AM designed the study, conducted the research, performed the statistical analyses of the results, wrote the paper, and approved the final version to be submitted for publication; Melchior C, Gourcerol G, Bridoux V, and Vérin E recruited the patients, conducted the research, participated in drafting the final version of the paper, and approved the final version to be submitted for publication; Bildstein C and Boueyre E collected and analyzed the data and approved the final version to be submitted for publication.

Institutional review board statement: This study was reviewed and approved by local Ethics Committee (No. E2016-64).

Informed consent statement: The study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice and applicable regulatory requirements. No informed consent was required under French legislation since it was a retrospective study with no interventions other than routine patient care.

Conflict-of-interest statement: None of the authors has a conflict of interest regarding this study.

Data sharing statement: No additional data are available.

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Abstract

AIM

To investigate compliance with transanal irrigation (TAI) one year after a training session and to identify predictive factors for compliance.

METHODS

The compliance of one hundred eight patients [87 women and 21 men; median age 55 years (range 18-83)] suffering from constipation or fecal incontinence (FI) was retrospectively assessed. The patients were trained in TAI over a four-year period at a single institution. They were classified as adopters if they continued using TAI for at least one year after beginning the treatment or as non-adopters if they stopped. Predictive factors of compliance with TAI were based on pretreatment assessments and training

progress. The outcomes of the entire cohort of patients who had been recruited for the TAI treatment were expressed in terms of intention-to-treat.

RESULTS

Forty-six of the 108 (43%) trained patients continued to use TAI one year after their training session. The patients with FI had the best results, with 54.5% remaining compliant with TAI. Only one-third of the patients who complained of slow transit constipation or obstructed defecation syndrome continued TAI. There was an overall discontinuation rate of 57%. The most common reason for discontinuing TAI was the lack of efficacy (41%). However, 36% of the patients who discontinued TAI gave reasons independent of the efficacy of the treatment such as technical problems (catheter expulsion, rectal balloon bursting, instilled water leakage or retention, pain during irrigation, anal bleeding, anal fissure) while 23% said that there were too many constraints. Of the patients who reported discontinuing TAI, the only predictive factor was the progress of the training (OR = 4.9, 1.3-18.9, P = 0.02).

CONCLUSION

The progress of the training session was the only factor that predicted patient compliance with TAI.

Key words: Neurogenic bowel dysfunction; Fecal incontinence; Constipation; Obstructed defecation; Transanal irrigation

Core tip: Less than 50% of the trained patients continued to use transanal irrigation (TAI) for the treatment of their defecation disorders one year after their training sessions. We showed for the first time that the only predictive factor for TAI discontinuation was the progress of the training. This suggested that the first training session should be better structured in order to promote more realistic expectations of treatment efficacy, side-effects, and constraints in order to reduce the discontinuation rate.

Bildstein C, Melchior C, Gourcerol G, Boueyre E, Bridoux V, Vérin E, Leroi AM. Predictive factors for compliance with transanal irrigation for the treatment of defecation disorders. *World J Gastroenterol* 2017; 23(11): 2029-2036 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i11/2029.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i11.2029

INTRODUCTION

Chronic constipation and fecal incontinence (FI) are common disorders with debilitating symptoms[1,2]. There is a hierarchy of management strategies for constipation and FI. Conservative (education, behavioral therapy, biofeedback) and pharmacological (oral and rectal laxatives) management measures are effective for most constipation and FI patients[1,2]. However, when these measures fail to control symptoms, additional strategies, including transanal irrigation (TAI), are required to improve symptoms and quality of life. TAI involves the instillation of water into the colon and rectum using a disposable balloon catheter. The water and the contents of the descending colon, sigmoid, and rectum are then evacuated in a controlled manner[3]. TAI was first introduced into clinical practice by Shandling and Gilmour for children with neurogenic bowel dysfunction (NBD) related to spina bifida[3]. Based on its clinical efficacy in treating children with NBD, TAI was then applied to adults with NBD[4] and to children and adults with functional bowel disorders for whom traditional treatments had failed[5]. While short-term trials indicated that constipation and FI are significantly improved by TAI, compliance is difficult to maintain over time[6,7]. The predictive factors of mid-term and long-term compliance with TAI in adults have been poorly described to date[6,7].

The purposes of the present study were (1) to investigate compliance with TAI of adults suffering from constipation or FI; and (2) to identify specific predictive factors of compliance with TAI based on pretreatment assessments and training progress.

MATERIALS AND METHODS

Patients and data collection

A retrospective review of adult patients with constipation or FI refractory to first-line treatments and their compliance with a Peristeen® TAI (Coloplast A/S, 3050 Humlebæk, Denmark) bowel management program over a four-year period (2010-2014) in a single institution was conducted. There were no predefined physiological, radiological, or psychosocial criteria to propose TAI to patients except the failure of the first-line treatment (education, behavioral therapy, biofeedback, oral and rectal laxatives) and the possibility of using a rectal probe.

Data were prospectively collected and included patient demographics, main indication for TAI (constipation or FI), type of FI (active, passive, or mixed), type of constipation (slow transit constipation or obstructed defecation disorder) based on Rome criteria[8], and the etiology of the constipation or FI. We also interviewed each patient using a validated FI score (Cleveland Clinic Incontinence Score, CCIS 0-20)[9], a constipation severity score (Kess score, 0-39)[10], or an NBD score (NBD, 0-47)[11], as appropriate. Because of the heterogeneity of the population (patients with constipation or FI), each patient was classified as having a severe defecation disorder based on the severity score of their main symptom (Kess score > 20 if constipation was the main symptom[10], CCIS > 10 if FI was the main symptom[12], or NBD score > 14 for a

neurological disease[11]). The evaluation of the patients prior to the treatment was conducted based on their heterogeneous background pathologies. However, for most patients, the evaluation included anorectal physiology tests, defecography, and a colonic transit time study performed as previously described[13-15]. Anal manometry was performed using a water-perfused system. Maximal anal resting and squeeze pressures were recorded. Rectal sensation to balloon distension with air was assessed. The smallest amount of distension felt by the patient (the threshold of conscious rectal sensation) and the maximum tolerable volume were determined[14].

Intervention

The Peristeen® irrigation system was used[6]. Patients were prepared using information leaflets, DVDs, and meetings with specialist nurses where the system was presented and explained (training session). TAI attempts were performed under the supervision of specialist nurses using a previously described procedure[6]. The modalities of the TAI training session were evaluated with respect to the volume of water used for the irrigation and the number of pressings necessary to inflate the balloon. The nurses evaluated the progress of the training sessions and reported any difficulties (expulsion of catheter, fluid leakage, no evacuation of stools during irrigation). During the first month following the training sessions, the patients were asked to perform washouts daily or every two days. Appointments were scheduled for 1, 3, 6, and 12 mo after the training sessions to discuss the various treatment modalities (frequency of enema administration, volume of water used, *etc.*). The patients were encouraged to contact the specialist nurses in the event of problems.

Outcome measures

We assessed the outcomes based on compliance with TAI one year after the training sessions. To identify TAI users one year later, we mailed a questionnaire to patients who were introduced to TAI from January 2010 to December 2014 and who were not seen at the 12-mo follow-up. The patients were asked if they were still using the TAI system, how they used it, and whether they had experienced any technical problems or complications. If they were no longer using TAI, they were asked to explain why they had stopped and when.

Statistical analysis

The proportion of patients in each outcome was expressed in terms of intention-to-treat (ITT). The ITT approach was designed to analyze the patients who had attended a TAI training session, including those who had failed the first session and those who were lost at follow-up. The purpose was to assess the outcomes of the entire cohort of patients who had been recruited for the TAI treatment.

To analyze the predictive factors of the outcomes of TAI, the patients were divided into two groups based on their compliance with TAI one year after their training session. They were classified as non-adopters if they stopped using TAI within one year of the start date or as adopters if they had continued the therapy. Patients who were lost at follow-up or who failed the training session were not included in the analysis of predictive factors.

The χ^2 test was used to compare qualitative variables. The Mann-Whitney *U* test was used to compare quantitative variables. A multivariate analysis was performed to identify relationships between disease background factors and the outcome of TAI. Variables with a $P < 0.15$ in the univariate analysis were used for the stepwise logistic regression. Results are expressed as odds ratios and 95%CI. Data are expressed as medians or mean values with a range or standard deviation. Statistical significance was set at $P < 0.05$.

RESULTS

One hundred eight patients with defecation disturbances [87 women and 21 men; median age 55 years (range 18-83)] over a four-year period were introduced to TAI at our institution. TAI was used to treat isolated constipation in 51 patients, relieve constipation associated with retentive FI in 47, and treat isolated FI in 10. The main complaints, type of constipation and FI, main causes, and prior surgical procedures for defecation disorders are given in Table 1.

The study protocol complied with the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in *a priori* approval by the institution's human research committee (N°E2016-64).

Training session

Five patients (4.6%) withdrew during the first training session, 4 because of repeated expulsion of the rectal catheter during irrigation and water leakage around the rectal catheter, and 1 because of difficulty emptying the instilled water. Nine patients (8.3%) needed more than one training session (2 sessions for 8 patients and 3 sessions for 1 patient) because they did not feel comfortable performing the TAI after one session. Following a satisfactory home-use training session, 92 patients (85.2%) were able to auto-administer TAI, while 11 patients (10.2%) required assistance either from a nurse (7) or a member of the family (4). Seventy percent of the patients performed TAI at least 2 to 3 times a week.

Outcome of TAI at the one-year follow-up

The outcomes of the patients based on compliance with TAI one year after the training session are summarized in a flow diagram (Figure 1). At the one-

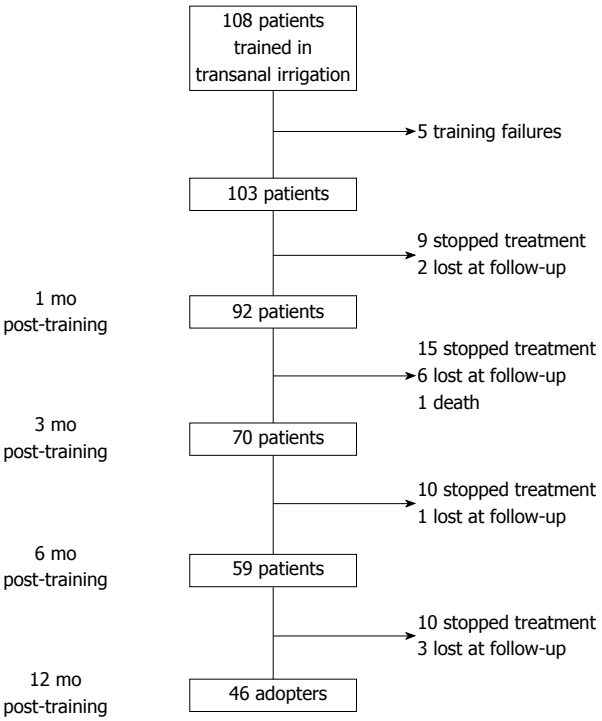

Figure 1 Flow chart of the 108 patients based on compliance with transanal irrigation at the one-year follow-up.

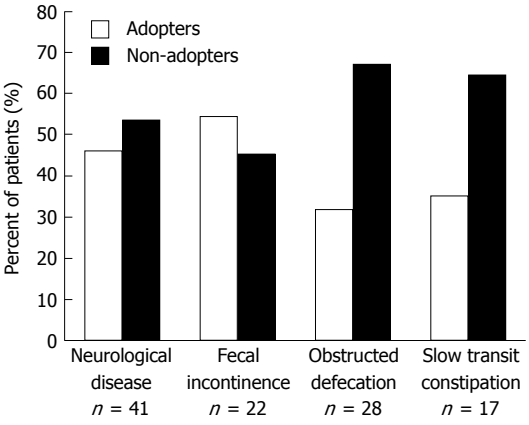

Figure 2 Successful outcome (intention-to-treat analysis) defined by compliance with transanal irrigation based on the etiology of the defecation disorders in 108 patients.

year follow-up, 46 of the 108 patients still maintained TAI at home and were considered as adopters (ITT 42.6%) while 62 had discontinued TAI and were considered as non-adopters (ITT 57.4%), including 44 who had discontinued TAI prior to the one-year follow-up, 5 who had failed during the first training session, 12 who were lost at follow-up, and 1 who died for a reason unrelated to TAI. The successful outcomes with TAI (ITT analysis) are presented in Figure 2 based on the etiology of the defecation disorder. The median follow-up after the successful start of TAI was 16 (1-67) mo.

The main reasons for discontinuing TAI following a successful training session were technical problems

Table 1 Predominant symptoms, main causes of constipation/fecal incontinence, and prior surgical procedures for constipation/fecal incontinence in the 108 patients trained in transanal irrigation *n* (%)

	Study group (*n* = 108)
Predominant symptoms	
Constipation	65 (60.2)
Fecal incontinence	43 (39.8)
Type of constipation	
Slow transit constipation	15 (15.3)
Obstructed defecation	47 (48)
Mixed constipation	36 (36.7)
Type of FI	
Active FI	12 (21)
Passive FI	42 (73.7)
Mixed FI	3 (5.3)
Main causes of constipation/FI	
Neurological disease (multiple sclerosis, spina bifida, spinal cord injury, Parkinson disease):	41 (38)
Slow transit constipation	17 (15.7)
Obstructed defecation syndrome	28 (25.9)
FI	22 (20.4)
Pudendal neuropathy	11
Sphincter lesion	5
Low rectal/reservoir compliance	5
Idiopathic	1
Prior surgical procedures	32 (29.6)
Rectal resection with colo-anal anastomosis	3
Colonic resection with colo-rectal anastomosis	3
Colonic resection with ileo-rectal anastomosis	1
Hysterectomy	1
Cystopexy	2
Rectopexy	4
Starr	1
Sacral nerve stimulation	15
Anorectal malformation	1
Rectoplasty	1

FI: Fecal incontinence.

(catheter expulsion, rectal balloon bursting, instilled water leakage or retention, pain during irrigation, anal bleeding, anal fissure) (16 patients, 36.4%), inefficacy (18 patients, 40.9%), and too many constraints (10 patients, 22.7%). Constraints were mainly related to the time spent performing the irrigation. The median time the patients used TAI before discontinuing was 3 mo (range, 0.2-11). At the last follow-up, the discontinuation of TAI had led to the resumption of a medical treatment for 21 patients (42.8%) and an invasive surgical procedure for 18 (36.7%): Malone antegrade continence enema, *n* = 6; sigmoid colostomy, *n* = 4; ileostomy, *n* = 1; coloproctectomy, *n* = 1; rectopexy, *n* = 2; sacral nerve stimulation, *n* = 3; and artificial bowel sphincter, *n* = 1. Eight patients preferred resuming traditional water or sodium phosphate enemas.

Side-effects

Of the 46 adopters, 13 (28.3%) complained of the time spent on bowel management while 25 (54.3%) reported 47 minor and self-limiting adverse events.

Table 2 Comparison of background variables, baseline demographics, severity scores, anorectal physiological testing, defecography, and colonic transit times of adopters and of non-adopters who discontinued transanal irrigation during the first year *n* (%)

Variables	Adopters *n* = 46	Non-adopters *n* = 44	*P* value
Age (yr)	55.9 ± 13.9	51 ± 17.8	0.29
Gender			
Female	36 (78)	38 (86)	0.46
Male	10 (22)	6 (14)	
BMI	25.4 ± 4.3	23.6 ± 4.9	0.05
Main symptom			
Constipation	22 (48)	28 (64)	0.19
FI	24 (52)	16 (36)	
Type of constipation	*n* = 42	*n* = 39	
Slow transit	8 (19)	6 (15.4)	0.48
Obstructed defecation	17 (40.5)	21 (53.8)	
Mixed	17 (40.5)	12 (30.8)	
Type of FI	*n* = 28	*n* = 24	0.82
Urge	7 (25)	5 (21)	
Passive	19 (68)	18 (75)	
Mixed	2 (7)	1 (4)	
Main causes of constipation/FI			
Neurological disease	19 (41.3)	14 (31.8)	0.47
Slow transit constipation	6 (13)	8 (18.2)	0.70
Obstructed defaecation syndrome	8 (17.4)	11 (25)	0.53
FI	13 (28.3)	11 (25)	0.91
Prior surgical procedures	11 (24)	17 (39)	0.13
	n = 42	*n* = 40	
Severe defecation disorders according to severity scores	32 (76)	31 (77)	0.99
Anorectal manometry:	*n* = 38	*n* = 33	
Resting pressure (cmH_2O)	66.6 ± 30.7	57.2 ± 27.3	0.20
Squeeze pressure (cmH_2O)	57.8 ± 56.5	59.6 ± 67.7	0.81
Threshold volume (mL)	21.9 ± 15.7	15.7 ± 6.9	0.16
Maximum tolerated volume (mL)	225.1 ± 98.6	234.5 ± 145.6	0.83
Rectal compliance (mL/cmH_2O)	4.1 ± 2.4	4.2 ± 4	0.51
Colonic transit time	*n* = 31	*n* = 33	
Right colon (h)	28.1 ± 19.7	29.9 ± 18.4	0.54
Left colon (h)	35.5 ± 23.6	38 ± 25.4	0.60
Recto-sigmoid colon (h)	23.6 ± 21.8	29 ± 26.6	0.53
Total (h)	90.2 ± 35.4	88.4 ± 44.3	0.97
Defecography			
Rectal prolapse	*n* = 29	*n* = 22	
or recto-anal procidentia	9 (31)	6 (27)	0.22

Values are expressed as mean ± SD. BMI: Body mass index; FI: Fecal incontinence.

The most common events reported were leakage of irrigation fluid around the catheter (16 events, 34%), pain on catheter insertion or water instillation (14 events, 29.9%), catheter expulsion (9 events, 19.1%), rectal balloon bursting (5 events, 10.6%), and instilled water retention (3 events, 6.4%). No cases of bowel perforation were reported at follow-up.

Predictors of compliance with TAI

To identify predictive factors of compliance with TAI, we compared the characteristics of the 44 non-adopters who had discontinued TAI during the first year (excluding patients who were lost at follow-up or who had failed the training session) with those of the 46 adopters.

Predictive factors based on baseline assessments: We did not find any significant differences between adopters and non-adopters related to age, gender, body mass index, main symptom (constipation or FI), severity of main symptom based on severity scores, type of constipation or FI, underlying pathology, or previous colo-rectal surgeries (Table 2). The anorectal physiological parameters, colonic transit times, and defecography results of the adopters and non-adopters were similar (Table 2).

Predictive factors based on TAI modalities: Only the satisfactory progress of the first training session was related to the outcome of TAI. Non-adopters were more likely to experience a first training session complicated by technical problems (expulsion of catheter, fluid leakage, or no evacuation of stools during irrigation) than adopters ($P = 0.02$) (Table 3). The multivariate analysis confirmed that the success of the first training session was the only predictive factor of mid-term compliance with TAI (odds ratio = 4.9, 1.3-18.9; $P = 0.02$). The number of sessions required to learn the TAI

Table 3 Comparison of transanal irrigation training modalities for adopters and for non-adopters who discontinued transanal irrigation during the first year *n* (%)

Variables	Adopters *n* = 46	Non-adopters *n* = 44	*P* value
Training sessions			
Number of training sessions	1.2 ± 0.4	1.1 ± 1	0.30
Volume of instilled water	607.9 ± 214.8	517.4 ± 241.7	0.07
Numbers of balloon pressings	3.2 ± 1.3	3.2 ± 1.8	0.55
Auto-administration	40 (87)	42 (95.5)	0.30
Assisted administration	6 (13)	2 (4.5)	
Complicated first training session	4 (9)	13 (29)	0.02
Current sessions	*n* = 45	*n* = 24	
Frequency of irrigation:			
2-3/wk or more	35 (78)	22 (92)	0.35
Less than 2-3 wk	10 (22)	2 (8)	
Total number of side-effects	1.04 ± 1.2	1.1 ± 1	0.51
Percent of patients:			
with side-effects	25 (54)	29 (66)	0.37
with constraints	13 (28)	16 (36)	

Values are expressed as mean ± SD.

technique, the type of administration (self-administered or assisted), the frequency of use of the TAI system, and the number of side-effects observed during TAI were not predictive factors of discontinuation (Table 3).

DISCUSSION

Our main aim was to investigate compliance with TAI by adults with constipation or FI one year after a TAI training session. Based on ITT, 43% of the 108 patients were still using TAI one year after their training session. The patients with FI had the best results, with 54.5% remaining compliant with TAI. Only one-third of the patients who complained of slow transit constipation or obstructed defecation syndrome continued TAI.

Studies on adults with defecation disorders of mixed etiology have reported variable response rates to TAI[5]. The proportion of patients with a positive outcome with TAI ranges from 30%[16] to 78%[17]. The large difference in reported response rates may be due to three main inherent factors of the study designs: (1) the success criteria; (2) the TAI system and training modalities; and (3) the duration of the follow-up. Nevertheless, the results of the present study were relatively consistent with those of a previous study by Christensen *et al*[6], who used similar success criteria. In the study by Christensen *et al*[6], patients still using TAI at follow-up, patients in whom symptoms had resolved during treatment and thus no longer needed the treatment, and patients whose bowel symptoms had been successfully treated but who had died for reasons not related to treatment were regarded as having a successful TAI outcome. They reported a success rate of 47% for the entire cohort, 51% for patients with FI, and 34% for patients with slow transit constipation or obstructive defecation syndrome[6]. They also reported that TAI was successful in 63% of patients with a neurological disorder, which is at odds with our results showing that only 46% of such patients were compliant with TAI at the one-year follow-up[6]. This inconsistency may be due to different treatment durations. Indeed, Christensen *et al*[6] determined the success of TAI at the last follow-up (ranging from 1 to 116 mo). However a high drop-out rate over time is common in patients with a neurological disorder. For example, only 35% of such patients continue TAI for 3 years[7]. These discrepancies indicate that it is important to evaluate all patients over the same time frame. We decided to use a one-year follow-up assessment since (1) the risk of underestimating the percentage of non-compliant patients is limited given that most dropouts occur at the beginning of the treatment[6]; and since (2) few patients are lost at follow-up.

We confirmed that FI patients are relatively good candidates for TAI, with 54.5% remaining compliant one year after the training session despite the constraints and technical problems, which is in agreement with previous studies[6,18,19]. This suggested that patients are willing to suffer the side-effects because some of their colorectal dysfunctions are resolved. While the results of ITT FI by sacral nerve stimulation, for example, have been reported to be slightly better one year after implantation (66% ITT)[20], TAI requires no surgical intervention and has minimal side effects. TAI may thus be suitable as a second-line treatment for incontinent patients, especially those with retentive FI.

In the present study, like others[6,18], we observed an overall discontinuation rate of 57%. The most common reason for discontinuing TAI was the lack of efficacy (41%). However, 59% discontinued because of technical problems or constraints. Regardless of the reason, discontinuation could lead to a delay in the effective management of defecation disorders and a decrease in the cost-effectiveness of TAI. We thus

focused our patient selection efforts in order to identify predictive factors of non-compliance. In previous studies, several predictive factors of non-compliance have been suggested, including gender, mixed constipation and FI symptoms, prolonged colonic transit time in patients with NBD[7], anal insufficiency, and low rectal volume in a population with defecation disorders of different origins[6]. However, no consistent, reliable patterns indicating a greater risk of discontinuing TAI have been identified. In the present study, technical problems that occurred during the first training session were the only predictive factor for TAI discontinuation. Patients who experienced moderate technical problems during the training session that were not sufficient to discontinue the treatment after the session were five times more likely to be non-adopters because of the inefficacy of TAI or persistent technical problems. No other studies have investigated the modalities of training sessions as predictive factors of a successful outcome. If our results are confirmed with a larger patient cohort, this could help optimize the management of these patients by, for example, using more training sessions with specialized nurses in a clinical or home setting in the early treatment phase.

The present study had several main limitations. First, it was a retrospective study. Second, several subgroups of patients were required to identify predictive factors of non-compliance due to the heterogeneity of the population. As such, our population may have been underpowered for such an analysis. Third, we did not use the symptom severity scores to evaluate TAI efficacy. However, the poor correlation between the subjective perception of patients of their functional bowel disorders and the symptom severity scores is well known[21]. Consequently, the continuation of a binding treatment such as TAI by patients for at least 1 year appeared to us to be as clinically relevant as severity scores in reaching the conclusion that TAI provides a significant clinical benefit to patients.

With the exception of progress during the first training session, no predictive factors for compliance with TAI were identified. This suggested that the first training session should be better structured in order to promote more realistic expectations about treatment efficacy, side-effects and, especially, constraints in order to reduce the discontinuation rate.

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COMMENTS

Background

Transanal irrigation (TAI) is an interesting therapeutic option for defecation disorders that are refractory to first-line medical treatments. However, mid- and long-term compliance with TAI is disappointing. The main reasons and the predictive factors of compliance with TAI in adults have been poorly described to date.

Research frontiers

A better understanding of why many patients with defecation disorders who are treated with TAI discontinue the therapy after the training session should help reduce the rate of failure.

Innovations and breakthroughs

Like others, the authors found that less than 50% of the trained patients continued to use TAI one year after their training session. They failed to identify any consistent, reliable patterns indicating a greater risk of discontinuing TAI. However, we showed for the first time that (1) 59% of the patients who reported discontinuing TAI gave reasons independent of the efficacy of the treatment (technical problems or constraints) and that (2) the only predictive factor for TAI discontinuation was the progress of the training.

Applications

Presented results indicated that the first training session should be better structured in order to promote more realistic expectations of treatment efficacy, side-effects, and, especially, constraints in order to reduce the discontinuation rate. In the future, authors plan to schedule several successive hospital or home training sessions with a specialized nurse for patients whose first session led to complications in order to improve their compliance.

Terminology

The first training session is important to predict patient compliance with TAI.

Peer-review

This paper on TAI is interesting, well written, with exhaustive tables and figures. The limitations are already outlined by the authors at the end of the discussion section.

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ORIGINAL ARTICLE

Retrospective Study

Is endoscopic ultrasonography essential for endoscopic resection of small rectal neuroendocrine tumors?

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Institutional review board statement: The study protocol was approved by the Institutional Review Board at the Pusan National University Yangsan Hospital (IRB number 05-2015-043).

Informed consent statement: Patients were not required to give informed consent for study participation because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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Abstract

AIM

To evaluate the importance of endoscopic ultrasonography (EUS) for small (≤ 10 mm) rectal neuroendocrine tumor (NET) treatment.

METHODS

Patients in whom rectal NETs were diagnosed by endoscopic resection (ER) at the Pusan National University Yangsan Hospital between 2008 and 2014 were included in this study. A total of 120 small rectal NETs in 118 patients were included in this study. Histologic features and clinical outcomes were analyzed, and the findings of endoscopy, EUS and histology were compared.

RESULTS

The size measured by endoscopy was not significantly different from that measured by EUS and histology (r = 0.914 and r = 0.727 respectively). Accuracy for the depth of invasion was 92.5% with EUS. No patients showed invasion of the muscularis propria or metastasis to the regional lymph nodes. All rectal NETs

were classified as grade 1 and demonstrated an L-cell phenotype. Mean follow-up duration was 407.54 ± 374.16 d. No patients had local or distant metastasis during the follow-up periods.

CONCLUSION

EUS is not essential for ER in the patient with small rectal NETs because of the prominent morphology and benign behavior.

Key words: Neuroendocrine tumor; Small; Rectal; Endoscopic ultrasonography; Histology; Endoscopy

Core tip: Small rectal neuroendocrine tumors (NETs; ≤ 10 mm) that are confined to the mucosa or submucosa can be managed by endoscopic resection because of their low risk of metastatic spread. According to the 2015 guidelines of the National Comprehensive Cancer Network, when we evaluate rectal NET, endorectal magnetic resonance or endoscopic ultrasonography (EUS) is recommended. However, EUS may not be essential for evaluation of small rectal NET because of its prominent morphology and benign behavior.

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INTRODUCTION

Rectal neuroendocrine tumors (NETs) have rapidly increased in incidence, with more than a 10-fold increase occurring over the last 30 years[1]. However, the rectum remains one of the most frequent sites of digestive NETs[1,2]. The treatment of rectal NETs depends on the tumor size and depth of invasion[1]. Recent consensus guidelines on the management of rectal NETs suggest that small tumors (≤ 10 mm) that are confined to the mucosa or submucosa can be managed by endoscopic resection (ER) because of their low risk of metastatic spread[3,4].

Endoscopic ultrasonography (EUS) was found to be useful for measuring the size and local staging of rectal NETs, which is essential information for determining appropriate treatment[5-8]. Endoscopic size measurement using forceps or other accessories is also possible because the majority of rectal NETs are located in the mucosa or submucosa and have the easily recognized features of a nodular shape with a yellowish color. According to a previous study of 237 patients with rectal NETs < 10 mm, none had metastasis to the regional lymph node (LN) or invasion of the muscularis propria[9]. Furthermore, EUS with miniprobe, which is commonly used for the evaluation of small rectal NETs, offers limited assessment of regional LNs because of its low penetration depth. Thus, endoscopic evaluation without EUS may be sufficient in the management of small rectal NETs, but this has not been well established.

This study was designed to evaluate the biologic behavior of small NETs, to analyze the accuracy of endoscopy and EUS as compared with pathologic findings, and to determine the clinical impact of EUS in the choice of treatment strategy for small rectal NETs.

MATERIALS AND METHODS

Study design

This retrospective study was performed at a single tertiary referral center. Patients in whom rectal NETs were diagnosed by ER at the Pusan National University Yangsan Hospital between 2008 and 2014 were considered for study inclusion. Among the 132 rectal NETs that were treated with ER, 118 patients with a total of 120 small (≤ 10 mm) rectal NETs were enrolled in this study. Two of the patients had two small rectal NETs each. Twelve rectal NETs were excluded from study, and these included 5 patients without EUS examination, 4 patients with no visualization by EUS, and 3 patients with a tumor size > 10 mm. All rectal NETs were found incidentally on a screening colonoscopy, and all patients underwent computed tomography (CT) to assess the presence of metastasis to the perirectal lymph nodes or liver. CT investigations revealed that none of the tumors was associated with either regional LN or distant metastasis.

Rectal NETs were defined as tumors located within 15 cm of the anal verge, while tumors located more than 15 cm above the anal verge were regarded as colonic NETs. Rectal NETs were treated by ER, including endoscopic submucosal dissection, endoscopic mucosal resection (EMR) and EMR with suction methods, and were diagnosed by histologic analysis.

The study protocol was approved by the Institutional Review Board at the Pusan National University Yangsan Hospital (IRB number 05-2015-043).

Measurements

Medical records were reviewed retrospectively to extract clinical information, such as endoscopic, EUS and histologic findings. We consistently described the size, color and shape characteristics of the tumors according to the endoscopic record; however, if the endoscopic description of the tumor size was vague, it was retrospectively re-estimated.

The size and depth of invasion for all NETs was examined by endoscopy, EUS and histologic examination. The NET size was estimated by measuring the diameter of the lesion, and using an open or closed biopsy forceps

as a size reference on endoscopic examination. The biopsy forceps was then closed for use in determining the consistency and mobility of the mass.

Immediately after the endoscopic examination, EUS examination was performed by the UM-DP20-25R miniature ultrasonic probe (Olympus Medical Systems Corp, Tokyo, Japan) with a frequency of 20 MHz and the EU-M2000 sonogram processing equipment (Olympus). On EUS examination, the cross-sectional size of the lesion was measured electronically and the location of the lesion was identified as the EUS layer of origin. We also examined the appearance of the NET on EUS (hypoechoic or hyperechoic, homogenous or inhomogenous), and determined whether or not tumor invasion of the proper muscle layer had occurred. After EUS examination, ER was performed. The type of ER was determined by the size of the NET and by the endoscopists' experience and preference. All endoscopy, EUS and ER procedures were performed by two experienced endoscopists (Kim HW, Choi CW).

We did not perform biopsy before ER because biopsy can induce fibrosis and complicate removal of the submucosal lesion. After endoscopic removal, all resected specimens were evaluated histologically using light microscopy at both low and high power magnifications. Histologic examination of the NETs was performed, and included determination of tumor size, mitotic count, Ki-67 index, presence of lymphovascular invasion and margin status. The grading system used for NETs was that of the European Neuroendocrine Tumor Society[10]. NETs were classified as grade 1, grade 2 or grade 3 according to the mitotic count and the Ki-67 index. The three tumor categories were defined as follows[11]: grade 1, mitotic count < 2 per 10 high-power fields (HPFs) and/or ⩽ 2% on the Ki-67 index; grade 2, mitotic count 2-20 per 10 HPFs and/or 3%-20% on the Ki-67 index; and grade 3, mitotic count > 20 per 10 HPFs and/or > 20% on the Ki-67 index.

Examination following ER

Medical records were reviewed to determine the clinical outcomes. The first visit to the outpatient clinic was usually performed within 1 to 2 wk after endoscopic treatment and included a confirmation of the pathologic report as well as an assessment for any complications, such as bleeding and perforation. All patients were recommended surveillance colonoscopies and abdominal CT, with the first follow-up at 6 mo and the second follow-up at 30 mo after endoscopic treatment. All patients underwent the first 6-mo follow-up examination, but some patients did not undergo the second follow-up examination.

Statistical analysis

Continuous variables were reported as mean ± SD or as median (range), and categorical variables were reported as frequency (*i.e.*, %). The diagnostic accuracy of EUS was compared with that of histology in the subset of cases where tissue was obtained. The association among the size estimates by endoscopy, size measurements by EUS and histology was evaluated by calculating the Pearson correlation coefficients with unadjusted significance levels (correlation analysis, r value > 0.7: considered to be strongly correlated). Comparison of diagnostic certainty by endoscopy, EUS and histology was performed by using the Wilcoxon signed-rank test (Wilcoxon signed-rank test, P > 0.10: not statistically significant). All data analyses were performed by the Statistical Package for the Social Sciences (SPSS) software (version 18.0; SPSS, Chicago, IL, United States).

RESULTS

One hundred and eighteen patients [76 men and 42 women, with a mean age of 50.7 ± 11.4 years (range 18-77 years)] with a total of 120 rectal NETs were enrolled in this study. Two patients had two rectal NETs each. For most of the tumors, endoscopic morphology showed sessile or slightly elevated lesions (n = 110, 91.7%), with the others being flat lesions (n = 10, 8.3%). Some tumors had central depression (n = 8, 6.7%). The types and proportions of ERs were conventional EMR (n = 3, 2.5%), EMR with suction methods (n = 70, 58.3%) and endoscopic submucosal dissection (n = 47, 39.2%).

On histologic evaluation, all tumors were classified as grade 1 and as either enteroglucagon type or L-cell type. Microscopic invasion was observed in the histologic findings for 1 case [both lymphatic and vascular invasion (n = 1)]. Lymphovascular invasion was found in 1 patient who had a 6-mm tumor that required additional surgical therapy of low anterior resection. There was no recurrence during the follow-up periods. The mean follow-up period was 407.54 ± 374.16 d (range 154-2148 d) for all patients. Of the 120 lesions evaluated, 23 had follow-up at ⩾ 24 mo. The demographics of the lesions are shown in Table 1.

Size and depth measurements

The sizes of the rectal NETs were estimated by endoscopy, EUS and histologic findings, and the results were 5.47 ± 1.78 mm, 5.53 ± 1.76 mm and 5.54 ± 2.15 mm respectively. Overall, the sizes estimated by the three different methods were similar and not statistically different (Table 1). The mean size differences between the endoscopic and EUS measurements, between endoscopy and histology, and between EUS and histology were 0.065 ± 0.650 mm (maximum error range 1.5 mm), 0.071 ± 1.407 mm (maximum error range 3.0 mm) and 0.006 ± 1.393 mm (maximum error range 3.5 mm) respectively. None of the size differences were statistically significant (Table 2).

There was very good correlation between the sizes

Table 1 Clinical data of the small rectal neuroendocrine tumors *n* (%)

Parameter	Total (*n* = 120)
Tumor size in mm, mean ± SD	
Endoscopy	5.47 ± 1.78
EUS	5.53 ± 1.76
Histology	5.54 ± 2.15
Endoscopic morphology	
Sessile or slightly elevated	110 (91.7)
Flat	10 (8.3)
Central depression	8 (6.7)
Resection method	
Conventional EMR	3 (2.5)
EMR with suction methods	70 (58.3)
ESD	47 (39.2)
Histologic grade	
1	120 (100)
2	0
3	0
Histologic type	
Enteroglucagon or L-cell	120 (100)
Enterochromaffin or enterochromaffin-like cell	0
Microscopic invasion	
Lymphatic and vascular	1 (0.8)
Lymphatic	0
Vascular	0
Follow-up duration	
6-12 mo	84 (70.00)
12-24 mo	13 (10.83)
24-36 mo	16 (13.33)
⩾ 36 mo	7 (5.83)
Follow-up in day, median (range)	196 (154-2148)
Follow-up in day, mean ± SD	407.54 ± 374.16

EUS: Endoscopic ultrasound; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

Table 2 Comparison among the sizes measured by endoscopy, endoscopic ultrasonography and histology

Measurement technique	Wilcoxon signed-rank test
Endoscopy and EUS	$P = 0.215$
Endoscopy and histology	$P = 0.540$
EUS and histology	$P = 0.933$

EUS: Endoscopic ultrasonography.

Table 3 Correlation coefficient among the sizes measured by endoscopy, endoscopic ultrasonography and histology

Measurement technique	Correlation coefficient
Endoscopy and EUS	0.914 ($P < 0.01$)
Endoscopy and histology	0.727 ($P < 0.01$)
EUS and histology	0.727 ($P < 0.01$)

EUS: Endoscopic ultrasonography.

estimated by endoscopy and by EUS ($r = 0.914$, $P < 0.001$), and the size measurements of both endoscopy and histology, and EUS and histology, were well correlated ($r = 0.727$, $P < 0.001$ and $r = 0.727$, $P < 0.001$ respectively) (Figure 1 and Table 3).

Table 4 Comparison of depth of invasion measured by endoscopic ultrasonography and histology *n* (%)

Depth of invasion	EUS	Histology
2nd layer (muscularis mucosa)	9 (7.5)	2 (1.7)
3rd layer (submucosa)	111 (92.5)	118 (98.3)
4th layer (muscularis propria)	0 (0)	0 (0)
EUS accuracy	111 (92.5)	

EUS: Endoscopic ultrasonography.

The locations of the rectal NETs estimated by EUS were found at the second layer ($n = 9$, 7.5%) and the third layer ($n = 111$, 92.5%), but none were found at the fourth layer. The accuracy of EUS as compared to histology was 92.5% (Table 4). Involvement of the muscularis propria was not observed by either EUS or histology in any of the cases.

DISCUSSION

The overall incidence of rectal NETs is rapidly increasing[1], and the incidence in South Korea, in particular, has reached 48% among gastroenteropancreatic NETs[12]. Since most small rectal NETs will follow an indolent course with very infrequent local and distant metastases, endoscopic treatment is usually recommended. Endoscopic treatment for rectal NETs is considered a curative treatment for lesions ⩽ 10 mm in diameter and without lymphovascular invasion or metastasis. However, metastasis was reportedly detected in 9.7% of cases of ⩽ 10 mm rectal NETs[13], and the reported range of rates of distant metastasis in patients with rectal NETs is 2%-8%[10,14,15]. In our study, lymphovascular invasion was found in 1 patient. However, more studies are needed to verify the low metastasis rate in recent studies.

Risk factors for metastasis include tumor size, muscularis propria invasion, histologic grade (Ki-67 index and mitotic count), lymphovascular invasion, neural invasion, and atypical endoscopic features[10,16]. In particular, presence of a > 10-mm tumor size and lymphatic invasion strongly corresponded to LN metastasis, with the rate of LN metastasis increasing to 16% with either one of these two risk factors, and further increased to as high as 77% in patients with both risk factors[17].

In order to ascertain the presence of local or distant metastasis before the endoscopic treatment of rectal NETs, EUS and CT scan are routinely performed. EUS is especially useful for measuring the size and performing local staging of rectal NETs, which is essential for determining appropriate treatment[5-8]. Yet, the clinical value of EUS, particularly of EUS with miniprobe used for small rectal NETs, decreases in conjunction with the very low risk of LN metastasis, limited scope of evaluation for regional LNs, and ease of endoscopic measurement because of the well-demarcated margins of rectal NETs. Moreover, the

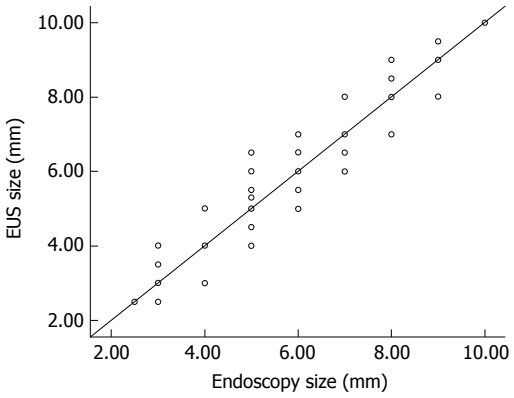

Figure 1 Correlation between the sizes of neuroendocrine tumors measured by endoscopy and endoscopic ultrasonography (r = 0.914). EUS: Endoscopic ultrasonography.

predictive ability of EUS regarding other risk factors, such as histologic grade and lymphovascular invasion, is minimal. Considering these issues, an effective evaluation of regional LN metastasis is possible using a combination of endoscopy and histology.

Recent reports have suggested that L-cell phenotype and small tumor size predict a favorable clinical outcome for rectal NETs[18,19]. In a Korean study, the frequency of the L-cell phenotype as detected by tumor immunoreactivity for L-cell markers (*i.e.*, glucagon-like peptide 1, pancreatic peptide and peptide YY) was 79%[18]. In our study, the frequency of enteroglucagon or L-cell phenotype, determined by histologic pattern and chromogranin A immunoreactivity, was 100%. We did not evaluate tumor immunoreactivity for L-cell markers, since performance of these studies involves high costs and some previous studies have suggested that about 80% of rectal NETs are L-cell type with typical trabecular pattern and reduced/absent chromogranin A immunoreactivity[20]. Therefore, given the highly frequent detection of the L-cell phenotype, EUS evaluation for regional LN metastasis and invasion of the muscularis propria is not essential for small rectal NETs.

Several studies have examined the utility of EUS in the assessment and management of rectal NETs[2,5-8,21]. However, no previous studies have compared the size measurement of rectal NETs by endoscopy to either measurements by EUS or by histology; although, one study compared endoscopy and EUS in the evaluation of gastrointestinal subepithelial masses, including carcinoid tumor[22].

Our study examined the accuracy of endoscopy and EUS in evaluating 120 rectal NETs in 118 patients. The purpose of this study was to determine whether endoscopy could (1) measure the size of tumors as accurately as EUS; (2) estimate the invasion of the muscularis propria or metastasis to regional LNs; and (3) perform an adequate evaluation of small rectal NETs prior to ER.

In our study, the endoscopic size estimation was similar to the measurements of EUS and histology, with no greater than a 3.5 mm difference between them. The measuring methods were also strongly correlated (Table 3 and Figure 1), and these findings showed the accuracy of endoscopy as well as the accuracy of EUS for small rectal NETs. Although previous reports on endoscopic size estimation have suggested that this method is inaccurate, primarily due to underestimation of the lesion size[23-25], the findings of our study and of Hwang *et al*[22] have suggested that endoscopy is quite accurate in determining the size of subepithelial masses if the size is estimated using a known size reference, such as an open biopsy forceps. We found a strong correlation between size measurements by endoscopy and EUS (r = 0.914, P < 0.001), with a mean difference in size measurement of 0.065 ± 0.650 mm. The correlation between size measurements by endoscopy and histology was also significant (r = 0.727, P < 0.001). Therefore, the size of small rectal NETs could be as accurately estimated by endoscopic examination as by EUS, if used with a known size reference.

As shown in Table 4, the diagnostic accuracy of EUS for invasion depth was 111/120 (92.5%), and invasion of the muscularis propria was not present in any of the rectal NETs. Therefore, although the evaluation of invasion depth by EUS is very effective, these findings showed that using EUS to evaluate invasion depth is not essential for small rectal NETs.

According to Kasuga *et al*[26], factors such as a tumor size of 10 mm or more, the presence of central depression, depth of tumor invasion, lymphatic invasion and venous invasion were all significantly associated with a higher incidence of LN metastasis on univariate analysis. Multivariate analysis revealed that a tumor size of 10 mm or more and the presence of venous invasion were independently predictive of LN metastasis.

Our study has some limitations. First, the patients' information could be inaccurate because of the selection bias related to the retrospective nature of the study. Second, the accuracy of endoscopic measurement differs depending on the endoscopists' level of experience. Third, the number of rectal NETs in our study is relatively small. Since there is generally a very low risk of LN metastasis and invasion of the muscularis propria, a study with a greater number of small rectal NETs is necessary to capture more accurate results. Fourth, the short follow-up interval may have influenced the results of our study because rectal NETs progress very slowly.

In conclusion, though the EUS estimation of tumor size and depth of invasion in small rectal NETs was very accurate and useful, the endoscopic estimation was equivalent. Overall, the accuracy of the endoscopic measurement, along with the lack of muscularis propria involvement in all of the rectal NETs of our study, suggested that histologic evaluation after ER is more significant than EUS evaluation before ER. Therefore,

EUS may not be an essential factor in deciding the treatment strategy for small rectal NETs.

COMMENTS

Background

Endoscopic ultrasonography (EUS) is commonly used in cases of neuroendocrine tumors (NETs) to evaluate the size of the tumor, invasion of the muscularis propria and metastasis of regional lymph nodes. This study was designed to investigate the biologic behavior of small (≤ 10 mm) rectal NETs and the clinical impact of EUS for endoscopic resections (ER).

Research frontiers

No previous studies have compared the size measurement of rectal NETs obtained by using endoscopy to measurements from either EUS or histology; although, one study compared endoscopy and EUS in the evaluation of gastrointestinal subepithelial masses. The size measured by endoscopy was not significantly different from that measured by EUS and histology. Moreover, the small rectal NETs had an indolent course and a low metastasis rate, compared with a few prior reports. The results of this study suggest the utility of performing EUS in small rectal NETs selectively.

Innovations and breakthroughs

EUS is helpful for measuring accurate tumor size and depth of invasion; however, it is not always necessary for deciding the treatment strategy. In this report, small rectal NETs were evaluated carefully by endoscopy and, then, if the tumor was deemed appropriate for endoscopic removal and no risk factors were present, it could be removed without EUS. It is important to identify risk factors and histologic results after ER.

Applications

This study suggests that EUS may not be necessary in deciding the treatment strategy for small rectal NETs ≤ 10 mm. If the lesion is strongly suspected with small rectal NET, ER can be chosen based on endoscopic features and size measurement without evaluating EUS.

Terminology

EUS is an endoscopic procedure that enables observation of the chest and abdominal organs, including the gastrointestinal tract.

Peer-review

The authors of this paper highlight the importance of EUS for deciding treatment strategy of small rectal NETs. EUS may not be essential for ER in the patient with small rectal NETs because of the relatively exact size measurement and prominent morphology obtained through endoscopy and the benign behavior of this tumor type. EUS can be applied selectively in patients with risk factors, and further clinical trials in a large population of patients with small rectal NETs will be valuable.

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ORIGINAL ARTICLE

Retrospective Study

Pancreatic hardness: Correlation of surgeon's palpation, durometer measurement and preoperative magnetic resonance imaging features

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Author contributions: Hong TH and Choi JI contributed equally to this work; Hong TH, Choi JI, You YK and Choi MH collected and analyzed the data; Choi JI, and Choi MH designed and supervised the study; Hong TH, Choi MH wrote the manuscript; Rha SE revised the manuscript for important intellectual content; Lee YJ provide administrative support; Park MY reviewed the statistical methods; all authors have read and approved the final version to be published.

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Abstract

AIM

To evaluate the correlation between subjective assessments of pancreatic hardness based on the palpation, objective measurements using a durometer, and magnetic resonance imaging (MRI) findings for assessing pancreatic hardness.

METHODS

Eighty-three patients undergoing pancreatectomies were enrolled. An experienced surgeon subjectively evaluated the pancreatic hardness in the surgical field by palpation. The pancreatic hardness was also objectively

evaluated using a durometer. Preoperative MRI findings were evaluated by a radiologist in terms of the apparent diffusion coefficient (ADC) values, the relative signal intensity decrease (RSID) of the pancreatic parenchyma, and the diameter of the pancreatic parenchyma and duct. Durometer measurement results, ADC values, RSID, pancreatic duct and parenchyma diameters, and the ratio of the diameters of the duct and parenchyma were compared between pancreases judged to be soft or hard pancreas on the palpation. A correlation analysis was also performed between the durometer and MRI measurements.

RESULTS

The palpation assessment classified 44 patients as having a soft pancreas and 39 patients as having a hard pancreas. ADC values were significantly lower in the hard pancreas group. The ductal diameter and duct-to-pancreas ratio were significantly higher in the hard pancreas group. For durometer measurements, a correlation analysis showed a positive correlation with the ductal diameter and the duct-to-pancreas ratio and a negative correlation with ADC values.

CONCLUSION

Hard pancreases showed lower ADC values, a wider pancreatic duct diameter and a higher duct-to-pancreas ratio than soft pancreases. Additionally, the ADC values, diameter of the pancreatic duct and duct-to-pancreas ratio were closely correlated with the durometer results.

Key words: Pancreas; Texture; Hardness; Magnetic resonance imaging; Fistula

Core tip: The texture of the pancreas is an important predictive factor for the development of postoperative complications following pancreatic surgery. Durometric measurements correlated well with surgeons' palpation-based assessment of the hardness of pancreas and histologic evaluation for fibrosis and fat content. If we could estimate the texture of the pancreas preoperatively, it would be very helpful for surgeons to prepare for the possibility of postoperative pancreatic leakage or fistula. Preoperative magnetic resonance imaging (MRI) can be used to predict the hardness of pancreas. Apparent diffusion coefficient (ADC) values measured in MRI were significantly lower in the hard pancreas than soft pancreas. The ductal diameter and duct-to-pancreas ratio were significantly higher in the hard pancreas. For durometer measurements, a correlation analysis showed a positive correlation with the ductal diameter and the duct-to-pancreas ratio and a negative correlation with ADC values.

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INTRODUCTION

The texture of the pancreas is an important predictive factor for the development of postoperative complications following pancreatic surgery. Postoperative pancreatic leakage or fistula formation is more frequent in soft pancreases than hard pancreases[1-5]. The pancreatic texture is determined by a combination of fatty infiltration and fibrotic change. A decreased pancreatic fat content and increased fibrosis are related to a harder pancreatic texture. A hard pancreas resulting from fibrosis has a high suture-hold capacity and low pancreatic juice secretion; thus, pancreatic leaks or pancreatic fistulae occur less frequently in patients with a hard pancreas[3,4,6-8].

The texture of the pancreas can be evaluated based on the histologic characteristics of the pancreas, including the degrees of fibrosis and fatty infiltration. However, a considerable amount of time is necessary to acquire histologic results. Therefore, the most commonly used method to assess pancreatic hardness in practice is the surgeon's subjective determination of pancreatic hardness during the operation. Given that a surgeon's palpation-based determination may not be reproducible, objective measurement using a durometer was suggested[9]. Durometric measurements correlated well with surgeons' palpation-based assessment and histologic evaluation for fibrosis and fat content[6,9]. One additional benefit of the durometer is that the durometer is easy to use, and quantitative assessment is possible.

The surgeon's palpation-based determination and durometer measurement can both be performed intraoperatively. If we could estimate the texture of the pancreas preoperatively, it would be very helpful for surgeons to prepare for the possibility of postoperative pancreatic leakage or fistula. Magnetic resonance imaging (MRI) is one possible candidate for the preoperative evaluation of the texture of the pancreas. Diffusion-weighted imaging (DWI) can be applied to the pancreas to measure pancreatic fibrosis[10-12]. The degree of fibrosis is negatively correlated with apparent diffusion coefficient (ADC) values[10,11]. An increase in the fat content is noted in soft pancreases, and the fat content of the pancreas can be estimated using in- and opposed-phase MRI[13]. As pancreatic hardness is caused by increased fibrosis, parenchymal atrophy and duct dilatation resulting from fibrosis are also important parameters to predict pancreatic hardness[14]. Therefore, the aim of this study was to evaluate the correlation among the subjective palpation-based assessment, objective measurements using a

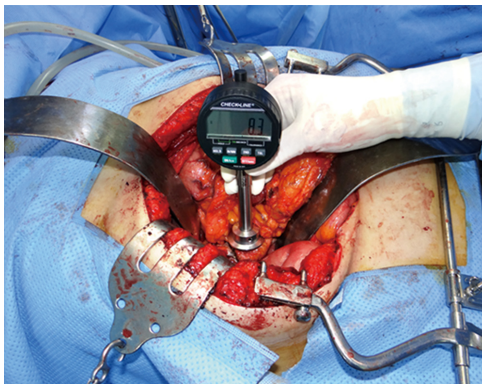

Figure 1 Measurement of pancreatic hardness by durometer during the operation. A Rex Durometer (Rex Gauge, Buffalo Grove, IL, United States) was placed perpendicular to the pancreatic parenchyma where no tumor was located to measure the pancreatic hardness.

Table 1 Magnetic resonance imaging sequences and parameters

	3-T system		
	T2WI HASTE	T1WI	DWI
TR	800	3.2	4500
TE	95	1.3	56
ETL	70	1	
Thickness (mm)	6	2.8	6
Slice gap	0	0	0
FOV (mm^2)	380 × 309	380 × 309	400 × 313
Matrix size	320 × 156	384 × 250	120 × 94
NEX	2	1	47
b factor (s/mm^2)			0, 500

TR: Repetition time; TE: Echo time; ETL: Echo train length; FOV: Field of view; HASTE: Half-Fourier acquisition single-shot turbo spin-echo; NEX: Number of excitations; DWI: Diffusion-weighted image; T1WI: T1-weighted image; T2WI: T2-weighted image.

durometer and MRI parameters for assessing pancreatic hardness.

MATERIALS AND METHODS

Study population

This retrospective study was approved by the Institutional Review Board, and the requirement for informed consent was waived. From September 2014 to November 2016, 145 patients underwent some resection of the pancreas, including Whipple's operation, pylorus-preserving pancreatoduodenectomy (PPPD), and distal pancreatectomy. Imaging analysis could not be performed in 62 patients for following reasons: there was no preoperative MRI (n = 32), the preoperative MRI was performed at another hospital (n = 18), DWI was not included in the preoperative MRI (n = 6), the ADC value could not be measured due to little residual pancreatic parenchyma (n = 5), and in- and opposed-phase chemical shift images were not included in the MRI (n = 1). Ultimately 83 patients (50 men and 33 women) with a mean age of 66.6 ± 10.3 years old (range, 34-89) were enrolled in this study. PPPD, Whipple's operation, and distal pancreatectomy were performed by an expert surgeon in 67, 11 and 5 patients, respectively. The indications for surgery included pancreatic cancers (n = 41), common bile duct cancers (n = 30), cystic tumors of the pancreas (n = 8), neuroendocrine tumors (n = 2), pancreatic duct stricture (n = 1), and duodenal cancer (n = 1).

Measurement of pancreas stiffness (or intraoperative texture analysis)

The surgeon who performed the operation subjectively evaluated the pancreatic hardness by palpation during the operation before resection of the pancreas. The pancreatic hardness was classified into the following four categories: very hard, hard, soft or very soft. Objective measurement of the pancreatic hardness was performed by the same surgeon during the operation using a durometer. A Rex Durometer (Rex Gauge, Buffalo Grove, IL, United States) was placed perpendicular to the pancreatic parenchyma where no tumor was located (Figure 1). The unit of the durometer result was displayed using a 0- to 100-point scale in durometer units (DU).

MRI protocol

All MRI examinations were performed using a 3-T MR unit (Magnetom Verio; Siemens Healthcare, Erlangen, Germany) with an 8-channel phase-array coil. The detailed parameters of the routine MRI protocol are summarized in Table 1. MR examination consisted of fat-suppressed T2-weighted images, T1-weighted images, chemical shift in- and opposed-phase images, contrast-enhanced T1-weighted images with fat suppression and diffusion-weighted images.

Image analysis

A radiologist with 7 years of experience performed the image analysis. The ADC value of the pancreatic parenchyma was measured three times using an operator-defined irregular shaped ROI (50-100 mm^2) on axial images. The mean and minimum ADC values from the ROI with the smallest standard deviation among the three measurements were used for analysis. Estimation of the fat content in the pancreas was performed using a previously suggested method called relative signal intensity decrease (RSID)[13,15]. The signal intensity (SI) of the spleen was used to normalize the pancreatic SI. The RSID was calculated using the following formula: RSID = 100 × (Pin/Sin - Pop/Sop)/(Pin/Sin). Pin and Pop were the signal intensities of the pancreas during in-phase and opposed-phase imaging, respectively. Sin and Sop were the signal intensities of the spleen during in-phase and opposed-phase imaging. The diameters of the pancreatic duct and pancreatic parenchyma at the body-to-tail junction were measured to evaluate the severity of pancreatic atrophy, and the ratio of duct to

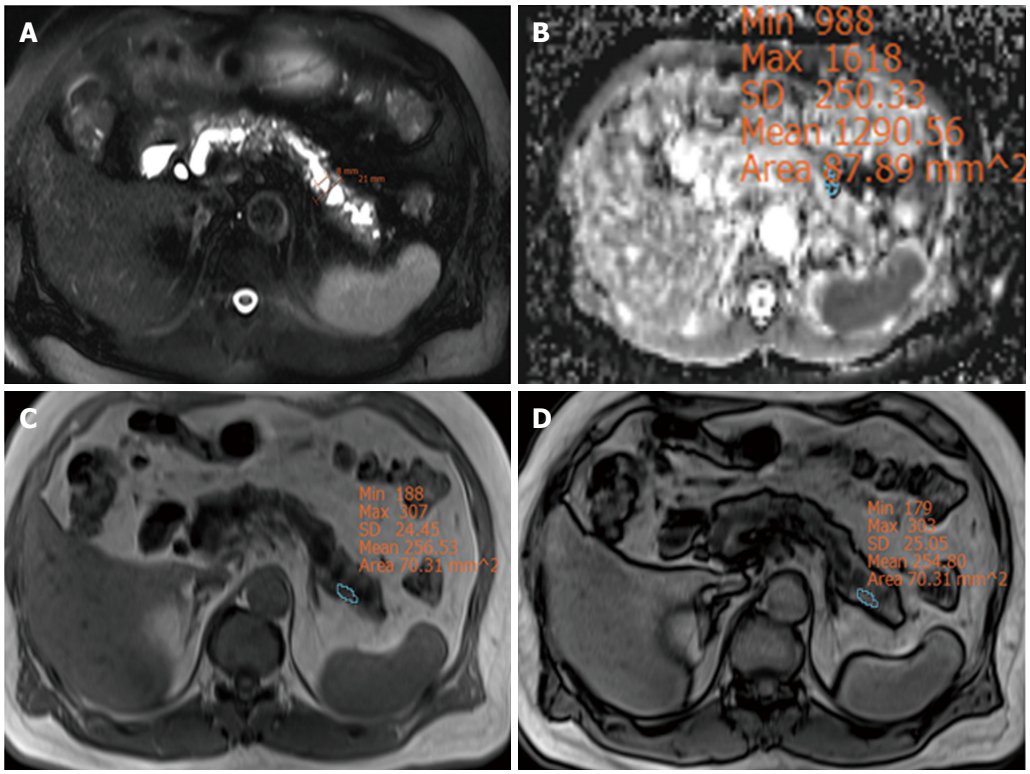

Figure 2 Pancreas cancer with a hard pancreas. Magnetic resonance imaging of a 66-year-old male with pancreas head cancer who was found to have a hard pancreas (durometer measurement: 18 DU). The diameters of the pancreatic duct and parenchyma were 8 mm and 21 mm, respectively (A). The apparent diffusion coefficient value of the pancreatic parenchyma was 1.290 mm^2/s (B). The signal intensity of the pancreas on in-phase imaging was 256.53 (C) and on opposed-phase imaging was 254.80 (D).

parenchyma was calculated.

Statistical analysis

The statistical review of the study was performed by a biomedical statistician. Statistical analysis was performed using SPSS 24.0 (IBM Corporation, Armonk, NY, United States). A P value < 0.05 was considered statistically significant.

Patients were classified into two groups as having a soft or hard pancreas according to the surgeon's subjective assessment of pancreatic hardness. Both very hard and hard pancreases were included in the hard pancreas group, while both soft and very soft pancreases were included in the soft pancreas group. The durometer measurement, ADC value, RSID, ductal diameter, parenchymal diameter and duct/parenchyma ratio (D/P ratio) were compared between the soft and hard pancreas groups using the Mann-Whitney U test. The Spearman correlation test was used to evaluate the correlation between the ADC value, RSID, diameters of the pancreatic parenchyma and pancreatic duct or D/P ratio and the durometer measurement. The association of each variable with subjective pancreatic hardness was evaluated using univariate logistic regression analysis. Variables with statistical significance or borderline significance (P < 0.15) were used in the multivariate logistic regression analysis.

RESULTS

Subjective analysis of pancreatic hardness and image analysis

The surgeon classified 39 patients as having a hard pancreas and 44 patients as having a soft pancreas. The duct diameter and D/P ratio in the hard pancreas group were significantly greater than in the soft pancreas group (P < 0.001). The mean and minimal ADC values in the hard pancreas group were significantly lower than in the soft pancreas group (P = 0.012, 0.004, respectively) (Table 2). The RSID was not different between two groups. Two representative cases are displayed in Figures 2 and 3.

Durometer and image analysis

The durometer measurement was significantly lower in the soft pancreas group [median 11, interquartile range (IQR) 8-13] than in the hard pancreas group (median 25, IQR 21-28) (P < 0.001). The mean and minimal ADC were negatively correlated with durometer measurement, and the correlation was statistically significant (P < 0.040, r = -0.280 and P < 0.010, r = -0.223, respectively). The ductal diameter showed a significant correlation with the durometer measurement (P < 0.005, r = 0.303). There was also significant correlation between the D/P ratio and the durometer measurement (P < 0.019, r = 0.257)

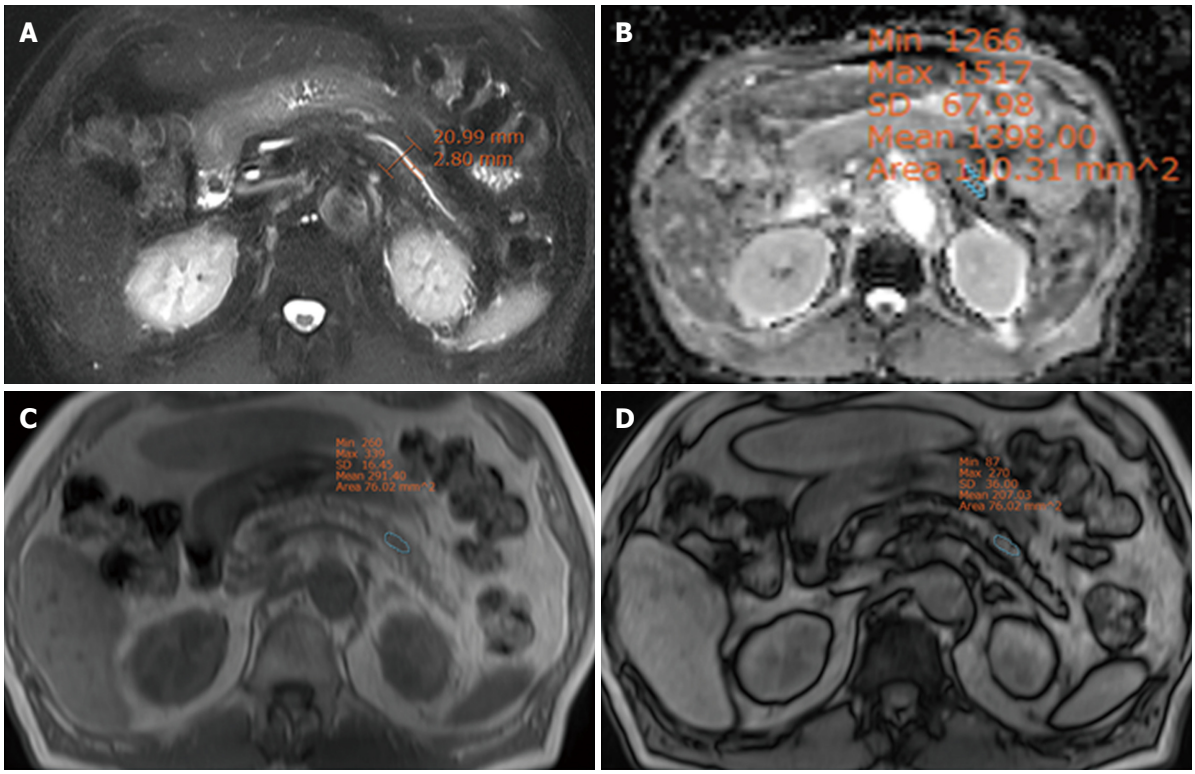

Figure 3 Pancreas cancer with a soft pancreas. Magnetic resonance imaging of a 74-year-old male with pancreas head cancer who was found to have a soft pancreas (durometer measurement: 8 DU). The diameters of the pancreatic duct and parenchyma were 3 mm and 21 mm, respectively (A). The apparent diffusion coefficient value of the pancreatic parenchyma was 1.398 mm^2/s (B). The signal intensity of the pancreas on in-phase imaging was 291.40 (C) and on opposed-phase imaging was 207.03 (D).

Table 2 Comparison of parameters between two groups based on the surgeon's subjective analysis of the pancreas

	Soft (n = 44)	Hard (n = 39)	P value
Parenchyma diameter	15 (13-20.75)	15 (13-20)	0.791
Duct diameter	3 (1-3)	5 (3-7)	< 0.001
D/P ratio	0.13 (0.08-0.23)	0.25 (0.18-0.55)	< 0.001
ADC_{mean} (mm^2/s)	1.62 (1.50-1.77)	1.45 (1.24-1.63)	0.012
ADC_{min} (mm^2/s)	1.51 (1.32-1.59)	1.27 (1.11-1.47)	0.004
RSID (%)	10.2 (5.9-22.5)	15.2 (7.8-22.8)	0.174

Data are presented as the median and interquartile range. D/P ratio: Duct/parenchyma ratio; ADC: Apparent diffusion coefficient; ADC_{mean}: Mean ADC value; ADC_{min}: Minimal ADC value; RSID: Relative signal intensity decreases.

(Figure 4). The diameter of the pancreatic parenchyma and RSID were not correlated with the durometer measurement (P = 0.724, r = 0.039 and P = 0.052, r = 0.214, respectively).

Factors associated with pancreatic hardness

Univariate logistic regression analysis showed that duct diameter, D/P ratio, and ADC_{min} were the factors that were significantly associated with subjective pancreatic hardness. Because the durometer measurement is an objective measurement of the hardness of the pancreas, it was excluded from the regression analysis. These three significant factors and ADC_{mean}, which had borderline significance in the univariate analysis, were used in the multivariate regression analysis. Only ductal diameter remained significant after multivariate regression analysis. These data are summarized in Table 3.

DISCUSSION

In this study, three methods to measure the hardness of the pancreas, including radiologic findings, durometer measurements and surgeon palpation, were well correlated with each other. Given that the durometer measurement during the operation correlated well with the surgeon's subjective measurement, the durometer could be considered a good objective measurement method. Among the studied radiological factors, ductal diameter was the factor that most highly correlated with durometer measurement. The mean and minimal pancreatic ADC values were negatively correlated with durometer measurement to a statistically significant extent. Therefore, preoperative radiologic evaluation can be useful to predict the texture of the pancreas.

The single radiological factor that remained associated with pancreatic hardness after multivariate regression was ductal diameter. More fibrosis of the pancreas makes the pancreas harder, and duct dilatation is aggravated as the pancreas atrophies and fibrosis progresses[16]. In our study, the diameter of the pancreatic parenchyma was not correlated with durometer measurement. Therefore, dilatation of

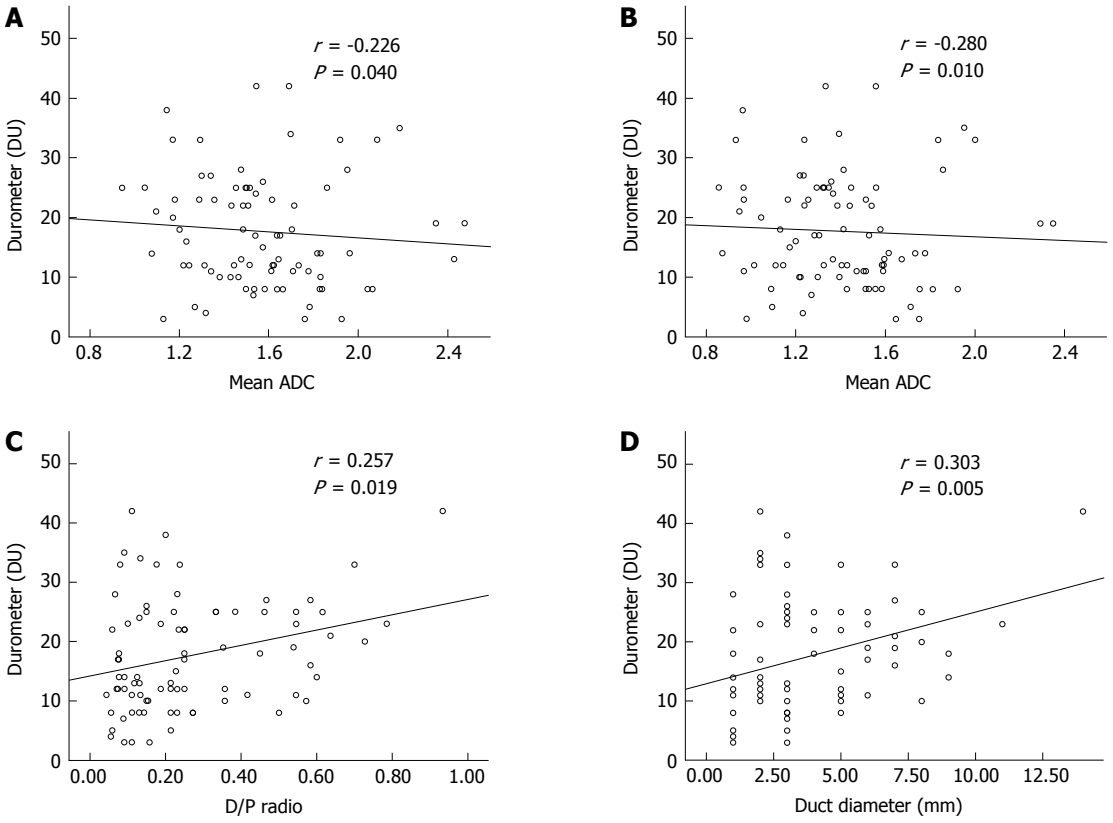

Figure 4 Scatter plots of durometer measurements *vs* apparent diffusion coefficient values. The durometer results are statistically significantly correlated with the mean apparent diffusion coefficient (ADC) (A), minimal ADC (B), duct-to-pancreas diameter ratio (C) and duct diameter (D).

Table 3 Univariate and multivariate regression analyses assessing the association between the factors and pancreatic hardness

	Univariate			Multivariate		
	Coefficient	SE	*P* value	Coefficient	SE	*P* value
Parenchyma diameter	-0.022	0.044	0.611	-		
Duct diameter	0.520	0.137	< 0.001	1.001	0.380	0.008
D/P ratio	4.819	1.442	0.001	-3.616	4.102	0.378
ADC_{mean} (mm^2/s)	-0.001	0.001	0.083	0	0.003	0.923
ADC_{min} (mm^2/s)	-0.002	0.001	0.017	-0.004	0.003	0.166
RSID (%)	0.013	0.014	0.356	-		

D/P ratio: Duct/parenchyma ratio; ADC: Apparent diffusion coefficient; ADC_{mean}: Mean ADC value; ADC_{min}: Minimal ADC value; RSID: Relative signal intensity decreases; SE: Standard error.

the duct is more important to predict fibrosis of the pancreas. As post pancreatectomy fistulae develop less frequently in fibrotic pancreases, duct dilatation may be a predictive marker for pancreatic hardness and risk of postoperative fistula[6].

There have been several studies regarding the prediction of the texture of the pancreas using preoperative MRI. Advanced MR techniques, such as intravoxel incoherent motion (IVIM) and MR elastography (MRE), have been applied to evaluate pancreas stiffness[17,18]. However, we used more common techniques, including DWI and in- and opposed-phase chemical shift images, because such emerging techniques are not generally available at all institutions. Additionally, the unique feature of our study is the correlation between the MRI measurements and the durometer measurements, which is the more intuitive and direct parameter for the hardness of the pancreas. Our results that the duct diameter and ADC were well correlated with the durometer measurement may be more useful clinically.

In this study, the ADC value was significantly correlated with durometer measurement. The ADC value was independently associated with pancreatic fibrosis and pancreatic stellate cell activity, which is known to produce desmoplasia in chronic pancreatitis and pancreas cancer[10,11]. Considering pancreatic hardness is associated with fibrosis, a low ADC value due to a hard texture can be explained by the decreased diffusion of water and the high density of

fibrin within the tissue. The usefulness of the ADC to evaluate for fibrosis or hardness of the tissue has already been proven in other organs such as the liver and kidney[19-21]. In our study, the minimal ADC value showed a higher degree of correlation than the mean ADC value. As the minimal ADC value indicates the most severe diffusion restriction, this measurement may represent the severity of the fibrosis more accurately than the mean ADC value.

Pancreatic hardness can be associated with fibrosis and fat content. In a previous study, the histological fibrosis stage but not the histological fat fraction differed among groups with different intraoperative pancreatic textures. However, the fat fraction on MRI was a significant factor that correlated with advanced fibrosis of the pancreas, and the fat fraction increased as the degree of fibrosis rose. Furthermore, the fat fraction on histologic examination was not correlated with a subjective assessment of pancreatic texture[18]. Our study showed similar results, and the RSID tended to be higher in the hard pancreas group than in the soft pancreas group, though the difference was not statistically significantly different. A larger proportion of fat in the tissue causes a greater signal decrease in opposed-phase compared with in-phase imaging; thus, a higher RSID means more fat in the tissue. Based on these results, we assumed that the fat fraction of the pancreas may be elevated with fibrosis and that the fat fraction may not independently affect pancreatic hardness.

There are several limitations in this study. First, advanced radiologic techniques such as IVIM and MRE were not applied. As they are relatively new and highly costly, they are not commonly used in many hospitals. Therefore, we thought that generally available techniques including DWI and chemical shift imaging were more useful clinically. Second, image analysis was performed by one radiologist, and the durometer measurements were performed by one surgeon. We could not evaluate the reproducibility of these radiologic and durometric analyses. However, repeated durometer measurement during the operation to evaluate reproducibility was impossible as we did not want to prolong operation time any more than strictly necessary. As the radiologic and durometric assessments were performed by experts, we believe that the reproducibility of the measurement was sufficiently high. Third, considering the retrospective nature of this study, selection bias cannot be avoided. Furthermore, many candidates were not enrolled because of the lack of MRI data. However, we enrolled almost all patients who had data for durometer measurement and MRI to tried to overcome this shortcoming.

In conclusion, hard pancreases showed lower ADC values, wider pancreatic duct diameters and higher duct-to-pancreas ratios than soft pancreases. Additionally, the ADC value, pancreatic duct diameter and duct-to-pancreas ratio also correlated well with the durometer results.

COMMENTS

Background

The texture of the pancreas is an important predictive factor for the development of postoperative complications following pancreatic surgery. Postoperative pancreatic leakage or fistula formation is more frequent in soft pancreases than hard pancreases. A hard pancreas resulting from fibrosis has a high suture-hold capacity and low pancreatic juice secretion; thus, pancreatic leaks or pancreatic fistulae occur less frequently in patients with a hard pancreas. A decreased pancreatic fat content and increased fibrosis are related to a harder pancreatic texture. The most commonly used method to assess pancreatic hardness in practice is the surgeon's subjective determination of pancreatic hardness during the operation. Durometric measurements correlated well with surgeons' palpation-based assessment and histologic evaluation for fibrosis and fat content. Magnetic resonance imaging (MRI) is one possible candidate for the preoperative evaluation of the texture of the pancreas. Diffusion-weighted imaging (DWI) can be applied to the pancreas to measure pancreatic fibrosis

Research frontiers

Because surgical resection is the only treatment expecting cure in pancreatic cancer, expanding indications of pancreatic surgery and reducing its complication is very important and many researches are published recently.

Innovations and breakthroughs

Hard pancreases showed lower apparent diffusion coefficient (ADC) values, a wider pancreatic duct diameter and a higher duct-to-pancreas ratio than soft pancreases. Additionally, the ADC values, diameter of the pancreatic duct and duct-to-pancreas ratio were closely correlated with the durometer results. Therefore, hardness of pancreas could be predicted preoperatively with MRI, which may be helpful surgeons to reduce surgery-related complications. However, there was no study correlating the hardness of pancreas to MRI findings.

Applications

If MRI is useful to predict the hardness of pancreas, it can help surgeons to reduce surgery-related complications. The authors expect future researches related to the complication rate of pancreatic surgery to the measurements from durometer and MRI.

Terminology

DWI is an imaging method that uses the diffusion of water molecules to generate contrast in MR images. It allows the mapping of the diffusion process of molecules, mainly water, in biological tissues, in vivo and non-invasively. ADC is a measure of the magnitude of diffusion (of water molecules) within tissue, and is commonly clinically calculated using MRI with diffusion weighted imaging. In- and opposed-phase: Because water and fat protons have slightly different resonance frequencies, their spins go in- and out-of-phase with each other as a function of time. On in-phase image, signals from water and fat are in the same direction and the signal is addition of these two signals. However, on opposed-phase, signals from water and fat are in the opposite direction and cancel each other.

Peer-review

This work is a review on assessing the pancreatic hardness by examining the pancreas using the durometer and MRI. Eighty three patients were involved in this study and the authors concluded that hard pancreases showed lower ADC values which correlated with the durometer results. This work has adequate number of patients and an error analyses. It is very well prepared and the conclusion is useful for the reader.

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ORIGINAL ARTICLE

Observational Study

Infection does not increase long-term mortality in patients with acute severe alcoholic hepatitis treated with corticosteroids

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Author contributions: Dhanda AD and Collins PL conceived and designed the study; Dhanda AD, Sinha A, Hunt V and Saleem S collected data; Dhanda AD conducted data analysis and drafted manuscript; Cramp ME and Collins PL edited, reviewed and approved the final article.

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Informed consent statement: Written informed consent was obtained from participants or, where they lacked capacity, assent was obtained from a personal or nominated consultee.

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Abstract

AIM

To determine whether infection in patients with acute severe alcoholic hepatitis (AAH) treated with corticosteroids is associated with increased mortality.

METHODS

Consecutive patients with AAH were treated with steroids and recruited to the study. Clinically relevant infections (body temperature > 38 ℃ or < 36 ℃ for more than 4 h, ascitic neutrophil count > 0.25 × 10^9/L, consolidation on chest radiograph or clinically relevant positive microbiological culture of bodily fluid) were recorded prospectively. Clinical and laboratory parameters were recorded and survival at 90 d and 6 mo was determined. Univariate analysis of factors associated with 90-d mortality was performed and significant variables included in a multivariate analysis.

RESULTS

Seventy-two patients were included in the final analysis (mean age 47.9 years, 26% female, mean discriminant function 53.0). Overall mortality in the group occurred in 15 (21%), 23 (32%) and 31 (43%) at day 28, day 90 and 1 year respectively. 36 (50%) had a clinically relevant infection during their hospitalisation (23 after initiation of steroids). The median time to development of incident infection after commencement of steroids was 10 d. The commonest site of infection was ascites (31%) and bacteraemia (31%) followed by urinary tract (19%) and respiratory tract (8%). Forty-one separate organisms were isolated in 33 patients; the most frequent genus was Escherichia (22%) and Enterococcus (20%). Infection was not associated with 90-d or 1 year mortality but was associated with higher creatinine, model for end-stage liver disease and Lille score. Baseline urea was the only independent predictor of 90-d mortality.

CONCLUSION

Clinically relevant infections are common in patients with AAH but are not associated with increased 90-d or 1 year mortality.

Key words: Alcoholic hepatitis; Escherichia; Infection; Lille score; Corticosteroids

Core tip: Corticosteroids are the only treatment shown to improve outcome in patients with acute severe alcoholic hepatitis (AAH) but may be associated with increased rates of infection and mortality. In this prospective cohort study of patients with AAH treated with corticosteroids rates of clinically relevant infections were accurately documented. Half of the study participants developed an infection during their hospitalisation with the commonest sites being ascites and bacteraemia. Infection was associated with higher creatinine, model for end-stage liver disease and lille score but not with higher 90-d or 1 year mortality. Infection is common in patients with AAH but is not associated with increased mortality.

Dhanda AD, Sinha A, Hunt V, Saleem S, Cramp ME, Collins PL. Infection does not increase long-term mortality in patients with acute severe alcoholic hepatitis treated with corticosteroids. *World J Gastroenterol* 2017; 23(11): 2052-2059 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i11/2052.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i11.2052

INTRODUCTION

Alcoholic hepatitis is an acute syndrome characterised by recent onset jaundice and coagulopathy in a patient with a history of prolonged and heavy alcohol consumption[1]. Despite improved recognition of and research interest in the condition, mortality remains high in patients with acute severe alcoholic hepatitis (AAH; traditionally defined as having a discriminant function > 32[2]) with 90 d and 1 year mortality of 29% and 56% respectively[3]. Data regarding cause of death is challenging to capture but a Danish registry study suggests that early mortality (within 84 d) is mostly liver related (58%) or due to infection (20%) while late mortality is also contributed to by cancer and alcohol and 16% is still due to infection[4]. Other than abstinence from alcohol the only treatment with a proven short-term survival benefit is corticosteroids (steroids)[5]. However, this benefit may be outweighed by the increased risk of infection posed by steroid treatment. Although some randomised controlled trials (RCTs) have reported higher rates of infection in steroid treated patients[3,6], it remains controversial whether increased infections result in increased mortality.

Increased risk of mortality was clearly described in a prospective cohort study in which infections that developed after initiation of steroid treatment were associated with increased 2 mo mortality[7]. However, adequately treated infections prior to commencement of steroids were not associated with increased mortality risk. A retrospective cohort analysis also demonstrated that infection at presentation or during hospitalisation was associated with increased 1 year mortality risk on univariate but not multivariate analysis[8]. However, only 43% of the cohort received steroids and the interaction between steroids and infection was not investigated. A recent sub-analysis from the United Kingdom STOPAH trial data found that prednisolone treatment was associated with increased risk of infection in the post-treatment period and that incident infection increased 28- and 120-d mortality but this was independent of steroid treatment[9]. A meta-analysis of data from 12 RCTs including a steroid arm did not demonstrate any increased infection or mortality risk associated with steroids except with the occurrence of fungal infections, which were uncommon (9 out of 1062 patients)[10].

The inconsistency of these data may be explained by poor recording of infections in clinical trials (which do not always specify prospective collection of infection information) and due to insufficient detail in retrospective analyses. Here, we add to the existing literature with a single centre prospective cohort of patients with AAH including long-term follow-up data.

MATERIALS AND METHODS

This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the NHS Health Research Authority (07/Q2007/09). Written informed consent was obtained from participants or, where they lacked capacity, assent was obtained from a personal or nominated consultee.

Table 1 Patient characteristics at baseline (day 0 of steroid treatment) and day 7 of steroid treatment (*n* = 72) and survival at day 28, day 90 and 12 mo

Age	47.9 ± 10.6
Male (%)	74
Baseline CRP (mg/L)	33 ± 26.8
Baseline bilirubin (μmol/L)	294 ± 142
Baseline albumin (g/L)	25 ± 7.7
Baseline INR	1.9 ± 0.5
Baseline PT (s)	19.2 ± 4.6
Baseline urea (μmol/L)	3.9 ± 2.7
Baseline creatinine (μmol/L)	90 ± 56.6
Baseline WBC ($\times 10^9$/L)	9.1 ± 4.5
Day 7 bilirubin (μmol/L)	251 ± 174
Baseline DF	53.0 ± 24.4
Baseline GAHS	8.1 ± 1.4
Baseline MELD	22.3 ± 6.7
Lille score	0.403 ± 0.350
Day 28 survival (%)	21
Day 90 survival (%)	32
12 mo survival (%)	43

CRP: C-reactive protein; INR: International normalised ratio; PT: Prothrombin time; WBC: White blood count; DF: Discriminant function; GAHS: Glasgow alcoholic hepatitis score; MELD: Model for end-stage liver disease.

Consecutive patients admitted to University Hospitals Bristol NHS Foundation Trust with AAH from October 2007 to September 2015 were prospectively recruited to this study. AAH was defined as new onset jaundice (within the previous 3 mo) with serum bilirubin > 80 μmol/L and coagulopathy in a heavy drinker [more than 10 units alcohol (80 g ethanol) daily in males and 7.5 units (60 g ethanol) in females within the previous 4 wk]. Additionally, discriminant function[2] was greater than 32.

Clinically relevant infections were recorded at first presentation and prospectively during hospital admission and were defined as a body temperature > 38 ℃ or < 36 ℃ for more than 4 h, ascitic neutrophil count > 0.25 × 10^9/L, consolidation on chest radiograph or clinically relevant positive microbiological culture of bodily fluid.

All patients were screened for infection on admission to hospital with chest radiograph, urinalysis, ascitic fluid analysis (where ascites was present) and peripheral blood cultures if body temperature was greater than 38 ℃. In patients with temperature < 38 ℃ and negative infection screen, oral prednisolone was commenced at a dose of 40 mg daily and continued for 28 d. Patients with a positive infection screen or body temperature > 38 ℃ were treated with broad spectrum intravenous antibiotics according to Trust protocol for at least 48 h before converting to oral antibiotics. In these patients the 28 d course of prednisolone was only started after temperature < 38 ℃ had been recorded for at least 48 h.

Patients who developed clinically relevant infection after initiation of steroid treatment (incident infections) were treated within 12 h with intravenous broad spectrum antibiotics according to Trust protocol for at least 48 h before being converted to oral antibiotics. Steroids were not discontinued during or after incident infections except in those in whom active treatment was withdrawn and palliative care was initiated.

Routine laboratory data were collected at baseline, day 7 and 28 of steroid treatment and survival status was recorded at day 90, 6 and 12 mo. Survival was determined by accessing Trust databases which are linked to community databases and where necessary by direct contact with the patient's General Practitioner. Alcohol consumption at follow-up was determined by face-to-face or telephone consultations carried out by the Alcohol Liaison Team at regular intervals after hospital discharge.

Patients who did not receive prednisolone or in whom investigators were blinded to their treatment (where they also participated in the United Kingdom STOPAH clinical trial) were excluded from this analysis.

Statistical analysis

Patient characteristics, baseline laboratory parameters, day 7 serum bilirubin levels and composite scores [discriminant function (DF), Glasgow Alcoholic Hepatitis Score (GAHS), Model for End-stage Liver Disease (MELD) and Lille score[11]] were compared between survivors and non-survivors at day 90 by univariate analysis with Mann-Whitney *U* tests for continuous data and Fisher Exact tests for categorical data. Terms that were found to be significant at the 5% level of significance were then included in a multivariate regression model which used survival at day 90 as the dependent variable.

Kaplan-Meier survival analysis was also performed at day 90 and 1 year. Survival was compared between patients with clinically relevant infection on admission, post-steroid initiation and at any time by log-rank test.

RESULTS

A total of 116 participants were recruited to the study; 44 were excluded as treatment allocation was blinded due to participation in the STOPAH trial (*n* = 42) or they did not receive prednisolone (*n* = 2; 1 with concurrent active hepatitis C infection and 1 with a borderline DF which improved to less than 32 in 24 h without treatment). Therefore 72 patients were included in the final analysis (mean age 47.9 years, 26% female, mean DF 53.0; Table 1). Overall mortality in the group occurred in 15 (21%), 23 (32%) and 31 (43%) at day 28, day 90 and 1 year respectively.

During the period of recruitment to the STOPAH trial in our centre (April 2011 to December 2013), 27 patients who met the STOPAH trial selection criteria were not recruited to it either due to patient choice or because steroids were commenced prior to screening and were hence included in the present study. These patients represent a similar population to the study

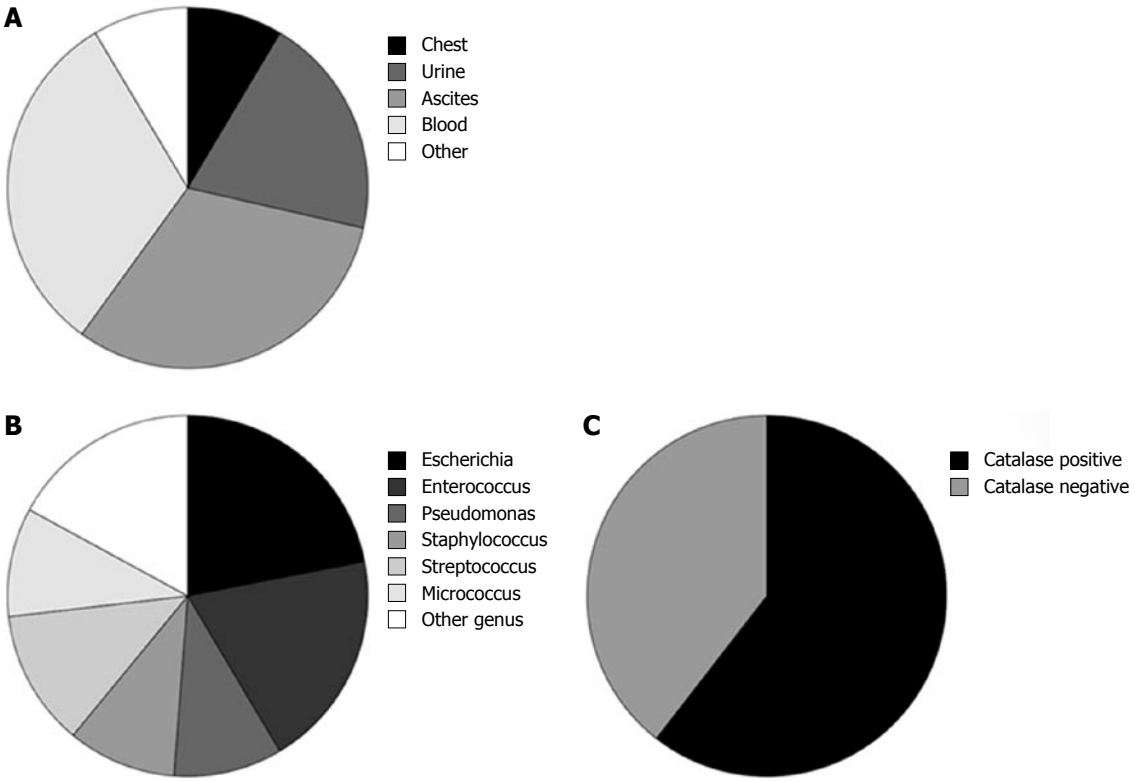

Figure 1 Site and causative bacterial genus of all clinically relevant infections during hospital admission for acute severe alcoholic hepatitis. A: The site of clinically relevant infections (n = 36). The "Other" category includes gastrointestinal tract and cutaneous; B: The causative bacterial genus where identified by microbiological analysis in 41 separate clinically relevant infections. The "Other Genus" group includes Acinebacter (n = 2), Haemophilus (n = 1), *Clostridium difficile* (n = 2) and norovirus (n = 1); C: Catalase status of identified bacteria (n = 40).

participants recruited outside the STOPAH trial recruitment period in terms of age (49 *vs* 47, P = 0.87), gender (26% *vs* 27% female, P = 1.0) and disease severity (DF 53.7 *vs* 52.7, P = 0.46). Additionally, there were no statistical differences between patients in the current study and those recruited to the STOPAH clinical trial from our centre (n = 42). Age was similar (48 *vs* 51, P = 0.14) as was DF (53.0 *vs* 53.1, P = 0.88), 90 d mortality (23% *vs* 32%, P = 0.48) and 1 year mortality (43% *vs* 42%, P = 1.0).

Clinically relevant infections occur in half of patients with AAH

In total 36 patients (50%) had a clinically relevant infection during their hospital stay with 8 (11%) on admission, 7 (10%) prior to initiation of steroids and 23 (32%) after initiation of steroids (including 2 who also had a separate infection on admission). On admission, bacteraemia was present in 3 patients (all *Escherichia coli*), spontaneous bacterial peritonitis (SBP) in 2, respiratory infection in 1 and urinary tract infection and SBP in 1. No obvious source of infection could be identified in 1 patient.

Of the 7 patients who developed a clinically relevant infection after admission but prior to initiation of steroids 3 were due to bacteraemia, 2 urinary tract infections, 1 gastrointestinal (GI) infection and 1 in whom no proven source of infection was found.

In those that developed incident infections after initiation of steroids there were 9 cases of SBP, 4 urinary tract infections, 2 respiratory infections, 5 bacteraemias and 5 infections at other sites (2 ear infections, 2 GI tract infections and 1 cellulitis). A source of infection was not determined in 2 patients. Four of these patients had non-concurrent infections at more than one site.

Overall bacteraemia and SBP were the most common sites of infection (11 each) followed by urinary tract (7) and respiratory tract (3; Figure 1A and Supplementary Table). The median time to commencement of steroids from hospital admission was 2 d (mean 2.4 d) in all patients. In those with infection identified at the time of admission the median time to commencement of steroids was 3 d (mean 5.0). The median time to the first incident infection after commencement of steroids was 10 d (range 2-42 d).

Commonest causative organisms are catalase positive

The causative bacterial genus was isolated from 41 separate clinically relevant infections in 33 individual patients. The commonest genus was *Escherichia* (9; 22%) followed by *Enterococcus* (8, 20%), *Streptococcus* (5; 12%) and *Staphylococcus*, *Pseudomonas* and *Micrococcus* (4 each; 10%; Figure 1B). Seven patients had non-concurrent infections with more than 1 organism and in 3 patients 2 organisms were identified

Table 2 Univariate analysis of variables at baseline and day 7 of steroid treatment to assess association with day 90 outcome

	Day 90 outcome		*P* value
	Survivor (*n* = 49)	Non-survivor (*n* = 23)	
Age (yr)	45	53	0.01
Gender (% male)	73	74	1.00
Sepsis on admission (%)	10	13	0.50
Sepsis at any time (%)	51	48	1.00
Sepsis after steroids (%)	33	30	1.00
CRP (mg/L)	30	40	0.15
Baseline bilirubin (μmol/L)	301	279	0.52
Baseline albumin (g/L)	25	24	0.38
Baseline INR	1.8	2	0.14
Baseline PT (s)	18.6	20.5	0.17
Baseline urea (μmol/L)	3.2	5.5	< 0.01
Baseline creatinine (μmol/L)	90	91	0.93
Baseline WBC (× 10^9/L)	9	10	0.44
Day 7 bilirubin (μmol/L)	244	254	0.67
Baseline DF	51.1	57.2	0.41
Baseline GAHS	7.8	8.7	0.04
Baseline MELD	21.7	23.7	0.23
Lille score	0.38	0.46	0.38

Continuous variables were compared by Mann-Whitney *U* test and categorical data by Fisher Exact test. CRP: C-reactive protein; INR: International normalised ratio; PT: Prothrombin time; WBC: White blood count; DF: Discriminant function; GAHS: Glasgow alcoholic hepatitis score; MELD: Model for end-stage liver disease.

from the same specimen. Four other infective organisms were identified: *Acinetobacter* (2 cases), *Clostridium difficile* (2 cases), *Haemophilus* and norovirus. Of the 36 patients with clinically relevant infections, 23 (64%) had infections with catalase positive bacteria including 2 patients who had infections with both catalase negative and positive organisms (Figure 1C).

Clinically relevant infection is associated with high Lille score

Clinically relevant infection was not significantly associated with biochemical or haematological markers of infection at baseline or day 7 of steroid treatment: baseline C-reactive protein was 36 *vs* 30 g/L (P = 0.40), baseline white blood count (WBC) was 9.9 *vs* 8.3 × 10^9/L (P = 0.14) and day 7 WBC was 13.4 x10^9/L *vs* 10.5 × 10^9/L (P = 0.08) in patients with infection *vs* no infection respectively. However, presence of clinically relevant infection was associated with higher creatinine (105 *vs* 73 μmol/L, P = 0.01), MELD (23.9 *vs* 20.6, P = 0.04) and Lille score (0.51 *vs* 0.28, P = 0.01) than those without infection.

Urea is the only independent predictor of 90 d survival

Univariate analysis of patient characteristics, baseline and day 7 biochemistry and composite scores identified age, baseline urea and baseline GAHS as significantly different between survivors and non-survivors at day 90 (Table 2). GAHS was excluded from multivariate analysis due to co-linearity with the other variables. Applying age and urea to a multivariate regression model identified only urea as an independent predictor of 90-d outcome (P = 0.01; Table 3 and Supplementary Table).

Infection does not predict short- or long-term survival

In total 11 and 15 out of 36 patients who developed infection died at 90 d or 12 mo respectively compared to 12 and 16 out of 36 of the patients without infection. Kaplan-Meier survival analysis from the time of steroid commencement did not demonstrate a significant survival difference between groups at any time. The same was true in patients with clinically relevant infection at the time of hospital admission (P = 0.26 at 90 d and P = 0.67 at 1 year; no censored data; Figure 2A and B). There was neither a 90 d nor 1 year survival difference in patients with and without an incident infection after commencement of steroids (P = 0.78 at 90 d and P = 0.98 at 1 year; no censored data; Figure 2C).

DISCUSSION

In this cohort of patients with AAH in which clinically relevant infections were clearly defined and documented prospectively, 50% developed an infection during the study period which was not associated with increased mortality at 90 d or 1 year. 11% of patients presented with clinically relevant infection, 10% developed infection after admission but prior to steroid initiation and 32% developed an incident infection during steroid treatment but the timing of infection did not influence survival. In agreement with recently published studies, *Escherichia* and *Enterococcus* genii were the commonest bacterial pathogens isolated[12,13]. The commonest site of infection was ascites (31%) and 31% had bacterial septicaemia. In only 3 patients a site of infection could not be identified.

Although this is a single centre study, the patient cohort is representative of United Kingdom patients with AAH with study selection criteria similar to the recent STOPAH clinical trial[3]. Mean age in this study was 47.9 years (in comparison to 48.7 years in STOPAH), with 74% male (68% male in STOPAH) and mean discriminant function 53.0 (62.6 in STOPAH). Mortality is also comparable with the STOPAH study and other recent clinical trials with overall mortality of 21%, 32% and 43% at day 28, day 90 and 1 year respectively[3,6,14,15]. This study has the added advantage over multicentre studies of having standardised patient management by a small team of clinicians which reduces variability in patient outcome.

Data regarding infection in AAH are sparse in the literature and of varying quality. Uniquely, this study has robustly prospectively recorded clinically relevant infections. This is reflected in the higher rate of infections noted here compared to only 13% recorded in the prednisolone treated patients in the STOPAH trial, which was likely an underestimate of the true rate of infection since it relied on clinician judgement to report

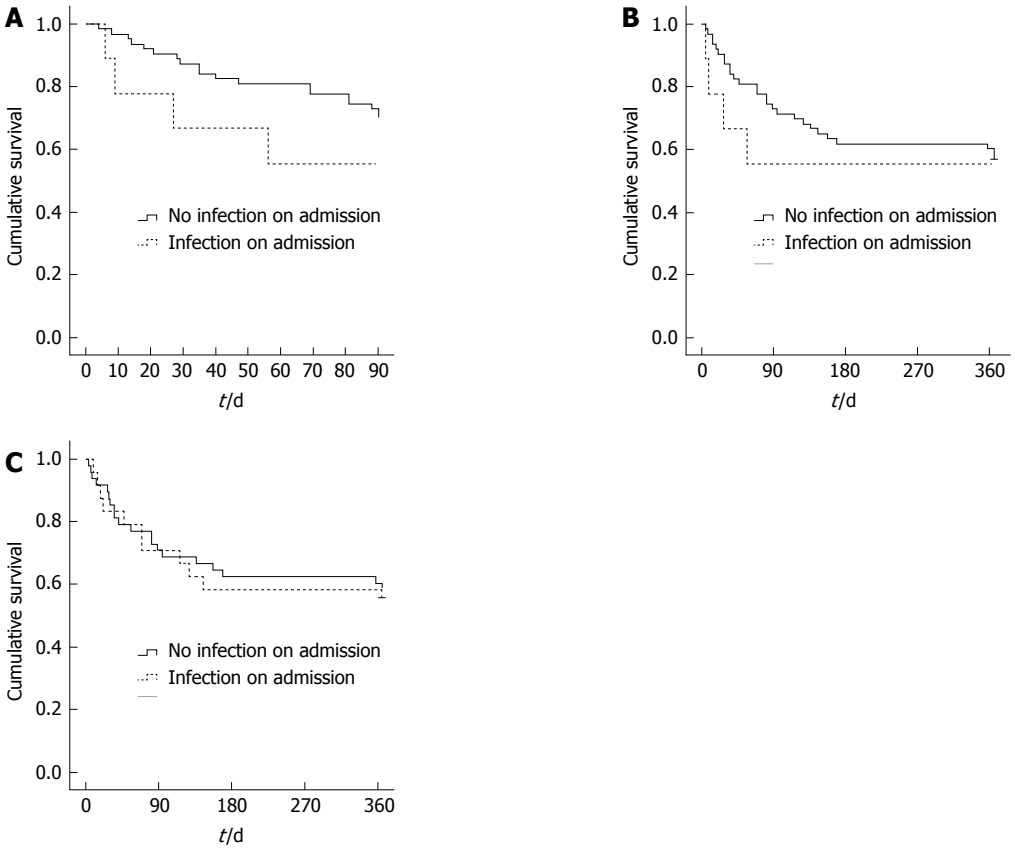

Figure 2 Clinically relevant infection does not significantly affect early or late mortality. Kaplan-Meier survival curves of AAH patients with (n = 9) and without (n = 63) clinically relevant infection at the time of hospital admission at 90 d (A) and 1 year (B); P = 0.26 and P = 0.67 respectively; C: One year survival of AAH patients with (n = 23) and without (n = 48) clinically relevant infection after commencement of steroid treatment (P = 0.93). AAH: Acute severe alcoholic hepatitis.

Table 3 Multivariate regression analysis with 90 d outcome as the dependent variable and age and baseline urea as independent variables

	R^2	Adjusted R^2	B	Std. Error	F	Sig
Model	0.205	0.182		0.425	8.87	< 0.001
Constant			-0.351	0.234		0.138
Urea			0.054	0.020		0.010
Age			0.010	0.005		0.071

The model generated accounted for approximately 20% of the dependent variable (R^2 = 0.205). The model demonstrated that urea was the only statistically significant predictor of 90 d outcome.

it as a serious adverse event[3].

A meta-analysis of 12 RCTs with sufficient infection data (including the STOPAH study) described incident infections occurring in 20% of patients without a higher rate in steroid treated patients[10]. The commonest infection was sepsis of unknown source followed by respiratory tract infections. It concluded that infection after steroid commencement was not associated with increased mortality at 28 d. However, the effect on longer term mortality was not assessed and most of the 12 studies were historic from more than 20 years ago highlighting the fact that many recent studies have not collected high quality data on infections.

A French single centre prospective study of 246 patients reported infection in 26% at presentation and 24% developed an incident infection after commencement of steroid treatment[7]. In agreement with the current study SBP was once again the commonest infection on admission but respiratory tract infections were more common in those with incident infection. Patients who had an infection at admission had a similar outcome to other patients but those who developed infection on steroid treatment had a reduced 60-d survival. However, they demonstrated that the biochemical response to steroids (using the Lille score) and not infection itself was the most important determinant of survival. A more recent RCT also concluded that treatment non-responders rather than responders were at higher risk of death from infection (14% *vs* 4%[6]). In the present study, clinically

relevant infection at any time during hospital admission was associated with a significantly higher Lille score. Conversely, Lille non-responders (Lille score > 0.45) had a trend to increased rates of clinically relevant incident infection (48% *vs* 24%, $P = 0.07$) but the Lille score itself was not identified as an independent predictor of outcome. These data suggest there is an interaction between biochemical response to treatment and infection which influences survival. However, it cannot be determined whether infection itself has an effect on liver biochemistry or whether treatment non-response increases susceptibility to infection.

An increased infection rate is seen in patients with AAH due to generalised immune dysfunction. Recent studies have investigated the immune defect in more detail and describe impaired monocyte oxidative burst, phagocytic capacity as well as increased T cell exhaustion[12,16]. The current data support these findings since the majority of bacterial isolates were able to produce catalase which is protective against phagocytic oxidative burst. Interestingly these effects were independent of steroid treatment[12] suggesting that steroids do not increase susceptibility to infection through this mechanism.

Local clinical protocols in our liver unit mean all patients with clinically relevant infection are treated rapidly (within 12 h) after first identification of infection. Additionally, in those presenting with infection, steroids were withheld until at least 48 h of intravenous broad spectrum antibiotics had been received and they were free from clinical signs of infection for this period. This protocol was adhered to in all patients in this study. This proactive antibiotic treatment policy may partly explain why no increased risk of mortality is associated with infection in these patients with AAH.

This prospective observational cohort study demonstrates that clinically relevant infections that are sought and treated early with broad spectrum antibiotics do not influence short- or long-term mortality in patients with severe AAH treated with steroids. However, long-term mortality of patients with AAH remains high primarily through alcohol recidivism and progressive liver disease[3,4]. Continuing efforts to improve long-term outcome from severe AAH should be directed to modify behaviour after hospital discharge.

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COMMENTS

Background

Acute severe alcoholic hepatitis (AAH) is a serious complication of chronic heavy alcohol misuse resulting in progressive liver failure with a high mortality. Corticosteroids are the only treatment with a proven survival benefit but may be associated with higher rates of infection. The current study describes clinically relevant infections in a prospective cohort of patients at a large single National Health Service hospital in the United Kingdom.

Research frontiers

The existing literature reports increased rates of infection associated with corticosteroid treatment but there are conflicting data regarding whether this is linked to higher mortality. This information is particularly relevant since it will help guide clinician treatment of AAH.

Innovations and breakthroughs

The current study collected infection data prospectively using a clear definition of clinically relevant infection. This is an improvement on previous studies which have less robust definitions of infections, collected infection data retrospectively or relied on clinician discretion to report relevant infections.

Applications

This study demonstrates that although clinically relevant infections are common in AAH patients they are not associated with higher mortality if actively sought and treated. These data will provide clinicians with more confidence to select appropriate patients with AAH for treatment with corticosteroids.

Terminology

AAH is defined as new onset jaundice (within the previous 3 mo) with serum bilirubin > 80 μmol/L and coagulopathy in a heavy drinker [more than 10 units alcohol (80 g ethanol) daily in males and 7.5 units (60 g ethanol) in females within the previous 4 wk]. Clinically relevant infections were defined as a body temperature > 38 ℃ or < 36 ℃ for more than 4 h, ascitic neutrophil count > 0.25×10^9 L, consolidation on chest radiograph or clinically relevant positive microbiological culture of bodily fluid.

Peer-review

Alcoholic hepatitis is still a clinical challenge, despite improved nutrition management. The study carries important information also regarding the aetiology of infectious complications, which would provide help on proper antibacterial choice. The predictive value of urea is also very important in this setting.

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ORIGINAL ARTICLE

Observational Study

Factors associated with residual gastroesophageal reflux disease symptoms in patients receiving proton pump inhibitor maintenance therapy

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Abstract

AIM

To elucidate the factors associated with residual gastroesophageal reflux disease (GERD) symptoms in patients receiving proton pump inhibitor (PPI) maintenance therapy in clinical practice.

METHODS

The study included 39 GERD patients receiving maintenance PPI therapy. Residual symptoms were assessed using the Frequency Scale for Symptoms of GERD (FSSG) questionnaire and the Gastrointestinal Symptom Rating Scale (GSRS). The relationships between the FSSG score and patient background factors, including the CYP2C19 genotype, were analyzed.

RESULTS

The FSSG scores ranged from 1 to 28 points (median score: 7.5 points), and 19 patients (48.7%) had a score of 8 points or more. The patients' GSRS scores were significantly correlated with their FSSG scores (correlation coefficient = 0.47, $P < 0.005$). In erosive esophagitis patients, the FSSG scores of the CYP2C19 rapid metabolizers (RMs) were significantly higher than the scores of the poor metabolizers and intermediate metabolizers (total scores: 16.7 ± 8.6 *vs* 7.8 ± 5.4, $P < 0.05$; acid reflux-related symptom scores: 12 ± 1.9 *vs* 2.5 ± 0.8, $P < 0.005$). In contrast, the FSSG scores of the CYP2C19 RMs in the non-erosive reflux disease patients were significantly lower than those of the other patients (total scores: 5.5 ± 1.0 *vs* 11.8 ± 6.3, $P < 0.05$; dysmotility symptom-related scores: 1.0 ± 0.4 *vs* 6.0 ± 0.8, $P < 0.01$).

CONCLUSION

Approximately half of the GERD patients receiving maintenance PPI therapy had residual symptoms associated with a lower quality of life, and the CYP2C19 genotype appeared to be associated with these residual symptoms.

Key words: Gastroesophageal reflux disease; CYP2C19; FSSG; Residual symptoms; Proton pump inhibitor

Core tip: The relationships between residual gastroesophageal reflux disease (GERD) symptoms in patients receiving proton pump inhibitor (PPI) maintenance therapy and patient background factors, including the CYP2C19 genotype, were evaluated. Approximately half of the GERD patients receiving maintenance PPI therapy had residual symptoms associated with a lower quality of life. Although the CYP2C19 genotype appeared to be associated with these residual symptoms, the impact in the erosive esophagitis patients was distinct from the impact in the non-erosive reflux disease patients.

Kawara F, Fujita T, Morita Y, Uda A, Masuda A, Saito M, Ooi M, Ishida T, Kondo Y, Yoshida S, Okuno T, Yano Y, Yoshida M, Kutsumi H, Hayakumo T, Yamashita K, Hirano T, Hirai M, Azuma T. Factors associated with residual gastroesophageal reflux disease symptoms in patients receiving proton pump inhibitor maintenance therapy. *World J Gastroenterol* 2017; 23(11): 2060-2067 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i11/2060.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i11.2060

INTRODUCTION

Gastroesophageal reflux disease (GERD) encompasses disorders in which gastric reflux leads to various symptoms and complications[1,2]. In GERD patients, reflux of gastric juice and other fluid causes not only mucosal injuries but also esophageal dysmotility associated with endogenous cytokines, which in turn leads to the appearance of GERD symptoms[3-6]. Proton pump inhibitors (PPIs) are used as first-choice drugs for GERD patients[7-9]. However, the efficacy of PPIs differs among patients depending on background factors, including genetic polymorphisms of cytochrome P450 (CYP) 2C19, which participates in the metabolic clearance and effectiveness of PPIs[10-15]. Moreover, non-erosive reflux disease (NERD) is more difficult to treat using PPIs than erosive esophagitis (EE)[16-18]. In clinical practice, GERD patients are not necessarily treated using strategies that take into account their individual backgrounds. Therefore, some cases might be treated for a long time without sufficient effects[19]. However, few reports have evaluated residual GERD symptoms in patients receiving maintenance therapy tailored to CYP2C19 genetic polymorphisms and endoscopic findings.

In this study, we investigated the residual symptoms of GERD patients receiving PPI maintenance therapy in clinical practice using the Frequency Scale for Symptoms of GERD (FSSG) questionnaire and the Gastrointestinal Symptom Rating Scale (GSRS). Additionally, we evaluated the relationships between the FSSG score and patient background factors, including the CYP2C19 genotype.

MATERIALS AND METHODS

Subjects

This study was conducted between February 2011 and March 2012. The study protocol was approved by the ethics committee of Kobe University Hospital, and all patients provided written informed consent before enrollment. Thirty-nine GERD patients receiving maintenance PPI therapy at Kobe University Hospital (Kobe-shi, Hyogo, Japan) were enrolled in this study. The FSSG questionnaire[20,21], which consists of 12 questions related to 7 acid reflux symptoms (RS) and 5 dysmotility-like symptoms (DS), was used to assess the GERD symptoms. The total FSSG score, FSSG-RS score, and FSSG-DS score were defined as the scores obtained by adding the scores from the 12 questions, the 7 acid reflux symptom-related questions, and the 5 dyspeptic symptom-related questions, respectively.

Additionally, the GSRS questionnaire[22,23] was used to assess the patients' health-related quality of life (QOL). The CYP2C19 genotypes were determined using the polymerase chain reaction-restriction fragment length polymorphism technique with allele-specific primers using a DNA sample extracted from each patient's peripheral blood leukocytes. Based on the finding of the wild-type allele or the two mutated alleles (*2 and *3), the patients were classified as rapid metabolizers (RM: homozygous for the wild-

Table 1 Clinical characteristics of gastroesophageal reflux disease patients

Background factors	The number of applicable subjects for the factor
Erosive esophagitis/NERD	19/20
Gender (male/female)	17/22
Atrophic gastritis (+/-)	20/19
Hiatal hernia (+/-)	24/15
Alcohol consumption habit (+/-/unknown)	16/21/2
Smoking habit (+/-/unknown)	4/33/2
PPI types (omeprazole/lansoprazole/rabeprazole/unknown)	5/11/22/1
PPI dose (half/full/double/unknown)	8/26/4/1
Concomitant drug against GERD (+/-)	14/25
Ca antagonist (+/-)	15/24
ASA (+/-)	7/32
NSAIDs (+/-)	2/37
CYP2C19 genotype (RM/IM/PM/unknown)	13/20/5/1
Maintenance PPI therapy period (< 6 mo/6-12 mo/> 12 mo)	3/1/35
Age (mean ± SD)	68.5 ± 11.9
BMI (mean ± SD)	22.7 ± 3.1

GERD: Gastroesophageal reflux disease; NERD: Non-erosive reflux disease; PPI: Proton pump inhibitor; ASA: Acetylsalicylic acid; NSAIDs: Non-steroidal anti-inflammatory drugs; CYP2C19: Cytochrome P450 2C19; RM: Rapid metabolizer; IM: Intermediate metabolizer; PM: Poor metabolizer; BMI: Body mass index.

type allele), intermediate metabolizers (IM: carrier of only one mutated allele), or poor metabolizers (PM: homozygous for the mutated allele)[24-27]. We examined the patients' medical records and investigated their clinical characteristics, including gender, age, BMI, alcohol consumption habits, smoking habits, PPI doses, use of concomitant drugs against GERD, use of Ca antagonists, acetylsalicylic acid (ASA) and non-steroidal anti-inflammatory drugs (NSAIDs), and endoscopic findings (atrophic gastritis and hiatal hernia). Hiatal hernia was diagnosed based on a proximal translocation of the esophagogastric junction of more than 2 cm above the diaphragmatic hiatus. Patients with an atrophic mucosa endoscopically graded as C-2, C-3, O-1, O-2, and O-3 according to the Kimura-Takemoto classification[28] were defined as positive for atrophic gastritis. Patients with esophageal mucosal breaks were classified into the EE group [Modified Los Angeles (LA) classification grades A, B, C and D], and the remaining patients were classified into the NERD group (grades N and M)[3,29,30].

Statistical analysis

Spearman's rank correlation coefficient was used to assess the correlation between the FSSG and GSRS scores in the GERD patients. A bivariate analysis (Mann-Whitney *U* test, Pearson's correlation coefficient, or Kruskal-Wallis test) was performed to assess differences in the FSSG scores (total score, RS score or DS score) and background factors in the EE and NERD patients. All statistical analyses were performed using JMP 10 (SAS Institute, Cary, NC, United States). $P <$ 0.05 indicated statistical significance.

RESULTS

Clinical characteristics of the GERD patients

The clinical characteristics of the GERD patients are shown in Table 1. The patients were divided into 19 EE patients and 20 NERD patients according to the endoscopic findings. The mean age of the patients was 68.5 ± 11.9 years, with a range from 40 to 84 years. Prokinetic agents were the most common concomitant drugs used together with PPIs for the GERD patients (25.6%, 10/39). Other common concomitant drugs were histamine H_2-receptor antagonists (H_2RAs), gastro-protective agents and kampo medicine (herbal medicine).

Correlation between the FSSG and GSRS scores

The total FSSG scores of the patients ranged between 1 and 28 points. The average total FSSG score was 10.6 ± 7.3 points, and the median was 7.5 points. Nineteen (11 EE and 8 NERD) patients (48.7%) had a total FSSG score of more than 8 points, and 11 (6 EE and 5 NERD) patients (28.2%) had a score of more than 16 points (Figure 1). As shown in Figure 2, the total FSSG scores significantly correlated with the total GSRS scores ($P < 0.005$). Similarly, significant correlations were observed in both the EE and NERD patients ($P < 0.01$ and $P < 0.05$).

Correlation between the CYP2C19 genotype and the FSSG score in the EE and NERD patients

Tables 2 and 3 show bivariate analyses of the factors associated with the FSSG scores in the EE and NERD patients, respectively. The CYP2C19 genotype was a significant factor associated with the FSSG score in both the EE and NERD patients. Subjects with the CYP2C19 RM genotype in the EE patient group had significantly higher FSSG scores than the EE subjects with the other CYP2C19 genotypes (16.7 ± 8.6 *vs* 7.8 ± 5.4, $P = 0.0415$). In contrast, the subjects with the CYP2C19 RM genotype in the NERD patient group had significantly lower FSSG scores than the NERD subjects with the other CYP2C19 genotypes (5.5 ± 1.0 *vs* 11.8 ± 6.3, $P = 0.0151$) (Figure 3).

We also examined the correlation between the CYP2C19 genotypes and the FSSG-RS or FSSG-DS scores in the EE and NERD patients. In the EE patients, the FSSG-RS scores of the subjects with the CYP2C19 RM genotype were significantly higher than those of the subjects with the other CYP2C19 genotypes (11 ± 1.9 *vs* 3.8 ± 0.8, $P = 0.0044$). In contrast, the FSSG-DS scores of the NERD patients with the CYP2C19 RM genotype were significantly lower than the scores of the NERD subjects with the other CYP2C19 genotypes (1.3 ± 0.4 *vs* 5.2 ± 0.8, $P = 0.0069$).

Significant differences in the FSSG-RS scores in the EE patients (RM: 11.0 ± 1.9, IM: 3.6 ± 0.9, PM:

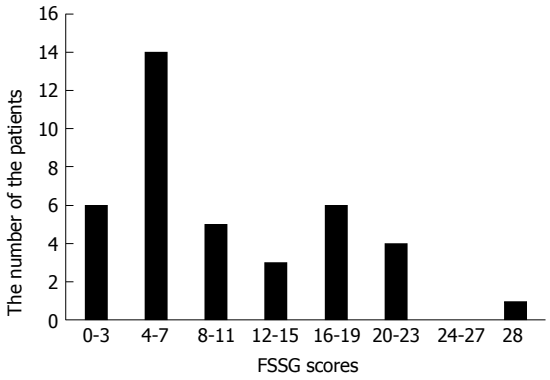

Figure 1 Distribution of frequency scale for symptoms of gastroesophageal reflux disease scores in the present study. FSSG: Frequency scale for symptoms of gastroesophageal reflux disease.

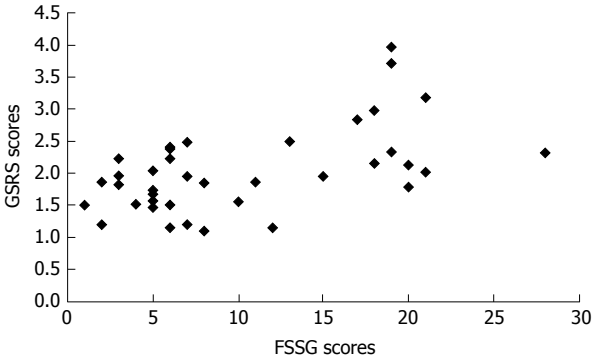

Figure 2 Correlation between the frequency scale for symptoms of gastroesophageal reflux disease and gastrointestinal symptom rating scores. FSSG: Frequency scale for symptoms of gastroesophageal reflux disease; GSRS: Gastrointestinal Symptom Rating Scale.

4.5 ± 1.5, P = 0.0147) and the FSSG-DS scores in the NERD patients (RM: 1.3 ± 0.4, IM: 4.7 ± 0.8, PM: 7.0 ± 2.3, P = 0.0177) were also observed in the bivariate analyses among the three CYP2C19 genotypes.

DISCUSSION

This study clarified the actual state of residual GERD symptoms in patients receiving PPI maintenance therapy in clinical practice and assessed the relationships between background factors, including the CYP2C19 genotype, and residual GERD symptoms.

The FSSG questionnaire was used to assess residual GERD symptoms in this study because this questionnaire has been reported to be useful for the evaluation of these symptoms and has been used in clinical practice[20,21]. The investigation using the FSSG questionnaire revealed that approximately half of the GERD patients receiving PPI maintenance therapy had residual GERD symptoms that led to a lower health-related QOL. McColl *et al*[31] reported that the agreement between clinicians and patients in their assessments of the severity of reflux symptoms was poor and that clinicians tended to underestimate the severity of patients' GERD symptoms.

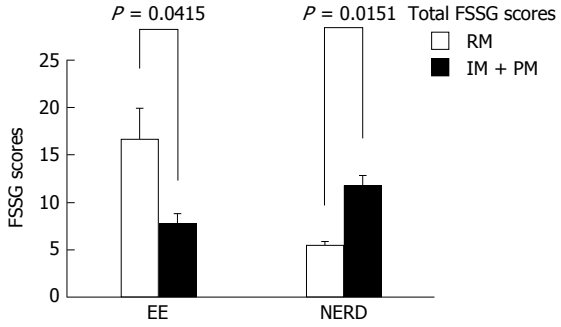

Figure 3 Correlation between the cytochrome P450 2C19 genotype and the total frequency scale for symptoms of gastroesophageal reflux disease score in the erosive esophagitis and non-erosive reflux disease patients. FSSG: Frequency scale for symptoms of gastroesophageal reflux disease; EE: Erosive esophagitis; NERD: Non-erosive reflux disease; RM: Rapid metabolizer; IM: Intermediate metabolizer; PM: Poor metabolizer.

In the analyses that divided the GERD patients into EE and NERD patients, the CYP2C19 genotype was the only significant factor associated with the total FSSG score in both groups of patients. CYP2C19 genetic polymorphisms have been reported to influence the effects of therapy on reflux symptoms and the healing of mucosal injuries[10,27,32]. However, few studies have evaluated the relationship between the CYP2C19 genotype and GERD symptoms in GERD patients (including NERD patients) receiving PPI maintenance therapy. In the present study, the impact of CYP2C19 genetic polymorphisms in EE patients was quite different from the impact in NERD patients. The total FSSG and FSSG-RS scores of the EE subjects with the CYP2C19 RM genotype were significantly higher than the scores of the EE subjects with the other CYP2C19 genotypes. These results are consistent with previous reports that insufficient acid inhibition is achieved by PPI in patients with the CYP2C19 RM genotype, which lowers the healing rate of EE[33,34]. Increasing the PPI dose in EE patients with the CYP2C19 RM genotype may improve their GERD symptoms.

In contrast, the total FSSG and FSSG-DS scores of the NERD subjects with the CYP2C19 RM genotype were significantly lower than the scores of the subjects with the other CYP2C19 genotypes in this study, suggesting that subjects with the CYP2C19 PM or CYP2C19 IM genotype receiving PPI maintenance therapy might suffer more frequently from GERD-related dyspeptic symptoms. However, this finding is difficult to explain because the CYP2C19 genotype has not been reported to be associated with the therapeutic outcomes in NERD patients[17]. Certain biological phenomena, such as reflux of digestive juices except gastric acid[4,35], mucosal hypersensitivity[36], esophageal dysmotility and psychological factors[37], are thought to cause symptoms in patients with NERD who are refractory to PPI therapy[38]. CYP2C19 gene polymorphisms might be a risk factor for PPI-refractory NERD through the biological phenomena mentioned above. An increase in the PPI dose in NERD patients

Table 2 Bivariate analysis of background factors associated with frequency scale for symptoms of gastroesophageal reflux disease scores in the erosive esophagitis patients

Background factors	FSSG scores of the applicable subjects for the factor [mean ± SD (the number)]	FSSG scores of the inapplicable subjects for the factor [mean ± SD (the number)]	*P* value
Gender (male)	11.4 ± 8.0 (9)	10.8 ± 8.1 (10)	0.9673
Atrophic gastritis	9.0 ± 9.0 (7)	12.3 ± 7.2 (12)	0.2696
Hiatal hernia	9.1 ± 7.2 (12)	14.6 ± 8.2 (7)	0.0817
Alcohol consumption habit	10.8 ± 8.1 (10)	11.4 ± 8.0 (9)	0.6225
Smoking habit	10.3 ± 8.7 (3)	11.3 ± 8.0 (16)	0.9552
Use of half dose of PPI	5.5 ± 0.7 (2)	11.3 ± 8.1 (16)	0.5251
Use of rabeprazole	11.2 ± 8.8 (12)	9.7 ± 6.1 (6)	0.9625
Concomitant drug against GERD	15.6 ± 8.5 (8)	7.8 ± 5.6 (11)	0.0512
Use of Ca antagonist	10.2 ± 5.7 (6)	11.5 ± 8.8 (13)	1.0000
Use of ASA	4.0 ± 2.8 (2)	11.9 ± 7.9 (17)	0.1610
Use of NSAIDs	8.0 (1)	11.3 ± 8.0 (18)	1.0000
CYP2C19 RM genotype	16.7 ± 8.6 (7)	7.8 ± 5.4 (12)	0.0415
Age	ρ = -0.4083		0.0826
BMI	ρ = -0.1490		0.5428

FSSG: Frequency scale for symptoms of gastroesophageal reflux disease; PPI: Proton pump inhibitor; ASA: Acetylsalicylic Acid; NSAIDs: Non-steroidal anti-inflammatory drugs; CYP2C19: Cytochrome P450 2C19; RM: Rapid metabolizer; IM: Intermediate metabolizer; PM: Poor metabolizer; BMI: Body mass index.

Table 3 Bivariate analysis of background factors associated with frequency scale for symptoms of gastroesophageal reflux disease scores in the non-erosive reflux disease patients

Background factors	FSSG scores of the applicable subjects for the factor [mean ± SD (the number)]	FSSG scores of the inapplicable subjects for the factor [mean ± SD (the number)]	*P* value
Gender (male)	6.5 ± 5.2 (8)	11.3 ± 6.2 (12)	0.0807
Atrophic gastritis	9.6 ± 6.7 (13)	9.0 ± 5.4 (7)	0.9365
Hiatal hernia	7.1 ± 5.0 (12)	12.9 ± 6.4 (8)	0.1510
Alcohol consumption habit	9.0 ± 6.8 (6)	10.2 ± 6.4 (12)	0.4221
Smoking habit	6.0 (1)	10.0 ± 6.5 (17)	0.6270
Use of half dose of PPI	7.2 ± 5.7 (6)	10.4 ± 6.3 (14)	0.2454
Use of rabeprazole	11.8 ± 7.0 (10)	7.0 ± 4.3 (10)	0.2095
Concomitant drug against GERD	10.5 ± 6.7 (6)	8.9 ± 6.1 (14)	0.5896
Use of Ca antagonist	9.0 ± 6.2 (9)	9.7 ± 6.4 (11)	0.5159
Use of ASA	9.6 ± 6.0 (5)	9.3 ± 6.4 (15)	0.8262
Use of NSAIDs	18.0 (1)	8.9 ± 6.0 (19)	0.2568
CYP2C19 RM genotype	5.5 ± 1.0 (6)	11.8 ± 6.3 (13)	0.0151
Age	ρ = 0.0963		0.6863
BMI	ρ = -0.3714		0.1069

FSSG: Frequency scale for symptoms of gastroesophageal reflux disease; NERD: Non-erosive reflux disease; PPI: Proton pump inhibitor; ASA: Acetylsalicylic Acid; NSAIDs: Non-steroidal anti-inflammatory drugs; CYP2C19: Cytochrome P450 2C19; RM: Rapid metabolizer; IM: Intermediate metabolizer; PM: Poor metabolizer; BMI: Body mass index.

with the CYP2C19 PM or CYP2C19 IM genotype is unlikely to improve their GERD symptoms. Regarding therapy for NERD patients, multifaceted approaches that include not only gastric acid suppression but also lifestyle improvement, clinical management of psychogenic factors and the use of concomitant drugs, such as kampo medicine, are desirable[39-42].

In Japan, the number of patients with GERD is expected to increase in the future due to the reduction of *Helicobacter pylori* infection, the spread of a Westernized diet, the increase in the aging population and the increase in gastric acid secretion in the younger generation[43-45]. Treatment of GERD symptoms is a clinically important issue because the health-related QOL of GERD patients is reduced in proportion to the extent of GERD symptoms, and ideally, tailored therapy based on the characteristics of each patient should be provided. To this end, quantifying GERD symptoms using a tool such as the FSSG questionnaire and making a differential diagnosis of EE and NERD are useful approaches. Furthermore, testing for the CYP2C19 genotype may be useful for treatment strategy decisions for PPI-refractory GERD patients. Other treatments that are not gastric acid inhibitory drugs may be required for PPI-refractory NERD patient with the CYP2C19 IM or PM genotype.

The present study has several limitations. The number of subjects was not large, which may have influenced the statistical analyses. The diagnosis of NERD was confirmed only by the medical history and the endoscopic findings. Further studies with a larger number of subjects are needed to clarify the relationship

between the CYP2C19 genotype and the residual symptoms of GERD patients receiving maintenance PPI therapy.

In conclusion, approximately half of the GERD patients receiving maintenance PPI therapy had residual symptoms associated with a lower quality of life. Although CYP2C19 genetic polymorphisms appeared to be associated with these residual symptoms, the impact of the genetic polymorphisms differed significantly between the EE and NERD patients. NERD patients with the CYP2C19 IM or PM genotype might require additional treatment other than PPIs. Further studies on the usefulness of the treatment strategy tailored to the CYP2C19 genotype are required for PPI-refractory GERD patients.

COMMENTS

Background

In the clinical practice of maintenance proton pump inhibitor (PPI) therapy for gastroesophageal reflux disease (GERD) patients, some cases might be treated for a long time without sufficient effects.

Research frontiers

Few reports have evaluated the relationship between the cytochrome P450 2C19 (CYP2C19) genotype and GERD symptoms in patients, including non-erosive reflux disease (NERD) patients, receiving PPI maintenance therapy.

Innovations and breakthroughs

In this study, the impact of CYP2C19 genetic polymorphisms differed significantly between the erosive esophagitis (EE) and NERD patients. In the EE patients, the Frequency Scale for Symptoms of GERD (FSSG) scores of the CYP2C19 rapid metabolizers (RMs) were significantly higher than the scores of the poor and intermediate metabolizers. In contrast, in the NERD patients, the FSSG scores of the CYP2C19 RMs were significantly lower than the scores of the other groups.

Applications

Testing of the CYP2C19 genotype may be useful when determining a treatment strategy for PPI-refractory GERD patients.

Terminology

Genetic polymorphisms of CYP2C19 participate in the metabolic clearance and effectiveness of PPIs.

Peer-review

This is a well-designed study that investigates a common problem facing gastroenterologists and other treating physicians of GERD. The manuscript is well written; the results are well explained, the discussion is exhaustive, and the limitations of the study are clearly stated.

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ORIGINAL ARTICLE

Observational Study

Quality of life after total *vs* distal gastrectomy with Roux-en-Y reconstruction: Use of the Postgastrectomy Syndrome Assessment Scale-45

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Abstract

AIM

To investigate the detrimental impact of loss of reservoir capacity by comparing total gastrectomy (TGRY) and distal gastrectomy with the same Roux-en-Y (DGRY) reconstruction. The study was conducted using an integrated questionnaire, the Postgastrectomy Syndrome Assessment Scale (PGSAS)-45, recently developed by the Japan Postgastrectomy Syndrome Working Party.

METHODS

The PGSAS-45 comprises 8 items from the Short Form-8, 15 from the Gastrointestinal Symptom Rating Scale, and 22 newly selected items. Uni- and multivariate analysis was performed on 868 questionnaires completed by patients who underwent either TGRY (n = 393) or DGRY (n = 475) for stage I gastric cancer (52 institutions). Multivariate analysis weighed of six explanatory variables, including the type of gastrectomy (TGRY/DGRY), interval after surgery, age, gender, surgical approach (laparoscopic/open), and whether the celiac branch of the vagus nerve was preserved/divided on the quality of life (QOL).

RESULTS

The patients who underwent TGRY experienced the poorer QOL compared to DGRY in the 15 of 19 main outcome measures of PGSAS-45. Moreover, multiple regression analysis indicated that the type of gastrectomy, TGRY, most strongly and broadly impaired the postoperative QOL among six explanatory variables.

CONCLUSION

The results of the present study suggested that TGRY had a certain detrimental impact on the postoperative QOL, and the loss of reservoir capacity could be a major cause.

Key words: Postgastrectomy syndrome; Quality of life; Gastric cancer; Gastrectomy; Patient-reported outcome

Core tip: The influence of postgastrectomy syndrome after total gastrectomy (TG) is believed to be more intense than that after distal gastrectomy (DG). However, the precise features and the degree of interference with quality of life after TG against DG have not been clarified. Then, we weighed DG against TG to determine pure influence of whether presence or absence of remaining stomach (as the reservoir capacity) by employing the same reconstruction route. Moreover, we reinforced the findings by multivariable analysis including other clinical factors, and defined the effect sizes of each variable in the unprecedented examination with large number cases using newly developed Postgastrectomy Syndrome Assessment Scale-45.

Takahashi M, Terashima M, Kawahira H, Nagai E, Uenosono Y, Kinami S, Nagata Y, Yoshida M, Aoyagi K, Kodera Y, Nakada K. Quality of life after total *vs* distal gastrectomy with Roux-en-Y reconstruction: Use of the Postgastrectomy Syndrome Assessment Scale-45. *World J Gastroenterol* 2017; 23(11): 2068-2076 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i11/2068.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i11.2068

INTRODUCTION

The five-year overall survival rate of stage I gastric cancer patients who undergo gastrectomy with sufficient lymphadenectomy has been shown to exceed 90% in Japan[1]. Maintaining quality of life (QOL) after surgery is an important issue for patients who are eventually cured, and several surgical procedures have been developed to minimize the influence of postgastrectomy syndromes (PGS).

The severity of PGS after total gastrectomy (TG) is clinically recognized to be greater than after distal gastrectomy (DG). And a functional analysis has demonstrated the significant interrelationship between weight loss and esophageal bile reflux after TG[2]. Recently, combined questionnaires using the EORTC QLQ-C30 and QLQ-STO22 have demonstrated the differences of several upper-gastrointestinal symptoms associated with distal, proximal and total gastrectomy[3]. However, other study using same combined QOL questionnaires have failed to reveal significant differences in QOL scores[4] among them. Such a discrepancy may have arisen from a lack of adequate instruments for evaluating the QOL in the postgastrectomy patients.

The Japan Postgastrectomy Syndrome Working Party (JPSWP) was founded to more closely investigate the symptoms and lifestyle changes of patients who had undergone gastrectomy. This group collaboratively developed a novel questionnaire [Postgastrectomy Syndrome Assessment Scale (PGSAS)-45] to evaluate the symptoms, living status, and QOL of gastrectomized patients[5]. In this study, we firstly focused the strength and extent of detrimental impact of TG against DG, in which the reservoir capacity is maintained by the remaining stomach, with the same Roux-en-Y reconstruction route, using multi-variate

analyses including other clinical factors influencing QOL[6].

MATERIALS AND METHODS

Patients

The following inclusion criteria were applied: (1) a pathologically confirmed diagnosis of stage IA or IB gastric cancer[7]; (2) first-time gastrectomy status; (3) age between ⩾ 20 and ⩽ 75 years; (4) no history of chemotherapy; (5) no evidence of recurrence or distant metastasis; (6) gastrectomy conducted ⩾ 1 year prior to study enrollment; (7) performance status (PS) ⩽ 1 on the Eastern Cooperative Oncology Group (ECOG) scale; (8) full ability to understand and respond to the questionnaire; (9) no history of other diseases or surgeries that might influence responses to the questionnaire; (10) absence of organ failure or mental illness; and (11) provision of written informed consent. The exclusion criteria included patients who had an additional malignancy and patients who underwent the concomitant resection of other organs (except for cholecystectomy or splenectomy).

QOL assessment

PGSAS-45 is a newly developed, multidimensional QOL questionnaire (QLQ) consists of a total of 45 questions, with 8 items from the Short-Form Health Survey (SF-8)[8], 15 items from the Gastrointestinal Symptom Rating Scale (GSRS)[9], and 22 clinically important items selected by the JPSWP[5]. These important items consists of newly selected 10 PGSAS specific items for symptoms (items 24-33), eight questionnaire items pertain to dietary intake (items 34-41), one item for social activity (item 42) and three items about level of satisfaction with daily life (items 43-45). For the 23 symptom items, a seven-grade (1-7) Likert scale was used. A five-grade (1-5) Likert scale was used for all the other items, except for items 1, 4, 29, 32, and 34-37. For items 1-8, 34, 35, and 38-40, higher scores indicate better conditions. For items 9-28, 30, 31, 33, and 41-45, higher scores indicate worse conditions.

Study methods

This study utilized continuous sampling from a central registration system for participant enrollment. The questionnaire was distributed to all eligible patients as they presented at the participating clinics. The patients were instructed to mail the completed questionnaires directly to the data center. All the QOL data from the questionnaires were matched with individual patient data collected *via* case report forms (CRFs) sent from the physicians in charge of the patients and stored in a database.

This study was registered with the University Hospital Medical Information Network's Clinical Trials Registry (UMIN-CTR; registration number 000002116). The study was approved by local ethics committees at each institution. Written informed consent was obtained from all the enrolled patients.

PGSAS-45 questionnaires were distributed to 2922 patients between July 2009 and December 2010. Among the 2520 (86%) patients who returned completed questionnaires, 152 were determined to be ineligible because of age (older than 75 years, n = 90), postoperative period > 1 year (n = 29), resection of other organs (n = 8), or other factors (n = 25). The data and responses from the remaining 2368 patients (81%) were eligible for subsequent analyses. In the current study, data from 868 patients who underwent either total gastrectomy (TGRY, n = 393) or distal gastrectomy (DGRY, n = 475), all with Roux-en-Y reconstruction, were retrieved from the database and analyzed. Other data from 1500 patients who underwent distal gastrectomy with Billroth-I reconstruction (DGBI, n = 909), pylorus preserving gastrectomy (PPG, n = 313, proximal gastrectomy (PG, n = 193) or local resection (LR, n = 85) were excluded for this study.

Refinement of the main outcome measures in the PGSAS-45

Based on the data from the completed PGSAS-45 questionnaires, the outcome measures were refined by consolidation and selection[5]. The 23 symptom items were consolidated into the 7 symptom subscales (SS) listed in Table 1. The main outcome measures for the assessment data included several subscales such as the total symptom score, quality of ingestion, level of dissatisfaction for daily life, a physical component summary (PCS) based on items derived from the SF-8, and a mental component summary (MCS), also based on SF-8 items. Each SS score, except the PCS and MCS, was calculated as the mean of the composed items, and the total symptom score was calculated as the mean of seven symptom SSs. In addition, the following parameters were selected as the main outcome measures: body weight changes, amount of food ingested per meal, necessity for additional meals, ability for working, dissatisfaction with symptoms, dissatisfaction at the meals, and dissatisfaction at working.

Statistical analysis

In comparing patient QOLs after TGRY and DGRY, statistical methods included the t-test and χ^2 test. All outcome measures that exhibited significant difference in univariate analysis were further analyzed using multiple regression analysis to eliminate confounding factors by adding time interval from surgery, age, gender, surgical approach, and celiac branch preservation to the type of gastrectomy. $P < 0.05$ was considered statistically significant. In the case of $P < 0.1$ by univariate analysis, Cohen's d was calculated. In the case of that P value of standardized regression coefficient (β) in multiple regression analysis was < 0.1,

Table 1 Uni-variate analysis of main outcome measures following total gastrectomy and distal gastrecrtomy procedures

Domains	Subdomains	Main outcome measures	TGRY		DGRY		Uni-variate analysis	
			mean	SD	mean	SD	*P* value	Cohen's *d*
Symptoms	PGSAS subscales (GSRS and PGSAS items)	Esophageal reflux subscale (items 10, 11, 13, 24)[2]	2.0	1.0	1.5	0.7	< 0.0001	0.58
		Abdominal pain subscale (items 9, 12, 28)[2]	1.8	0.8	1.7	0.8	0.0571	(0.13)
		Meal-related distress subscale (items 25-27)[2]	2.6	1.1	2.1	0.9	< 0.0001	0.56
		Indigestion subscale (items 14-17)[2]	2.3	0.9	2.0	0.8	< 0.0001	0.29
		Diarrhea subscale (items 19, 20, 22)[2]	2.3	1.2	2.1	1.1	0.0066	(0.19)
		Constipation subscale (items 18, 21, 23)[2]	2.1	0.9	2.1	1.0	⩾ 0.1	
		Dumping subscale (items 30, 31, 33)[2]	2.3	1.1	2.0	1.0	< 0.0001	0.31
	Total	Total symptom score (above seven subscales)[2]	2.2	0.7	1.9	0.7	< 0.0001	0.38
Living status	Body weight	Change in body weight	-13.8%	7.9%	-8.9%	6.6%	< 0.0001	0.66
	Meals(amount)	Ingested amount of food per meal	6.4	1.9	7.2	2.0	< 0.0001	0.42
		Necessity for additional meals	2.4	0.8	1.9	0.8	< 0.0001	0.57
	Meals (quality)	Quality of ingestion subscale[1] (items 38-40)[2]	3.8	0.9	3.8	0.9	⩾ 0.1	
	Social activity	Ability for working	2.0	0.9	1.8	0.9	0.0006	0.24
QOL	Dissatisfaction	Dissatisfaction with symptoms	2.1	1.0	1.8	0.9	< 0.0001	0.28
		Dissatisfaction at the meal	2.8	1.1	2.2	1.1	< 0.0001	0.57
		Dissatisfaction at working	2.1	1.1	1.7	1.0	< 0.0001	0.41
		Dissatisfaction for daily life subscale (items 43-45)[2]	2.3	0.9	1.9	0.9	< 0.0001	0.51
	SF-8	Physical component summary (PCS)[1] (items 1-8)[2]	49.6	5.6	50.8	5.6	0.0029	0.21
		Mental component summary (MCS)[1] (items 1-8)[2]	49.2	6.0	49.8	5.7	0.0974	(0.11)
					The interpretation of effect size			Cohen's *d*
					(none-very small)			(0.20 >)
					Small			0.20 ⩽
					Medium			0.50 ⩽
					Large			0.80 ⩽

Outcome measures with[1]: higher score indicating better condition. Outcome measures without[1]: higher score indicating worse condition. The main outcomes with[2] are integrated subscales. Each subscale is calculated as the mean of composed items or subscales, except PCS or MCS of SF-8.

the β value has shown in the table. Cohen's *d*, β, and R^2 (coefficient of determination) measure effect sizes. Interpretation of effect sizes were 0.2 < small, 0.5 < medium, and 0.8 < large in Cohen's *d*; 0.1 < small, 0.3 < medium, and 0.5 < large in β; and 0.02 < small, 0.13 < medium, and 0.26 < large in R^2. StatView software for Windows Ver. 5.0 (SAS Institute Inc.) was used for all statistical analyses.

RESULTS

Patient characteristics

The demographics of all the study participants enrolled from 52 institutions are listed in Table 2. Among the patients who underwent TGRY, the time interval from surgery until the current evaluation and the length of the Roux segment were significantly longer; age, incidence of applying an open approach, extent of lymph node dissection, and combined resection rate were significantly higher; and a posterior route of Roux-en-Y was significantly more common.

QOL assessments

PGSAS symptom subscales: Twenty-three symptom items comprising items derived from the GSRS and original items proposed and selected by the participating gastric surgeons during the establishment of the PGSAS were consolidated into seven symptom SSs (*i.e.,* the PGSAS symptom SSs). These SSs reflected the PGS symptom profile and revealed that the patients who underwent TGRY had significantly higher (*i.e.,* worse) scores (Table 1 and Figure 1A). The implication of the type of gastrectomy on each PGSAS symptom SS, in terms of effect size "Cohen's *d*", is shown in Table 1. To eliminate confounding factors, a multiple regression analysis was performed by adding the time interval from surgery, age, gender, surgical approach, and celiac branch preservation as explanatory variables. Cohen's *d* (by the univariate analysis) and β (by the multiple regression analysis) of the esophageal reflux SS, meal-related distress SS, indigestion SS and dumping SS were significantly declined by the extent of gastrectomy with medium to small effect size (*e.g.*, Cohen's *d* and β, Tables 1 and 3).

The total symptom score, which aggregated all seven PGSAS symptom SSs, denoted the severity of overall PGS symptom and its values of Cohen's *d*, β, and R^2 in the present study were 0.38, 0.216, and 0.059, respectively. This also indicated that the severity of overall PGS symptom after TGRY were significantly greater than those after DGRY (Tables 1 and 3).

Body weight changes: The body weight change more than one year after gastrectomy was -13.8% in the patients who underwent TGRY and -8.9% in those who underwent DGRY. The type of gastrectomy had a significant and medium implication on body weight loss as to effect size; the uni-variate analysis produced a Cohen's *d* of 0.66 (Table 1), and the multiple regression analysis produced a β of 0.315 (Table 3).

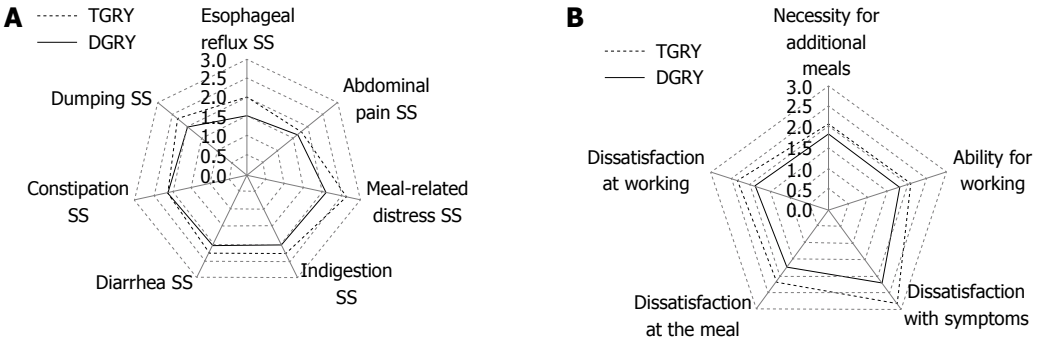

Figure 1 Rader charts of Postgastrectomy Syndrome Assessment Scale symptom subscales, life status, and dissatisfactions. A: Twenty-three symptom items consisting of Gastrointestinal Symptom Rating Scale and Postgastrectomy Syndrome Assessment Scale (PGSAS) specific items were consolidated into seven symptom subscales as PGSAS symptom subscales (SS). This radar chart demonstrated the worse conditions of postgastrectomy syndromes (PGS) in total gastrectomy (TGRY) group than that in distal gastrectomy (DGRY) group; B: The radar graph consisting of each score of items related to the life status and the dissatisfactions demonstrated the worse conditions of PGS in TGRY group than that in DGRY group.

Table 2 Patient characteristics

		TGRY	DGRY	*P* value
Number of patients		393	475	
Postoperative period	(mo)	35.0 ± 24.6[1]	31.7 ± 18.0[1]	0.0246[4]
Age		63.4 ± 9.2[1]	62.0 ± 9.1[1]	0.0244[4]
Gender	Male	276	318	⩾ 0.1[5]
	Female	113	154	
Preoperative BMI[2]		23.0 ± 3.3[1]	22.9 ± 3.0[1]	⩾ 0.1[4]
Postoperative BMI[2]		19.8 ± 2.5[1]	20.8 ± 2.7[1]	< 0.0001[4]
Approach	Open	293	320	0.0181[5]
	Laparoscopic	97	152	
Extent of lymph node dissection[3]	D0	0	0	0.0159[5]
	D1	4	3	
	D1a	28	60	
	D1b	192	246	
	D2	164	163	
Celiac branch of vagal nerve	Preserved	12	28	0.0523[5]
	Divided	371	442	
Combined resection	None	246	402	< 0.0001[5]
	Gallbladder	83	51	
	spleen	52	2	
	Miscellaneous	4	2	
Length of Roux-en-Y loop	(cm)	42 ± 4.2[1]	32.2 ± 7.1[1]	< 0.0001[4]
Route of Roux-en-Y	Anterior	179	292	< 0.0001[4]
	Posterior	206	175	

[1]Mean ± SD; [2]Body mass index; [3]According to Japanese gastric cancer treatment guidelines; [4]Unpaired *t*-test; [5]χ^2 test. TGRY: Total gastrectomy; DGRY: Distal gastrectomy.

Meals, social activity, and dissatisfaction: The ingested amount of food per meal, necessity for additional meals, ability for working, and dissatisfaction with symptoms at the meal and at working were important items included in the PGSAS-45. The scores on these items revealed that the patients who underwent TG experienced significantly worse living status and more dissatisfaction for their lives (Table 1 and Figure 1B).

The Cohen's *d* values for the necessity for additional meals, dissatisfaction at the meal and dissatisfaction for daily life SS were of medium effect size. All the other items related to dissatisfaction had small Cohen's *d* values, indicating that they were significantly influenced by the type of gastrectomy with small but clinically meaningful effect size (Tables 1 and 3). The dissatisfaction for daily life SS, which consisted of three dissatisfaction items, demonstrated a significant influence of PGS. The Cohen's *d*, β, and R^2 values for this SS were 0.51, 0.268, and 0.083, respectively (Tables 1 and 3).

SF-8: The SF-8, a useful and simple questionnaire for evaluating generic QOL, includes PCS and MCS as SSs. For the PCS, Cohen's *d* was 0.21, and β was 0.109. Thus, the PCS was significantly lower in the TGRY cases (with a small effect size), whereas the MCS did not differ between the TGRY and DGRY cases (Tables 1 and 3).

Table 3 Multi-variate analysis of main outcome measures

Domains	Subdomains	Main outcome measures	Multiple regression analysis											
			Type of gastrectomy [TGRY]		Postoperative period (mo)		Age (yr)		Gender [male]		Approach [laparoscopic]		Celiac branch of vagal nerve [preserved]			
			β	*P* value	β	*P* value	β	*P* value	β	*P* value	β	*P* value	β	*P* value	R^2	*P* value
Symptoms	Subscales (GSRS and PGSAS items)	Esophageal reflux SS[2]	0.298	< 0.0001		⩾ 0.1		⩾ 0.1	(-0.079)	0.0179	(-0.060)	0.0828		⩾ 0.1	0.096	< 0.0001
		Abdominal pain SS[2]	(-0.089)	0.0096	(-0.060)	0.0932		⩾ 0.1	-0.181	< 0.0001		⩾ 0.1		⩾ 0.1	0.045	< 0.0001
		Meal-related distress SS[2]	0.295	< 0.0001		⩾ 0.1	(-0.064)	0.0531	-0.114	0.0006		⩾ 0.1		⩾ 0.1	0.101	< 0.0001
		Indigestion SS[2]	0.166	< 0.0001	-0.114	0.0012		⩾ 0.1	(-0.083)	0.0138	(-0.071)	0.0436	(-0.063)	0.0639	0.057	< 0.0001
		Diarrhea SS[2]	0.107	0.0020	(-0.063)	0.0774		⩾ 0.1	(-0.092)	0.0074		⩾ 0.1		⩾ 0.1	0.024	0.0026
		Constipation SS[2]														
		Dumping SS[2]	0.189	< 0.0001		⩾ 0.1	-0.123	0.0006	-0.129	0.0003		⩾ 0.1		⩾ 0.1	0.071	< 0.0001
	Total	Total symptom score[2]	0.216	< 0.0001		⩾ 0.1		> 0.1	(-0.092)	0.0115		⩾ 0.1		> 0.1	0.059	< 0.0001
Living status	Body weight	Change in body weight	0.315	< 0.0001		⩾ 0.1	(-0.068)	0.0449	(-0.088)	0.0086		⩾ 0.1		⩾ 0.1	0.114	< 0.0001
	Meals (amount)	Ingested amount of food per meal[1]	0.202	< 0.0001		⩾ 0.1	(-0.060)	0.0838		> 0.1		⩾ 0.1		⩾ 0.1	0.048	< 0.0001
		Necessity for additional meals	0.273	< 0.0001		⩾ 0.1	-0.083	0.0142	(-0.093)	0.0057		⩾ 0.1		⩾ 0.1	0.090	< 0.0001
	Meals (quality)	Quality of ingestion SS[1,2]														
	Social activity	Ability for working	0.127	0.0003	(-0.079)	0.0289	0.163	< 0.0001	(-0.073)	0.0371		⩾ 0.1		⩾ 0.1	0.052	< 0.0001
QOL	Dissatisfaction	Dissatisfaction with symptoms	0.158	< 0.0001	(-0.083)	0.0199		⩾ 0.1	-0.105	0.0022		⩾ 0.1		⩾ 0.1	0.041	< 0.0001
		Dissatisfaction at the meal	0.291	< 0.0001	(-0.081)	0.0193		⩾ 0.1	(-0.072)	0.0313		⩾ 0.1		⩾ 0.1	0.093	< 0.0001
		Dissatisfaction at working	0.220	< 0.0001		⩾ 0.1		⩾ 0.1		> 0.1		⩾ 0.1		⩾ 0.1	0.051	< 0.0001
		Dissatisfaction for daily life SS[2]	0.268	< 0.0001	(-0.086)	0.0145		⩾ 0.1	(-0.087)	0.0097		⩾ 0.1		⩾ 0.1	0.083	< 0.0001
	SF-8	Physical component summary[1,2]	0.109	0.0016		⩾ 0.1	-0.124	0.0004	(-0.079)	0.0210		⩾ 0.1		⩾ 0.1	0.034	< 0.0001
		Mental component summary[1,2]		⩾ 0.1		⩾ 0.1		⩾ 0.1		⩾ 0.1		⩾ 0.1		⩾ 0.1		

The interpretation of effect size	r, β	R^2
(none-very small)	(0.100) >	(0.020) >
Small	0.100 ⩽	0.020 ⩽
Medium	0.300 ⩽	0.130 ⩽
Large	0.500 ⩽	0.260 ⩽

Outcome measures with[1]: higher score indicating better condition. Outcome measures without[1]: higher score indicating worse condition. The main outcomes with[2] are integrated subscales. If β is positive, the score of the outcome measure of the patients belonging to the category in [brackets] is higher in cases when the factor is a nominal scale, and he score of outcome measure of the patients with larger values is higher in cases when the factor is a numeral scale.

DISCUSSION

This study aimed to compare the effects of TGRY and DGRY on postoperative QOL in gastric cancer patients. This comparison was performed using the PGSAS-45, a questionnaire developed to measure QOL in postgastrectomy patients. In the series of our cross-sectional study, we found that there was a little difference in the

effect after DG between Billroth-I and Roux-en-Y procedure[10]. On the other hand, several clinical factors such as symptom severity, ability for working, and necessity for additional meals had a significant impact on postoperative QOL, while the influence of the extent of gastrectomy was unexpectedly small[6].

The influence of postgastrectomy syndrome is believed to be more intense after TG than after DG. However, the reasons for and degree of interference with QOL after TG *vs* DG have not been clarified. Most of prior reports compared the postoperative QOL between TG with Roux-en-Y and DG mainly with Billroth-I or II, in which the food or digestive juice passes through the other route[11,12]. Therefore, the detected differences between TG and DG in the previous studies may have designated the aggregated effect of both the preservation of proximal stomach and the reconstruction route. This study compared TGRY with DGRY to determine the pure influence of the remaining stomach (which approximates reservoir capacity).

Our results showed apparent differences in several main outcome measures associated with symptoms, daily living, and QOL. In multivariate analysis, 15 of 19 main outcome measures (with the exception of the constipation SS, indigestion SS, quality of ingestion SS and MCS) indicated significantly inferior conditions in the TGRY group. To identify which outcomes were most declined by the complete loss of reservoir capacity in TGRY, the effect sizes of these main outcome measures were compared. The variables shown to be adversely affected were (in decreasing order of severity) body weight loss, esophageal reflux SS, meal-related distress SS, dissatisfaction with meals, necessity for additional meals, and dissatisfaction with daily life SS. Thus, the PGSAS-45 was able to demonstrate the multifaceted influences on life conditions of total or distal gastrectomy.

Several reports have postulated that preserving the lower esophageal sphincter or cardia prevents esophageal reflux[11,12]. Moreover, esophageal reflux has been less frequently observed following DGRY than after DGBI[8,13-18]. Thus, preserving the proximal stomach with Roux-en-Y reconstruction should help to prevent esophageal reflux. A proximal gastrectomy is regarded as a potent alternative to avoid the serious detrimental impact of TG for early upper-third gastric cancer patients. Indeed, the recent study using the PGSAS-45 questionnaire revealed that proximal gastrectomy appeared to be valuable as a function-preserving procedure, however, declined the majority of symptom SSs such as esophageal reflux, abdominal pain, meal-related distress and ingestion in the same way as TG[19]. Another alternative to avoid the severe impairment of QOL after TG is to constitute the substitute stomach. Although Fein *et al*[20] and Iivonen *et al*[21] reported long-term benefits of RY pouch reconstruction after TG, there is no universal consensus regarding the optimal method of reconstruction following TG. A meta-analysis demonstrated several clinical advantages of pouch reconstruction after TG; patients with a pouch reportedly complained significantly less of dumping and heartburn, and they exhibited a significantly better postoperative food intake[22]. Thus, maintaining reservoir function after gastrectomy appears to be of utmost importance.

Further validation of our findings by prospective clinical trials using the PGSAS-45 for assessments at baseline and at various relevant time points is warranted.

Several investigators have assessed QOL following TG and have compared various surgical and reconstructive procedure[22], however, no large-scale QOL comparisons of TG and DG performed with the same reconstruction procedure, Roux-en-Y, have been published to date. The current study has the intrinsic limitation of being a retrospective study. Moreover, the patient-reported outcomes were assessed at a single time point, which could be any time at least one year after surgery. Nevertheless, the postoperative conditions of the patients more than one year after gastrectomy were generally stable[23], and the multivariate analysis identified that the type of gastrectomy (TGRY *vs* DGRY) was the factor with the greatest influence on several of the main outcome measures. The current study represents the first large-scale investigation of patient QOL following gastrectomy using the PGSAS-45 questionnaire with multivariate analysis.

In this study, the PGSAS-45 defined a certain impairment of QOL after TGRY compared to DGRY specifically. Therefore, it is important to closely monitor patients to enable early detection and management of PGS, and to provide appropriate nutritional support. The results of the present study defined that the sole loss of remaining stomach strongly and broadly impairs the QOL in postgastrectomy patients. This may suggest that reservoir reconstruction using a stomach substitute should be an optional alternative to maintain better QOL after TG.

ACKNOWLEDGMENTS

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The authors thank all the physicians who participated in this study and the patients whose cooperation made this study possible.

COMMENTS

Background

The influence of postgastrectomy syndrome (PGS) after total gastrectomy (TG)

is believed to be more intense than that after distal gastrectomy (DG). However, the precise features and the degree of interference with quality of life (QOL) after TG against DG have not been clarified. It was discussed by a comparison between TG and DG in the past report, however, even if it was the same DG. Most of prior reports compared the postoperative QOL between TG with Roux-en-Y (RY) and DG mainly with Billroth-I (BI) or II (BII), in which the food or digestive juice passes through the other route. Therefore, influence due to the physiological reconstruction might be mixed in the difference of both as well as influence of TG with RY (TGRY).

Research frontiers

Most of prior reports compared the postoperative QOL between TG with RY and DG mainly with BI or BII, in which the food or digestive juice passes through the other route. Several reports have postulated that preserving the lower esophageal sphincter or cardia prevents esophageal reflux. Moreover, esophageal reflux has been less frequently observed following DGRY than after DGBI. Thus, preserving the proximal stomach with Roux-en-Y reconstruction should help to prevent esophageal reflux.

Innovations and breakthroughs

This study was to have compared the DG with TG except influence of the reconstruction course. And we examined influence of the presence or absence of reservoir capacity by multivariable analysis including other clinical factors, and showed the statistical effect sizes. Moreover, this study was unprecedented examination with large number cases and used newly developed PGSAS-45 questionnaire evaluating patient QOL following gastrectomy. The PGSAS-45 defined a certain impairment of QOL after TGRY compared to DGRY specifically. Therefore, it is important to closely monitor patients to enable early detection and management of PGS, and to provide appropriate nutritional support. The results of the present study defined that the sole loss of remaining stomach strongly and broadly impairs the QOL in postgastrectomy patients.

Applications

The study results suggest that reservoir reconstruction using a stomach substitute should be an optional alternative to maintain better QOL after TG.

Terminology

Postgastrectomy syndrome is a group of disorders and complications following gastrectomy. It includes early dumping syndrome, late dumping syndrome, bile reflux gastritis, afferent loop syndrome, efferent loop syndrome, Roux syndrome, postvagotomy diarrhea, malabsorption, anemia, osteoporosis, gastroparesis, and weight loss.

Peer-review

The authors have conducted a well-written study based on sound methods. The manuscript flows easily and with clarity. The case selection and choice of the important variables were appropriate and clinically relevant. The graphs and the tables are to the point and summarizing the main results of the study in an appropriate fashion. The retrospective nature limits the study, and the analysis would benefit from a propensity score matching to match the cases better and hence give stronger inference.

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ORIGINAL ARTICLE

Prospective Study

Multi-analyte analysis of cytokines that predict outcomes in patients with hepatocellular carcinoma treated with radiotherapy

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Abstract

AIM

To analyze cytokine levels and to identify their association with outcome in patients with hepatocellular carcinoma (HCC) treated with radiotherapy (RT).

METHODS

Patients with HCC who were treated with RT were eligible for this prospective study. Blood samples were collected before and after RT, and serum cytokine levels including interleukin (IL)-1, IL-6, IL-8, IL-10, IL-12, and tumor necrosis factor-α were analyzed.

RESULTS

Between 2008 and 2009, 51 patients were enrolled in this study. Baseline IL-6 level was high in patients with a history of pre-RT treatment. Median survival was 13.9 mo with alpha-fetoprotein (AFP) as a significant factor (P = 0.020). Median failure-free survival (FFS) for infield, outfield-intrahepatic and extrahepatic failures were 23.3, 11.5 and 12.0 mo, respectively. Sex and baseline IL-6 level were associated with infield FFS, and baseline IL-10 level was correlated with outfield-intrahepatic FFS. For extrahepatic FFS, AFP was significant (P =

0.034). Patients with a baseline IL-6 level of ⩾ 9.7 pg/mL showed worse infield FFS (P = 0.005), and this significance was observed only in treatment-non-naïve patients (P = 0.022).

CONCLUSION

In addition to AFP, cytokines seem useful in predicting infield and outfield-intrahepatic failure. Serum cytokines could be useful biomarkers for predicting RT outcome in HCC.

Key words: Hepatocellular carcinoma; Radiotherapy; Cytokine; Interleukin-6

Core tip: A prospective study to identify associations between serum cytokine levels and radiotherapy (RT) outcomes was performed in 51 patients with hepatocellular carcinoma. Baseline serum interleukin (IL)-6 levels were higher in patients with treatment failure than in those without treatment failure. This significant difference was observed only in treatment-non-naïve patients. To predict RT outcomes, analysis of baseline serum IL-6 levels may be helpful.

Cha H, Lee EJ, Seong J. Multi-analyte analysis of cytokines that predict outcomes in patients with hepatocellular carcinoma treated with radiotherapy. *World J Gastroenterol* 2017; 23(11): 2077-2085 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i11/2077.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i11.2077

INTRODUCTION

The liver is an organ that is enriched with immune cells. Chronic inflammation caused by infection with hepatitis B or C virus or by steatohepatitis is a risk factor for the development of hepatocellular carcinoma (HCC). Continued cytokine-induced hepatocyte damage followed by hepatocyte regeneration leads to HCC development. The involvement of interleukin (IL)-1, IL-2, IL-6, IL-10, IL-12, tumor necrosis factor (TNF)-α, and transforming growth factor (TGF)-β in hepatocarcinogenesis has been reported. As levels of these cytokines are more increased in patients with HCC than in healthy individuals or patients with cirrhosis, they have been studied as biomarkers for the detection of HCC[1-5].

It has been reported that cytokine levels are associated with the radiation response of the tumor. In general, elevated pro-inflammatory cytokine levels before radiotherapy (RT) correlated with treatment resistance and poor treatment outcome in several cancers. Radiation-induced pro-inflammatory cytokine pathways within tumor cells may help the cells survive RT[6]. Recently, the application of RT in the treatment of HCC has increased[7]. However, there have been few studies on the role of cytokines in the RT response in patients with HCC.

Several biomarkers in cancer detection or treatment have been studied. As mentioned, cytokines play a role in cancer development and treatment response. Cytokine levels in serum or plasma are easily accessible and may be useful as biomarkers in screening for cancer or predicting outcomes[8]. The aim of this study was to analyze serum cytokine levels and to identify the significance of serum cytokines in treatment outcomes for patients with HCC treated with RT.

MATERIALS AND METHODS

This study was conducted prospectively and conformed to the ethical guidelines of the Declaration of Helsinki. It was approved by the Institutional Review Board at Yonsei Severance Hospital (IRB number: 4-2008-2012). All patients were enrolled after providing written informed consent. The number of patients was calculated using a one-sample t-test. Based on an expected dropout rate of 10%, we planned to enroll 109 patients. However, the study was closed early due to poor accrual. Ultimately, 51 patients were included in this study between September 2008 and October 2009.

RT was performed using 3D conformal RT or intensity-modulated RT. Blood samples were collected from patients before the start and at completion of the RT schedule. We selected serum cytokines, including IL-1, IL-6, IL-8, IL-10, IL-12 and TNF-α, for analysis that were known to be associated with HCC. Serum cytokine levels were measured using Cytokine Bead Array kits according to the manufacturer's instructions (BD Biosciences, San Jose, CA, United States) using a fluorescence-activated cell sorting (FACS) Verse flow cytometer (Becton Dickinson, Franklin Lakes, NJ, United States). The baseline level was defined as the value before RT.

We classified patterns of failure into three categories: infield failure, outfield-intrahepatic failure, and extrahepatic failure. Infield failure was defined as progression of the tumor within the RT field that covered the planning target volume. Intrahepatic failure was defined as progression of the tumor within the liver but outside of the RT field. Extrahepatic failure was a distant metastasis in lung, bone, *etc*. Overall survival (OS) and failure-free survival (FFS) were calculated from the first date of RT to the date of death and disease progression, respectively.

Statistical analysis

The statistical significance of differences in mean values according to patient and tumor characteristics was determined using Student's t-test or a one-way analysis of variance. Survival rates were evaluated

Table 1 Patient, tumor and treatment characteristics

Characteristic		*n*	Percent
Age (yr), median		54 (range, 32-75)	
Sex	Male	44	86.3
	Female	7	13.7
Viral type	B	44	86.3
	C	4	7.8
	NBNC	3	5.9
Child-Pugh class	A	49	96.1
	B	2	3.9
Modified UICC stage	II	4	7.8
	III	32	62.7
	IV	15	29.5
BCLC stage	A	14	27.5
	B	12	23.5
	C	25	49.0
PVT	No	31	60.8
	Yes	20	39.2
Multiplicity	No	25	49.0
	Yes	26	51.0
Tumor size in cm, median		8.5 (range, 1.0-19.6)	
AFP (IU/mL), median		141.9 (range, 1.53-83000)	
PIVKA-II (mAU/mL), median		600 (range, 12-2000)	
Treatment-naïve	Yes	29	56.9
	No	22	43.1
Total dose of RT (Gy), median		50.4 (range, 45-64.8)	

NBNC: Non-B non-C; UICC: International Union Against Cancer; BCLC: Barcelona Clinic Liver Cancer; PVT: Portal vein thrombosis; AFP: α-fetoprotein; PIVKA-II: Prothrombin induced by vitamin K absence-II; RT: Radiotherapy.

using a Kaplan-Meier analysis, and the correlation between FFS and clinical factors or serum cytokine level as a continuous variable was analyzed using the Cox hazard proportional model. Multivariate analysis was performed using the Cox stepwise regression model. The cut-off value of IL-6 was obtained from a receiver operating characteristic (ROC) curve based on the Youden index. Statistical analysis was performed using SPSS 20.0 (IBM, Armonk, NY, United States).

RESULTS

Patient, tumor and treatment characteristics

The median follow-up duration for the entire patient population was 13.3 mo (range, 1.6-63.5 mo).

The patient, tumor and treatment characteristics are described in Table 1. The median age was 54 years (range, 32-75 years). Most patients (92.2%) were diagnosed with International Union Against Cancer (UICC) stage Ⅲ or Ⅳ HCC, and half of the patients had Barcelona Clinic Liver Cancer (BCLC) stage C disease at the time of RT. Twenty-two patients (43.1%) had a history of pre-RT treatment and were defined as treatment-non-naïve patients. All treatment-non-naïve patients received transarterial chemoembolization (TACE) or transarterial chemoinfusion before RT. Additionally, surgery, radiofrequency ablation, holmium, or intra-arterial chemotherapy was performed in 7 patients. The median serum levels of alpha-fetoprotein (AFP) and PIVKA-II were 141.9 IU/mL (range, 1.53-83000 IU/mL) and 600 mAU/mL (range, 12-2000 mAU/mL), respectively. The median total prescribed dose of RT was 50.4 Gy (range, 45-64.8 Gy) and median duration of RT was 34 d (range, 25-56 d).

Serum cytokine as a prognostic biomarker

We evaluated the correlation between baseline serum cytokine levels and the characteristics of patients and tumors (Figure 1). Baseline IL-6 and IL-8 levels were found to increase with the UICC stage. When comparing stage Ⅱ with stage Ⅳ, there was a significant difference in the IL-8 level (P = 0.006), and IL-6 showed borderline significance (P = 0.051). Treatment-non-naïve patients showed higher baseline serum IL-6 levels than treatment-naïve patients (P = 0.028). There were no significant differences in serum cytokine levels according to sex, BCLC stage, portal vein thrombosis, tumor multiplicity, and pre-RT tumor marker level.

Serum cytokine as a predictive biomarker

Forty-five patients (88.2%) died during the follow-up period. Median OS was 13.9 mo (range, 2.2-63.5 mo) and 1-year and 2-year survival rates were 56.9% and 33.3%, respectively. The AFP level was only a significant factor for OS (P = 0.020, RR, 1.001, 95%CI: 1.000-1.002). There was no correlation between OS and baseline serum cytokine level (Table 2).

During the follow-up period, treatment failures occurred in 40 patients (79.4%), and infield, outfield-intrahepatic and extrahepatic failures were observed in 19, 31 and 31 patients, respectively. The median FFS for infield, outfield-intrahepatic, and extrahepatic failures were 23.3, 11.5 and 12.0 mo, respectively. The correlation of FFS with tumor characteristics and baseline cytokine level was described in Tables 3-5. For infield FFS, sex (P < 0.001, RR, 47.505, 95%CI: 7.384-305.601) and baseline serum IL-6 level (P < 0.001, RR, 1.019, 95%CI: 1.011-1.028) were statistically significant. Baseline serum IL-10 level was a significant factor for outfield-intrahepatic FFS (P = 0.026, RR, 0.830, 95%CI: 0.705-0.978), and AFP was associated with extrahepatic failure (P = 0.034, RR, 1.001, 95%CI: 1.000-1.003).

The cut-off value of baseline serum IL-6 level for infield FFS was 9.735 (Figure 2A; AUC 0.748, P = 0.003, 95%CI: 0.607-0.888). Patients with a baseline serum IL-6 level higher than 9.7 pg/mL showed worse infield FFS than those with a baseline serum IL-6 level less than 9.7 pg/mL (Figure 2B; P = 0.005).

As baseline serum IL-6 level significantly differed between treatment-non-naïve and treatment-naïve patients, we performed a subgroup analysis based on pre-RT treatment. The subgroup analysis indicated the significant difference in baseline serum IL-6 level was observed only in treatment-non-naïve patients (Figure 2C and D; P = 0.002).

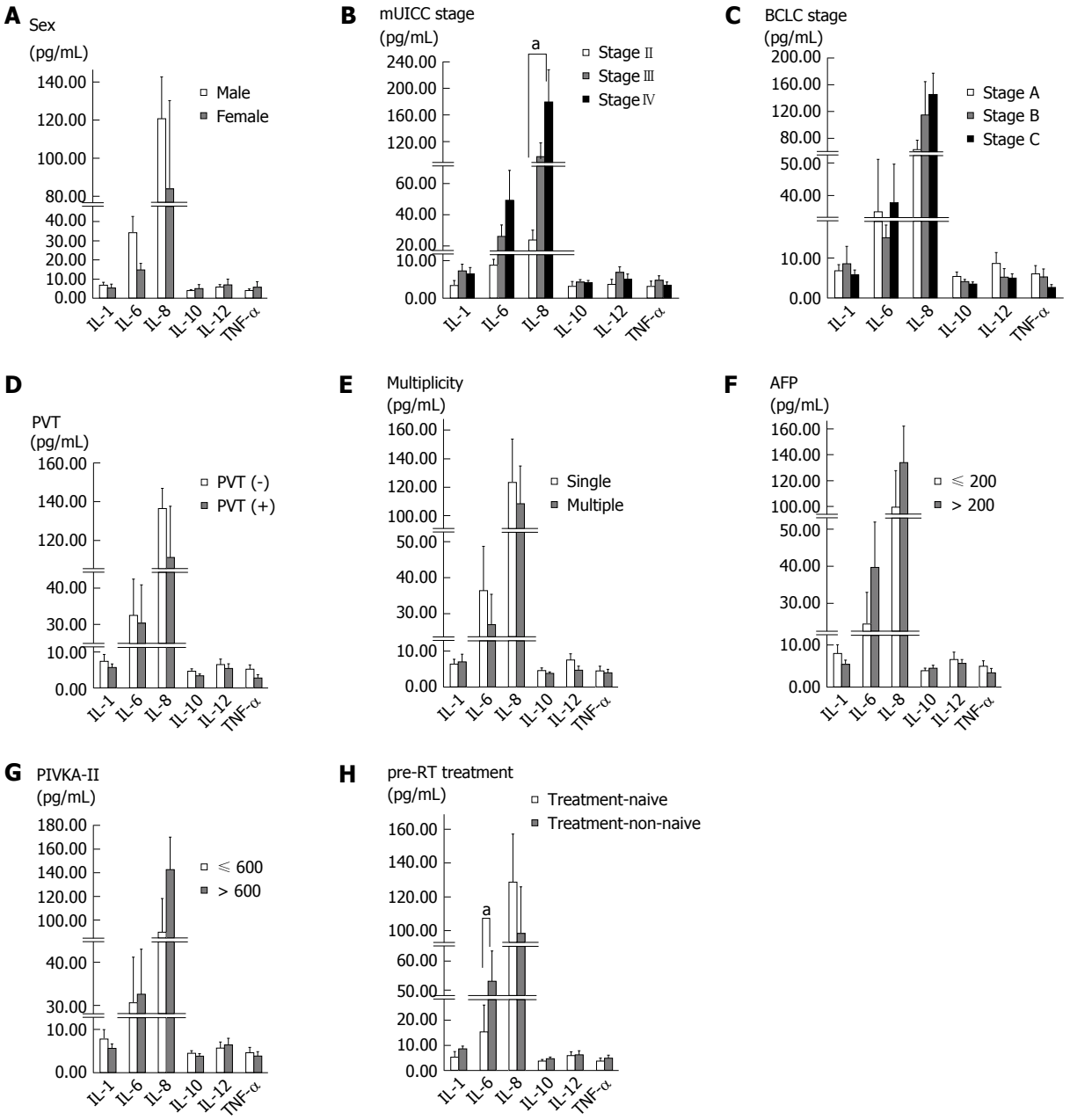

Figure 1 Baseline serum cytokine levels according to tumor characteristics. A, C, D, E, F and G: There were no significant differences in serum cytokine levels according to sex, BCLC stage, portal vein thrombosis, multiplicity and level of alpha-fetoprotein; B: When comparing stage Ⅱ with stage Ⅳ, interleukin (IL)-8 levels showed a significant difference (^aP = 0.006) and IL-6 showed a borderline significance (P = 0.051); H: Treatment-non-naïve patients showed higher baseline serum IL-6 levels than treatment-naïve patients (^aP = 0.028).

Variation of serum cytokine levels

We also analyzed serum cytokine levels on completion of RT (Table 6). After RT, serum IL-10 level had increased from 4.19 ± 0.41 to 5.83 ± 0.46 (P = 0.002), and serum IL-12 level had decreased from 6.10 ± 1.01 to 4.10 ± 0.59 (P = 0.018). However, these changes were not associated with survival or treatment failure. Variations in the serum levels of other cytokines after RT were not significant.

Treatment toxicity

The median value of the mean liver dose was 20.3 Gy (range, 5.7-34.9 Gy), and there were no cases of severe radiation-induced liver disease during RT and until 3 mo after the completion of RT. Grade 3-4 hematologic toxicities including anemia, neutropenia and thrombocytopenia were reported in 6, 12 and 9 patients, respectively. There were also 3 cases of hypoalbuminemia, 8 cases of hyperbilirubinemia, 10 cases of elevated liver enzymes, and 3 cases of gastrointestinal bleeding (> grade 3).

DISCUSSION

In this study, we investigated whether the serum levels of six cytokines (IL-1, IL-6, IL-8, IL-10, IL-12 and

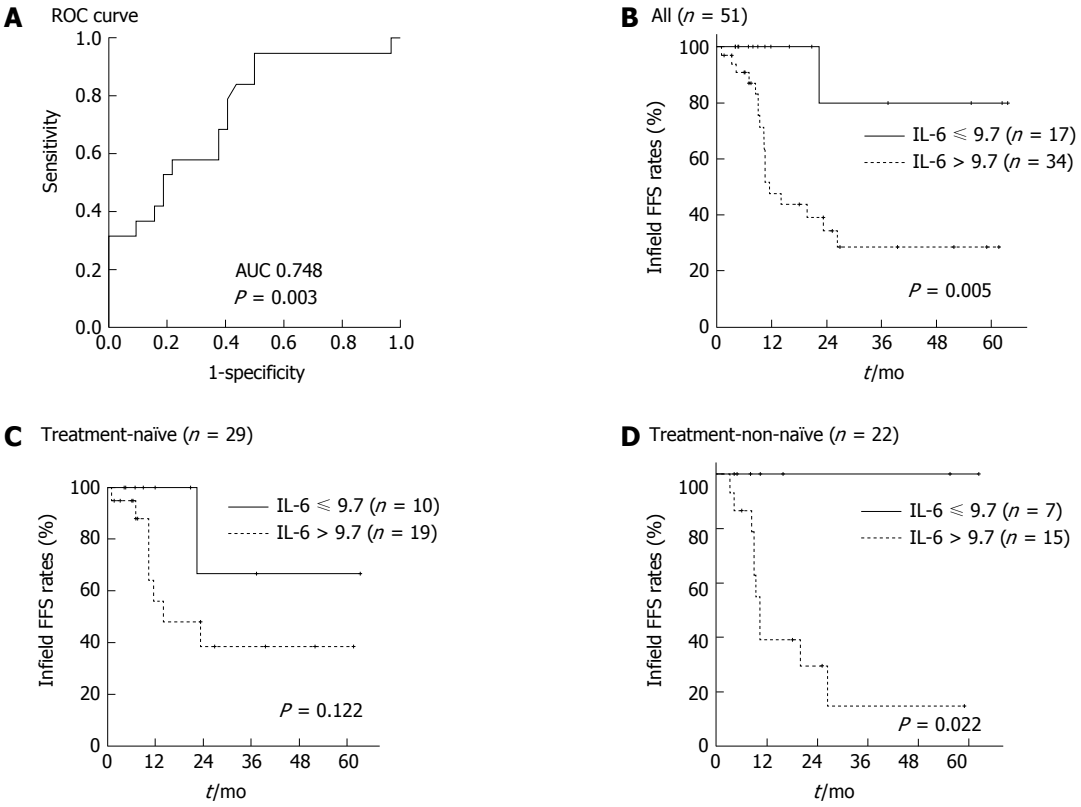

Figure 2 Infield failure-free survival according to cut-off value of baseline serum interleukin-6. A: The cut-off value of interleukin (IL)-6 was obtained from a receiver operating characteristic (ROC) curve based on the Youden index (AUC, 0.748, *P* = 0.003, 95%CI: 1.011-1.028); B: Patients with baseline serum IL-6 level higher than 9.7 pg/mL showed worse infield FFS than those with baseline serum IL-6 level lower than 9.7 pg/mL (47.5% *vs* 100%, *P* = 0.005); C and D: In subgroup analysis, the significant difference of infield FFS was observed only in treatment-non-naïve patients (*P* = 0.022).

Table 2 Univariate and multivariate analyses of clinical factors or cytokines for failure-free survival - overall survival

	Univariate		Multivariate	
	***P* value**	**RR (95%CI)**	***P* value**	**RR (95%CI)**
Sex	0.580	1.276 (0.538-3.027)		
Age	0.954	1.001 (0.970-1.033)		
Stage	0.861	1.052 (0.600-1.844)		
BCLC	0.224	1.252 (0.872-1.799)		
Size	0.308	1.037 (0.967-1.113)		
PVT	0.132	1.581 (0.871-2.871)		
LN metastasis	0.867	1.092 (0.389-3.061)		
Multiplicity	0.957	1.016 (0.561-1.841)		
AFP	0.020	1.001 (1.000-1.002)	0.020	1.001 (1.000-1.002)
PIVKA-II	0.211	1.022 (0.988-1.056)		
Pre-RT treatment	0.693	0.887 (0.487-1.608)		
Baseline IL-1	0.262	0.979 (0.942-1.016)		
Baseline IL-6	0.692	1.001 (0.996-1.007)		
Baseline IL-8	0.770	1.000 (0.998-1.002)		
Baseline IL-10	0.909	0.994 (0.890-1.109)		
Baseline IL-12	0.449	1.014 (0.978-1.052)		
Baseline TNF-α	0.573	0.984 (0.930-1.041)		

Age, size, AFP, PIVKA-II, and baseline cytokine levels were analyzed as continuous variables using the Cox hazard ratio model. BCLC: Barcelona Clinic Liver Cancer; PVT: Portal vein thrombosis; LN: Lymph node; AFP: α-fetoprotein; PIVKA-II: Prothrombin induced by vitamin K absence-II; RT: Radiotherapy; IL: Interleukin; TNF: Tumor necrosis factor.

TNF-α) would have clinical significance in patients with HCC who were treated with RT. We demonstrated that patients with a history of pre-RT treatment showed a higher baseline serum IL-6 level than treatment-naïve patients. A high baseline serum IL-6 level was also associated with infield failure. The subgroup analysis revealed that baseline serum IL-6 levels were associated with infield failure only in treatment-non-naïve patients.

IL-6 is a pro-inflammatory cytokine and one of

Table 3 Univariate and multivariate analyses of clinical factors or cytokines for failure-free survival - infield failure-free survival

	Univariate		Multivariate	
	P value	RR (95%CI)	*P* value	RR (95%CI)
Sex	0.001	10.262 (2.479-42.481)	< 0.001	47.505 (7.384-305.601)
Age	0.198	0.968 (0.921-1.017)		
Stage	0.082	2.106 (0.911-4.871)		
BCLC	0.441	1.257 (0.702-2.250)		
Size	0.903	1.007 (0.904-1.121)		
PVT	0.247	1.741 (0.681-4.452)		
LN metastasis	0.408	1.867 (0.425-8.191)		
Multiplicity	0.944	1.033 (0.412-2.591)		
AFP	0.653	0.999 (0.996-1.002)		
PIVKA-II	0.889	0.996 (0.947-1.048)		
Pre-RT treatment	0.290	1.627 (0.660-4.013)		
Baseline IL-1	0.895	0.997 (0.955-1.041)		
Baseline IL-6	0.000	1.011 (1.005-1.018)	< 0.001	1.019 (1.011-1.028)
Baseline IL-8	0.985	1.000 (0.997-1.003)		
Baseline IL-10	0.437	1.060 (0.915-1.227)		
Baseline IL-12	0.903	0.996 (0.938-1.059)		
Baseline TNF-α	0.864	0.993 (0.918-1.074)		

Age, size, AFP, PIVKA-II, and baseline cytokine levels were analyzed as continuous variables using the Cox hazard ratio model. BCLC: Barcelona Clinic Liver Cancer; PVT: Portal vein thrombosis; LN: Lymph node; AFP: α-fetoprotein; PIVKA-II: Prothrombin induced by vitamin K absence-II; RT: Radiotherapy; IL: Interleukin; TNF: Tumor necrosis factor.

Table 4 Univariate and multivariate analyses of clinical factors or cytokines for failure-free survival - outfield-intrahepatic failure-free survival

	Univariate		Multivariate	
	P value	RR (95%CI)	*P* value	RR (95%CI)
Sex	0.387	1.539 (0.579-4.086)		
Age	0.834	0.996 (0.959-1.035)		
Stage	0.601	0.834 (0.423-1.646)		
BCLC	0.383	1.210 (0.788-1.859)		
Size	0.534	0.974 (0.898-1.058)		
PVT	0.326	1.433 (0.699-2.940)		
LN metastasis	0.387	0.530 (0.126-2.230)		
Multiplicity	0.303	1.464 (0.709-3.021)		
AFP	0.649	1.000 (0.999-1.002)		
PIVKA-II	0.920	1.002 (0.963-1.043)		
Pre-RT treatment	0.745	1.126 (0.550-2.307)		
Baseline IL-1	0.573	1.015 (0.964-1.069)		
Baseline IL-6	0.271	0.950 (0.985-1.004)		
Baseline IL-8	0.942	1.000 (0.998-1.002)		
Baseline IL-10	0.026	0.830 (0.705-0.978)	0.026	0.830 (0.705-0.978)
Baseline IL-12	0.197	0.959 (0.901-1.022)		
Baseline TNF-α	0.240	0.951 (0.876-1.034)		

Age, size, AFP, PIVKA-II, and baseline cytokine levels were analyzed as continuous variables using the Cox hazard ratio model. BCLC: Barcelona Clinic Liver Cancer; PVT: Portal vein thrombosis; LN: Lymph node; AFP: α-fetoprotein; PIVKA-II: Prothrombin induced by vitamin K absence-II; RT: Radiotherapy; IL: Interleukin; TNF: Tumor necrosis factor.

the well-characterized pro-tumorigenic cytokines. It is upregulated in many cancers and is important for tumor cell proliferation, survival, differentiation, migration, invasion, metastasis and angiogenesis[9]. In the liver, IL-6 is involved in acute phase response, liver generation, and regulation of physiological and pathophysiological conditions. Similar to other cancers, IL-6 has been shown to be correlated with HCC development risk, advanced stage and poor survival[10-12]. In this study, serum IL-6 level showed borderline significance between patients with stage Ⅱ and Ⅳ disease, most likely due to the small number of patients with stage II disease (n = 4).

A history of pre-RT treatment was associated with a difference in baseline serum IL-6 level. Most of the treatment-non-naïve patients underwent TACE, and previous studies have demonstrated that serum IL-6 level increases after TACE and is correlated with post-TACE inflammation[13,14]. In these studies, the increase of serum IL-6 level occurred within several days after TACE and was attributed to a protective function of IL-6 against acute liver injury. In this study, the time interval between the last TACE and the start of RT was median 1.5 mo (range, 0.2-17.5 mo) and the

Table 5 Univariate and multivariate analyses of clinical factors or cytokines for failure-free survival - extrahepatic failure-free survival

	Univariate		Multivariate	
	P value	RR (95%CI)	*P* value	RR (95%CI)
Sex	0.153	2.043 (0.768-5.438)		
Age	0.502	0.987 (0.951-1.025)		
Stage	0.196	1.562 (0.795-3.070)		
BCLC	0.782	0.942 (0.615-1.441)		
Size	0.830	1.010 (0.926-1.101)		
PVT	0.988	0.994 (0.465-2.125)		
LN metastasis	0.936	0.952 (0.289-3.141)		
Multiplicity	0.622	1.197 (0.586-2.448)		
AFP	0.034	1.001 (1.000-1.003)	0.034	1.001 (1.000-1.003)
PIVKA-II	0.449	1.016 (0.976-1.057)		
Pre-RT treatment	0.878	1.057 (0.520-2.147)		
Baseline IL-1	0.725	1.005 (0.977-1.034)		
Baseline IL-6	0.529	1.002 (0.996-1.007)		
Baseline IL-8	0.546	1.001 (0.999-1.003)		
Baseline IL-10	0.309	1.062 (0.946-1.193)		
Baseline IL-12	0.726	1.009 (0.962-1.058)		
Baseline TNF-α	0.956	1.002 (0.942-1.066)		

Age, size, AFP, PIVKA-II, and baseline cytokine levels were analyzed as continuous variables using the Cox hazard ratio model. BCLC: Barcelona Clinic Liver Cancer; PVT: Portal vein thrombosis; LN: Lymph node; AFP: α-fetoprotein; PIVKA-II: Prothrombin induced by vitamin K absence-II; RT: Radiotherapy; IL: Interleukin; TNF: Tumor necrosis factor.

Table 6 Variation of serum cytokine levels before and after radiotherapy (mean ± SEM)

	Before RT (pg/mL)	After RT (pg/mL)	*P* value
IL-1	6.77 ± 1.18	9.13 ± 1.51	0.229
IL-6	31.63 ± 7.34	29.54 ± 6.60	0.794
IL-8	115.75 ± 19.84	159.73 ± 38.96	0.211
IL-10	4.19 ± 0.41	5.83 ± 0.46	0.002
IL-12	6.10 ± 1.01	4.10 ± 0.59	0.018
TNF-α	4.27 ± 0.79	3.33 ± 0.40	0.130

IL: Interleukin; TNF: Tumor necrosis factor; RT: Radiotherapy.

baseline serum level was not correlated with the time interval. Therefore, the elevated serum IL-6 level in treatment-non-naïve patients of our study cannot be explained only by a pre-RT history of TACE. After TACE, TACE-induced hepatic tissue injuries around the tumor provoke an inflammatory response and alter the immune system. These inflammatory cytokine responses, including IL-6, may also induce tumor recurrence or progression after TACE.

Among serum cytokines, only baseline serum IL-6 was a significant factor for treatment failure in the RT field in this study. The effect of serum IL-6 on RT and chemoradiotherapy (CRT) response has been reported in many cancers, including lung, prostate, esophagus, stomach, pancreas, and head and neck cancers[15-17]. In previous reports, an elevated serum IL-6 level before RT/CRT was associated with poor treatment outcome. A high serum IL-6 level is known to correlate with tumor aggressiveness and associated tumor factors that affect treatment outcome, such as large size and advanced stage. However, there have been few studies on the effect of serum IL-6 on RT response in HCC. Jang *et al*[11] reported that IL-6 was a strong inflammatory indicator for predicting the outcome in patients with HCC who received loco-regional therapy (mainly TACE). RT was considered in patients with portal vein thrombosis or extrahepatic metastasis, and only 13 patients received RT after TACE. A higher IL-6 level was correlated with shorter survival. Chen *et al*[18] investigated the role of IL-6 in the radiation response of liver tumors. IL-6 expression was positively linked to radiation resistance in both *in vivo* and *in vitro* experiments. The authors suggested that concurrent treatment with IL-6 inhibitors could be a potential therapeutic strategy for increasing the radiation response of tumors.

In a previous report, we described the results of hepatic arterial concurrent chemoradiotherapy (CCRT) for advanced HCC and found that pre-CCRT treatment history was a predictive risk factor for treatment failure, particularly infield failure[19]. Based on the results of the current study, this difference in treatment outcome might be explained by the fact that treatment-non-naïve patients had higher baseline serum IL-6 levels than treatment-naïve patients. Although application of RT has increased in the treatment of HCC, RT continues to be used as either complementary or salvage treatment after other treatment modalities such as TACE, radiofrequency ablation or surgery, rather than as a primary treatment. Therefore, assessment of baseline serum cytokine levels, particularly IL-6, could be helpful in predicting the treatment outcome in treatment-non-naïve patients.

To determine a useful criterion for predicting treatment outcomes in clinics, we identified the predictive cut-off value of baseline serum IL-6 level by ROC curve analysis. And 9.7 pg/mL as cut-off value was useful to predict infield failure in this study. Further

study is needed to verify this cut-off value in many patients with HCC treated with RT.

In this study, lower serum IL-10 level was correlated with worse outfield-intrahepatic FFS. IL-10 is known as a pleiotropic cytokine with dual roles in the immune system, including immune-suppression and immune-stimulation[20]. Many studies reported that serum IL-10 levels were higher in cancer patients than in healthy cohorts and that a higher IL-10 level was associated with worse prognosis[21]. However, other contradictory reports showed that patients with IL-10-negative tumors showed poor prognosis[22,23]. The inhibition of inflammatory cytokines and the stimulation of cytotoxic T cells by IL-10 have been further studied for cancer treatment using recombinant IL-10[24].

After RT, serum levels of IL-10 increased while the levels of IL-12 decreased. Several studies demonstrated changes in cytokine expression resulting from treatment of HCC. Cytokine levels are altered after treatment regimens including surgery or TACE; however, the direction of the change has not always been consistent between studies[25,26]. Although the change in the serum IL-10 or IL-12 level was not significantly associated with treatment failure in this study, these findings indicate that local RT might produce a systemic effect. It has been reported that local RT triggers systemic effects through the recruitment of biological effectors outside the radiation field[27].

The small number of patients was a limitation of this study and this limitation could affect statistical significance. Further large scale studies are needed to confirm our finding and to examine how radiation affects serum cytokine levels in HCC. In addition, this study was performed only in patients treated with RT. Further studies to identify serum cytokine levels in patients with HCC treated with other various treatment modalities might also be useful.

In conclusion, the current findings suggest that an assessment of serum cytokines may be helpful for predicting treatment outcome after RT in patients with HCC. In addition to tumor marker AFP, cytokines seemed to be useful in predicting infield failure (IL-6) and outfield-intrahepatic failure (IL-10). Serum cytokines could be useful biomarkers for predicting RT outcome in HCC.

COMMENTS

Background

The role of cytokines in carcinogenesis and treatment response in many cancers has been reported. Serum cytokine levels are easily accessible and may be useful as biomarkers in screening for cancers or predicting outcomes. Although the application of radiotherapy (RT) in the treatment of hepatocellular carcinoma (HCC) has increased, the role of cytokine in the RT response in patients with HCC is not well known.

Research frontiers

The study on the identification of significant cytokine in cancer screening or prediction of treatment outcome is an important area. As the application of RT for HCC increases, it has become important to find a useful biomarker to predict RT outcome in patients with HCC.

Innovations and breakthroughs

The association between cytokine levels and treatment response is known in many cancers. However, there are few studies on the role of cytokines in the RT response in patients with HCC. This study aimed to identify the significance of serum cytokines in the treatment outcomes for patients with HCC treated with RT. In this study, the authors collected samples prospectively and analyzed the serum cytokines before and after RT. We demonstrated that patients with a history of pre-RT treatment showed a higher baseline serum IL-6 level than treatment-naïve patients and a high baseline was also shown to be associated with infield failure, especially in treatment-non-naïve patients.

Applications

The results of this study suggest that the assessment of pre-RT serum cytokine levels, particularly IL-6, could be helpful in predicting treatment outcome in treatment-non-naïve paints.

Terminology

IL-6 is a pro-inflammatory cytokine and one of the well-characterized pro-tumorigenic cytokines. A high serum IL-6 level before RT or chemoradiotherapy was associated with poor treatment outcome in many cancers. Infield failure was defined as progression of the tumor within the RT field that covered the planning target volume.

Peer-review

This paper is relevant and adds to the literature. The use of cytokines is an active area of research and this group appears to be at the forefront of this investigation. Although a small number of patients limit the significance of this study, the results are clear and seem to promote further studies.

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CASE REPORT

Severe infection with multidrug-resistant *Salmonella choleraesuis* in a young patient with primary sclerosing cholangitis

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Author contributions: Ferstl PG was in charge of medical care of the patient, compiled the relevant patient data, wrote the manuscript, and conducted literature research; Reinheimer C and Jozsa K conducted literature research and took part in writing the manuscript on behalf of the microbiological topics; Zeuzem S, Kempf VAJ, and Waidmann O were involved in the critical review of the manuscript; Grammatikos G supervised patient care during hospital admission, compiled most of the relevant patient data, and was responsible for critical review of the manuscript.

Institutional review board statement: Since medical treatment was conducted according to highest clinical standards, the present case report is not of experimental character and does not require an ethical committee statement.

Informed consent statement: Patient data and dates of treatment were anonymized prior to writing the case report. The patient provided informed written consent prior to report submission.

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Abstract

Massive global spread of multidrug-resistant (MDR) *Salmonella* spp. expressing extended-spectrum beta-lactamase (ESBL) and additional resistance to fluoroquinolones has often been attributed to high international mobility as well as excessive use of oral antibiotics in livestock farming. However, MDR *Salmonella* spp. have not been mentioned as a widespread pathogen in clinical settings so far. We demonstrate the case of a 25-year-old male with primary sclerosing cholangitis who tested positive for MDR *Salmonella enterica* serotype Choleraesuis expressing ESBL and fluoroquinolone resistance. The pathogen was supposedly acquired during a trip to Thailand, causing severe fever, cholangitis and

pancreatitis. To our knowledge, this is the first report of *Salmonella enterica* serotype Choleraesuis in Europe expressing such a multidrug resistance pattern. ESBL resistance of *Salmonella enterica* spp. should be considered in patients with obstructive biliary tract pathology and travel history in endemic countries.

Key words: Biliary physiology; Infectious disease; Multidrug resistance; Primary sclerosing cholangitis; Salmonella choleraesuis

Core tip: We report a case of aggressive infection with a multidrug resistant strain of *Salmonella choleraesuis* in a patient with primary sclerosing cholangitis. Successful treatment involved repetitive ultrasound and endoscopic intervention, as well as multiple adjustments of the antibiotic regimen. This is the first case report addressing multidrug-resistant salmonellosis in patients with predisposing biliary disease in Europe. It illustrates how close interdisciplinary cooperation between clinicians and microbiologists is warranted in an era of emerging antibiotic resistance.

Ferstl PG, Reinheimer C, Jozsa K, Zeuzem S, Kempf VAJ, Waidmann O, Grammatikos G. Severe infection with multidrug-resistant *Salmonella choleraesuis* in a young patient with primary sclerosing cholangitis. *World J Gastroenterol* 2017; 23(11): 2086-2089 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i11/2086.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i11.2086

INTRODUCTION

Among a variety of pathogens that are known for colonizing the gallbladder, *Salmonella* spp. seem to benefit from its prevailing conditions in particular[1]. Bile has bactericidal properties, which *Salmonella* spp. manage to escape by several mechanisms[2]. Thus, the biliary system serves as a favorable reservoir for *Salmonella* spp. and they might bloom particularly well if the passage of bile into the intestine is impaired. In case of inflammation, the constitution of bile alters, thus supporting infection of the biliary tree with pathogenic Gram-negative organisms, *e.g.*, *Salmonella* spp.[3]. Up to now, this phenomenon has mostly been attributed to gallstones[4], which have been known to be a common cause of infectious cholangitis. However, only few case reports of patients suffering from primary hepatobiliary diseases such as Caroli's syndrome and shedding *Salmonella* are available up to now[5]. In case of severe cholangitis and acute pancreatitis in patients with a predisposition to cholestasis, *Salmonella* spp. should be considered as a causative pathogen. Since cases of infectious pancreatitis due to *Salmonella* spp, .*e.g.*, *Salmonella enterica* serotype Typhimurium, have been reported earlier[6], we demonstrate here an infection with *Salmonella enterica* serotype Choleraesuis. Immunocompromising diseases, *e.g.*, cirrhosis or inflammatory bowel disease, might promote invasive salmonellosis and bloodstream infection and lead to severe courses of infections[7].

CASE REPORT

A 25-year-old male student presented to the emergency department of the University Hospital Frankfurt with watery diarrhea at a frequency of ten stools per day, concomitant cramps in the lower abdomen, and fever up to 40 ℃ for six days. He had been diagnosed with primary sclerosing cholangitis and ulcerative colitis in 2005 and was on long-term medication with mesalazine and ursodesoxycholic acid. He had returned from a backpacking holiday to Thailand 16 d ago, which ended without any medical complaints or symptoms.

On admission, blood tests showed serum levels of bilirubin at 1.7 mg/dL, alkaline phosphatase (AP) at 276 U/L, alanine aminotransferase (ALT) at 56 U/L, lipase at 501 U/L, C-reactive protein (CRP) at 9.2 mg/dL, and 15000 leucocytes per milliliter of blood. Abdominal ultrasound showed an enlarged gallbladder, 11 cm in diameter, without any signs of cholecystitis or acute pancreatitis. Blood and stool cultures were taken and intravenous antibiotic therapy with ciprofloxacin 500 mg and metronidazole 400 mg three times daily was initiated. Stool cultures yielded detection of non-typhoidal *Salmonella* spp., hence antibiotic therapy was switched to ceftriaxone 2 g daily. Despite the adaptation of the antibiotic regimen, the patient's general condition worsened and he reported increasing abdominal pain located in the epigastrium. Fever continued and diarrhea suspended.

Repeated ultrasound of the abdomen displayed a mildly bloated pancreas with peripancreatic edema. Liver function tests showed rising bilirubin, ALT, AP, leucocytes, and CRP kept increasing. Antibiotic therapy was switched to imipenem 500 mg four times daily. Subsequently, an isolate of *Salmonella* group C with extended-spectrum beta-lactamase (ESBL) and additional resistance to fluoroquinolones was detected in stool cultures. Due to ongoing elevation of cholestatic enzymes and ultrasound evidence of a distended common bile duct (CBD), endoscopic retrograde cholangiopancreatography was performed, showing a high-grade stricture of the distal CBD (Figures 1 and 2). A 3 mm small stone and pus were extracted, and a stent was applied to the CBD. Fever ceased immediately and pain was easing during the following week. Gall cultures yielded detection of the same bacterial strain as was detected in the stool. Culture isolates were sent to the national reference center for salmonellosis in Wernigerode, Germany, in order to determine the exact species serotype. Imipenem was administered for 14 d and the patient was discharged with mild residual abdominal

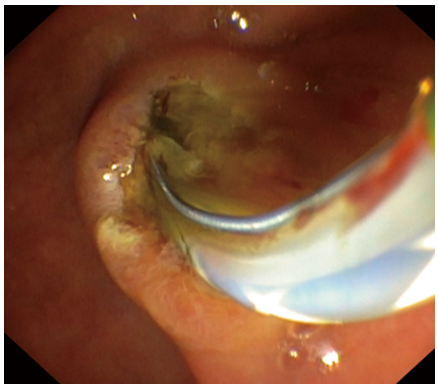

Figure 1 Endoscopic image of purulent discharge of the papilla of Vater.

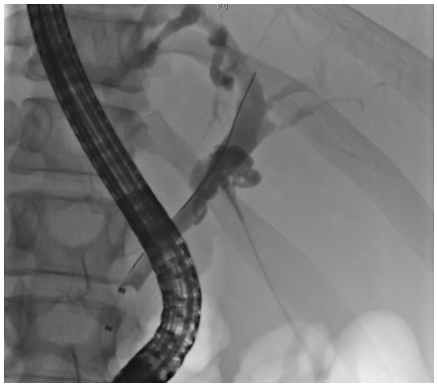

Figure 2 Endoscopic retrograde cholangiopancreatography showing a high-grade stricture of the distal common bile duct.

complaints. The following week, the reference center confirmed detection of *Salmonella enterica* serotype Choleraesuis [6,7,(c)1,5]. On readmission for planned stent extraction, the patient was free of symptoms and any *Salmonella* spp. in stool cultures. Thoracoabdominal magnetic resonance imaging showed no signs of mycotic aortic aneurism, which is frequently observed in *Salmonella* groups C and D infections[7].

Patient data and dates of treatment were anonymized prior to writing the case report. The patient provided informed written consent prior to report submission.

DISCUSSION

The rising global burden of multidrug-resistant (MDR) non-typhoidal *Salmonella* spp. has been attributed to increasing tourism to South-East Asia, where MDR non-typhoidal *Salmonella* spp. are endemic[8-10]. The prevalence of MDR non-typhoidal *Salmonella* spp. has been increasing worldwide for several years[7]. The global spread of these pathogens has been linked to use of antibiotics in livestock farming[11], consumption of raw or insufficiently cooked meat and vegetables, global food trade as well as travel to endemic areas[12,13]. The first case of serotype Choleraesuis resistant to both third-generation cephalosporins and fluoroquinolones has been reported in 2004[14]. Therefore, MDR *Salmonella* spp. are an issue of growing public health concern in Europe[11,13]. While antibiotic treatment is not recommended in asymptomatic shedders of *Salmonella* spp. or in uncomplicated gastroenteritis[15,16], MDR *Salmonella* is likely to have a critical impact in patients with obstructive biliary tract pathology and altered bile constitution. Since global burden of MDR *Salmonella* spp. keeps rising, this alarming development is reflected by our case report on travel-associated salmonellosis with serotype Choleraesuis expressing ESBL and additional resistance to fluoroquinolones.

We conclude that salmonellosis due to MDR *Salmonella* spp. should be considered in patients with immunosuppression or with hepatobiliary diseases, who can develop severe and complicated courses. Empiric treatment with carbapenems should be initiated in these patients upon clinical deterioration on common antibiotic regimens like fluoroquinolones and cephalosporins. Carbapenems cover MDR *Salmonella* spp., achieve higher concentrations within the pancreatic tissue, and thus reduce bacterial count[17]. Antibiotic treatment should be reserved for symptomatic patients. From the first day of treatment on, structured microbiological surveillance and close interdisciplinary cooperation between clinicians and microbiologists are warranted for best patient care.

COMMENTS

Case characteristics

A 25-year-old male with known primary sclerosing cholangitis and ulcerative colitis presented to our emergency ward with watery diarrhea at a frequency of ten stools per day, concomitant cramps in the lower abdomen, and fever up to 40 ℃.

Clinical diagnosis

The authors diagnosed a case of severe salmonellosis due to an isolate of *Salmonella choleraesuis* expressing extended-spectrum beta-lactamase (ESBL) and fluoroquinolone resistance, which could be detected in both bile and stool cultures.

Differential diagnosis

Initial differential diagnoses were infectious gastroenteritis, an atypical acute attack of ulcerative colitis, and obstructive cholangitis with febrile cholecystitis and pancreatitis.

Laboratory diagnosis

Blood tests showed serum levels of bilirubin at 1.7 mg/dL, alkaline phosphatase at 276 U/L, alanine aminotransferase at 56 U/L, lipase at 501 U/L, c-reactive protein at 9.2 mg/dL, and 15000 leucocytes/mL.

Imaging diagnosis

Ultrasound of the abdomen displayed a distended gallbladder, a mildly bloated pancreas with peripancreatic edema, and a distended common bile duct (CBD), while endoscopic retrograde cholangiopancreatography showed a high-grade stricture of the distal CBD with discharge of a small stone and pus.

Treatment

Upon unsuccessful antibiotic treatment with ciprofloxacin/metronidazole and later with ceftriaxone, the patient's condition and laboratory values improved rapidly under therapy with imipenem, which was administered for 14 d in total.

Experiences and lessons

ESBL resistance of *Salmonella enterica* spp. should be considered in patients with obstructive biliary tract pathology and travel history in endemic countries.

Peer-review

From an interdisciplinary perspective, this case report illustrates the features of multidrug-resistant non-typhoidal salmonellosis in a patient with primary sclerosing cholangitis, explains diagnostic pathways, and summarizes treatment recommendations for these patients.

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CASE REPORT

Dynamic enhanced computed tomography imaging findings of an inflammatory fibroid polyp with massive fibrosis in the stomach

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Abstract

Inflammatory fibroid polyp (IFP) is a rare benign lesion of the gastrointestinal tract. We report a case of computed tomography (CT) imaging finding of a gastric IFP with massive fibrosis. CT scans showed thickening of submucosal layer with overlying mucosal hyperenhancement in the gastric antrum. The submucosal layer showed increased enhancement on delayed phase imaging. An antrectomy with gastroduodenostomy was performed because gastric cancer was suspected, particularly signet ring cell carcinoma. The histopathological diagnosis was an IFP with massive fibrosis. The authors suggest that when the submucosal layer of the gastric wall is markedly thickened with delayed enhancement and preservation of the mucosal layer, an IFP with massive fibrosis should be considered in the differential diagnosis.

Key words: Inflammatory fibroid polyp; Gastric polyp; Gastric submucosal tumor; Signet ring cell carcinoma; Computed tomography imaging finding

Core tip: In our case, computed tomography imaging findings of markedly thickened submucosal layer with delayed enhancement made inflammatory fibroid polyp difficult to differentiate from malignant cancer,

particularly signet ring cell carcinoma.

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INTRODUCTION

Inflammatory fibroid polyp (IFP) is a rare non-neoplastic cellular proliferation, composed of fibrous tissue, blood vessels, and an inflammatory cell infiltrate dominated by eosinophils within an edematous and collagenous stroma[1]. The estimated incidence of IFP is 0.1% of all stomach polyps[2]. IFPs are most frequently found in the gastric antrum and are commonly ulcerated[1].

Several studies about gastric IFPs have reported its computed tomography (CT) findings and included only enhancement images of the single portal phase[3-8]. However, to the best of our knowledge, no previous report has been described about the CT findings of a gastric IFP showing delayed enhancement. Here, we report the dynamic enhanced CT imaging findings of a case of gastric IFP with massive fibrosis.

CASE REPORT

A 43-year-old female presented to the hospital with a 1-mo history of dyspepsia and epigastric pain. Physical examination was unremarkable. Blood chemistry findings were within normal range except for anemia with a hemoglobin level of 9.0 g/dL. Endoscopy showed an approximately 4 cm mass-like lesion with mucosal edema and superficial ulcer in the gastric antrum (Figure 1A). On endoscopic ultrasound (EUS), there was a heterogeneous hypoechoic submucosal mass-like lesion (Figure 1B). Only chronic inflammatory tissue without malignant cells was found on histological examination of biopsy specimens obtained through endoscopy. The patient had undergone an additional core biopsy of the submucosal lesion using EUS. However, fibrosis of submucosal lesion was extremely severe so failed biopsy using EUS. Also, the slight large size, non-pedunculated, intramural mass with fibrosis made it difficult for endoscopic resection. The patient underwent dynamic enhanced CT scan (LightSpeed16, GE Healthcare, Milwaukee, WI, United States) to evaluate the mass-like lesion in the gastric antrum. Portal phase contrast-enhanced CT showed marked wall thickening (about 2.9 cm in wall thickness, 7 cm in length) with overlying mucosal hyperenhancement in the gastric antrum (Figure 1C). The predominantly thickened wall was the submucosal layer, which showed hypoattenuation (about 85 HU) relative to the back muscles on portal phase imaging (Figure 1C) and hyperattenuation (about 115 HU) on 3-min delayed phase imaging (Figure 1D). The lesion did not show extension to adjacent organs such as the liver or pancreas and there was no lymph node enlargement (Figure 1E). These CT imaging findings were suggestive of a submucosal tumor or muscular hypertrophy of the stomach. However, a malignant tumor such as a signet ring cell carcinoma (SRC) could not be ruled out because of markedly thickened submucosal layer with delayed enhancement. Thus, an antrectomy with gastroduodenostomy was performed. Macroscopic inspection revealed the submucosal mass-like lesion measuring 4.5 cm × 4 cm × 3 cm (Figure 1F). Microscopic examination (original magnification × 12.5) demonstrated that the borders of the lesion were poorly demarcated and difficult to discern from the adjacent submucosal connective tissue (Figure 1G). Microscopic examination (original magnification × 100, × 400) showed fibroblastic cells with well-vascularized fibrotic stroma and infiltrate of chronic inflammatory cells, including many eosinophils (Figure 1H and I). No mitotic activity was identified. The final diagnosis was an IFP with prominent fibrotic and hyalinized stroma.

DISCUSSION

IFP is a rare benign lesion of the gastrointestinal (GI) tract that arises from the deep mucosa and submucosa[1,3]. IFPs can occur anywhere in the GI tract, but the gastric antrum is the most common site, followed by gastric body and fundus[4,5]. The size of gastric IFP is range from 1.2-6.5 cm and mean diameter about 2 cm. However, maximum diameter is up to 11cm and unexpected growth of IFP has been reported[1,3,4,7]. The size of our case is measuring 4.5 cm × 4 cm × 3 cm on histopathology and 7 cm length on CT that is slightly larger than usual case. The morphology features of gastric IFPs are commonly pedunculated, semi-pedunculated or sessile with covered normal mucosa. It can appear intramural mass with smooth, well-defined margins forming obtuse angles with the surrounding bowel wall[1,6,9]. The latter is consistent with our case. Clinical manifestations of gastric IFPs are mainly determined by their size and anatomical location. Patients may have symptoms such as abdominal pain, nausea, vomiting and frequent dyspepsia. If the lesions are located in the distal antrum or pylorus, they can cause gastric outlet obstruction. Massive GI bleeding with anemia can also result from IFPs with mucosal ulceration[6-8]. Our patient had history of dyspepsia, epigastric pain and mild anemia and these may be related to superficial ulcer of mucosa underlying intramural mass located in gastric antrum.

Although the exact etiology remains unknown, there are many postulate supposed to be related to IFPs including inflammation, trauma, previous surgery, infection or allergy. Some authors suggest that the

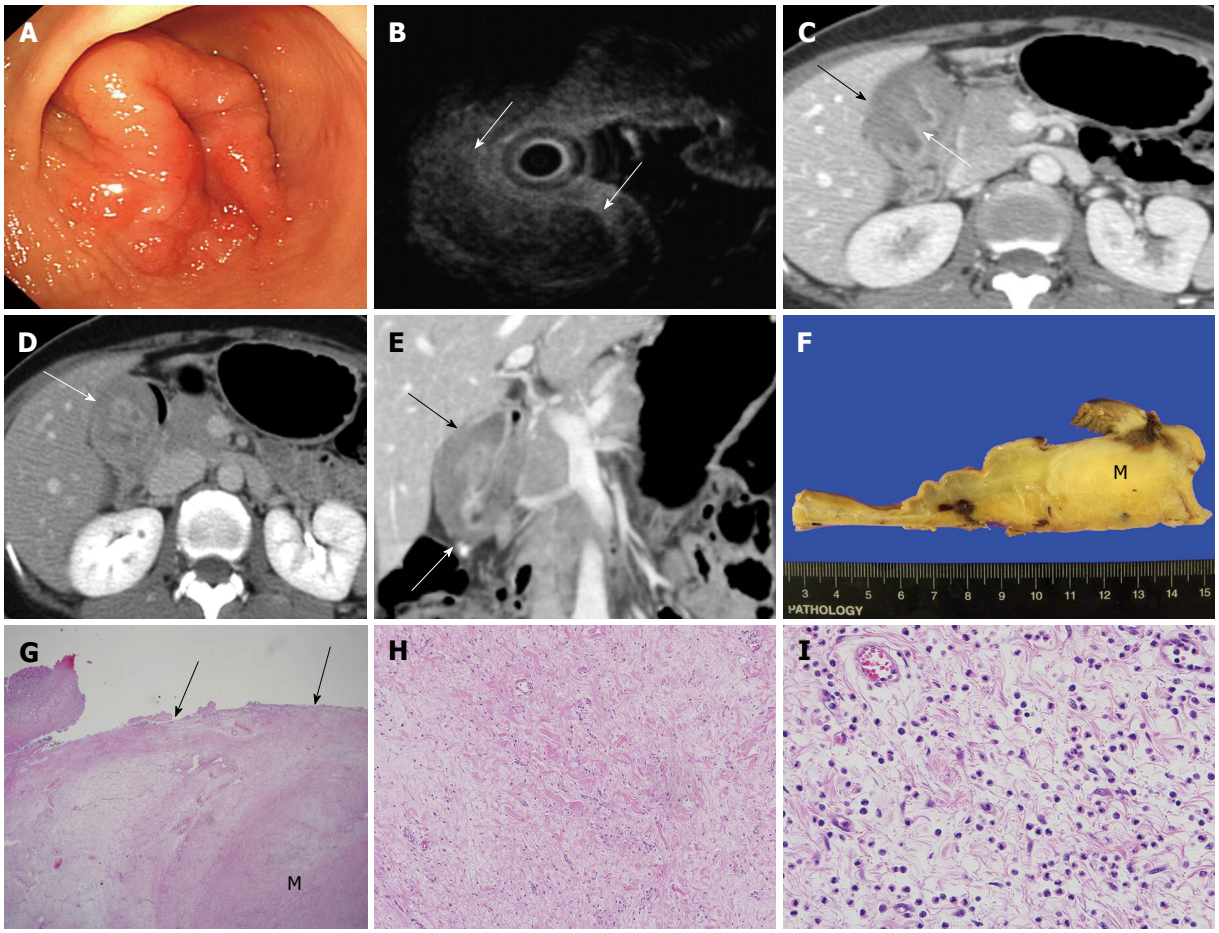

Figure 1 A 43-year-old female with an inflammatory fibroid polyp with massive fibrosis. A: Endoscopy showed an approximately 4 cm mass-like lesion with mucosal edema and superficial ulcer on the gastric antrum; B: On endoscopic ultrasound examination, an approximately 4 cm heterogeneous hypoechoic submucosal mass-like lesion (arrows) was seen; C: The portal phase of axial contrast-enhanced computed tomography (CT) scan showed a hypoattenuated marked wall thickening of the submucosal layer at the gastric antrum (black arrow) with preserved mucosal enhancement (white arrow); D: The 3-min delayed phase of axial contrast-enhanced CT scan demonstrated delayed enhancement (about 115 HU) of the submucosal layer at the gastric antrum (arrow); E: The coronal image of contrast-enhanced CT scan revealed that the lesion did not extend to the liver and demonstrated no perigastric fat infiltration (arrows); F: The surgical specimen demonstrated a submucosal tumor (M) measuring 4.5 cm × 4.0 cm × 3.0 cm and the overlying mucosa was intact; G: Microscopic examination (hematoxylin and eosin stain, magnification × 12.5) demonstrated a submucosal mass-like lesion (M). The borders of the submucosal mass-like lesion were poorly demarcated and difficult to discern from the adjacent submucosal connective tissue. The overlaying mucosa was intact (arrow); H: On microscopic examination (hematoxylin and eosin stain, magnification × 100), there were fibroblastic cells with well vascularized fibrotic stroma; I: Microscopic examination (hematoxylin and eosin stain, magnification × 400) showed infiltration of chronic inflammatory cells with many eosinophils present. No mitotic activity was identified.

IFP could be a result of inflammatory response of submucosa due to the localized damage to mucosa. Furthermore, inflammatory reactive process can be stimulated by bacterial, chemical, metabolic factors. Lately, several study have postulated that Helicobacter pylori infection is related with IFPs[1,3,7,9].

Endoscopy reveals only the presence of a submucosal lesion. Biopsy specimens using standard biopsy forceps may not offer useful histologic information because the lesion is in the submucosal layer. The EUS manifestations of IFP are an indistinct margin, hypoechoic subepithelial tumor, and located within the second and third layer (the deep mucosal and the submucosal layers) with an intact fourth layer (muscle layer)[6].

Typical CT findings of gastric IFP are smooth or lobulated contoured endoluminal mass. Most of the reviewed cases showed overlying mucosal hyperenhancement. An ulcer at the lesion surface and adjacent gastric wall thickening can be present. Lesion homogeneity and enhancement degree are varied[4]. The IFP enhancement patterns seem closely related to differences in the pathologic characteristics of the IFPs, such as the presence of inflammatory cells, particularly eosinophils, edematous and myxoid stroma, prominent blood vessels with perivascular fibroblastic proliferation, and hyalinization[4]. Some reports suggested that low attenuated areas in the IFP were histologically correlated with myxoid changes with edema[4,10]. In our case, the markedly thickened submucosal layer showed increase enhancement on the 3 min-delayed phase. There have been no previous reports on dynamic enhanced CT imaging finding of gastric IFP showing delayed enhancement. When compared with pathologic findings, the cause of delayed enhancement of thickened submucosal layer is

thought to be prominent fibrotic and well vascularized, hyalinized stroma.

The evolution of IFP is divided into four stages based on size and histology. First, nodular stage shows diameter range from 0.2-0.5 cm and major component is immature fibroblasts. Second, fibrovascular stage is most common, shows diameter range from 0.7-3.4 cm. This stage describes the characteristic onion-skin appearance of mature fibroblasts surrounding blood vessels or irregular, coarse arrangement. These typical findings are more frequently seen at younger stage and seen only peripheral areas of the senescent stage or even in mucosal layer in cases without superficial ulcer. Other components of second stage include eosinophil infiltration and numerous inflammatory cells. When the size of IFP became larger, the histopathological components become changes. Third, organized stage is sclerotic or edematous type and range from 2.5-12.0 cm. Major components of sclerotic stage are thick collagen bundle and prominent hyalinization. Edematous stage shows vascular proliferation, edema due to intracellular fluid. Although classic histopathologic findings of perivascular onion skinning and prominent eosinophils infiltration are well known, but IFPs evolve, they may manifest short fascicular growth pattern, a sparse eosinophils and remarkable hyalinization. Interestingly, as the size of the IFPs increased to find mixed histopathologic features more commonly, suggesting change of the original histologic characteristics[11,12]. Our case showed fibroblastic cells with well-vascularized fibrotic stroma and infiltrate of chronic inflammatory cells, including many eosinophils. This may be explained by mixed histologic pattern of fibrovascular and organized stages. Also superficial ulcer in mucosal layer, which may be contributed to the absence of characteristic onion-skin appearance of spindle cells.

Previous study, Advanced gastric cancer (AGC) showed delayed enhancement on CT (after 180 s for the start of infusion of contrast material) without regard to Borrmann's type. Correlation of the histopathologic findings with radiologic enhancement pattern, signet ring cell type showed good and delayed enhancement but mucinous type was poorly enhanced due to high mucin content[13]. Another previous study, when gastric wall thickening in AGC on CT, high-degree contrast enhancement was more usual in SRC than that with non-signet ring cell carcinoma. When histopathological findings are considered, malignant cells and immature fibrosis are causes of enhancement in SRC[14]. Since in our case, the lesion had marked wall thickening with delayed enhancement, malignant tumor could not be ruled out, particularly SRC. The operation was performed because the cancer was not excluded in the radiological findings. However, several CT features such as a preserved mucosal layer and lesions without perigastric fat infiltration or lymphadenopathy help the diagnosis IFP rather than malignant tumor.

The differential diagnosis of gastric IFPs include other submucosal lesions, such as leiomyoma, gastrointestinal stromal tumors (GIST), and heterotopic pancreas[4,9]. Leiomyomas typically present as homogenous masses with an endoluminal growth pattern and lower attenuation than back muscles; however, leiomyomas of the stomach are rare and if they develop, they are almost located in the gastric cardia[4,5,15]. GISTs are most commonly located in the gastric body. GISTs arise from the deep muscularis propria and have an exophytic, intramural, or mixed growth pattern. GISTs manifest with various enhancement patterns, and the degree of enhancement may be higher in hypervascular characteristic GISTs than in IFPs. Invasion into adjacent organs such as the pancreas and colon is only seen in enlarged, exophytic GISTs. Areas of hemorrhage, necrosis or cystic degeneration are frequently observed in focal low attenuated lesions of GISTs[4,5,15]. A heterotopic pancreas is typically located in the prepyloric antrum and duodenum. It presents as an ovoid or flat mass with an endoluminal growth pattern and higher attenuation than the back muscle with a prominent enhancement of the overlying mucosa[4,15].

In conclusion, dynamic CT in addition to endoscopy may be useful in the diagnosis of gastric IFP. Gastric IFP should be considered in the differential diagnosis if the submucosal layer of the gastric wall is markedly thickened with delayed enhancement and the mucosal layer is preserved without perigastric fat infiltration.

COMMENTS

Case characteristics

A 43-year-old female had a 1-mo history of dyspepsia and epigastric pain.

Clinical diagnosis

Physical examination was unremarkable. Abdomen was soft, flat with normoactive bowel sound. On endoscopy, gastric submucosal tumor was suspected.

Differential diagnosis

Gastric submucosal tumor was suspected on endoscopy but it was difficult to excluded malignant tumor on computed tomography (CT).

Laboratory diagnosis

Abnormal laboratory finding was only hemoglobin level, specifically 9.0 g/dL.

Imaging diagnosis

Dynamic enhanced CT imaging showed markedly gastric wall thickening (about 2.9 cm in wall thickness, 7 cm in length), predominantly submucosal layer with delayed enhancement.

Pathological diagnosis

The pathology showed a submucosal mass-like lesion with fibroblastic cells with well vasculized fibrotic stroma, infiltration of chronic inflammatory cells including many eosinophils.

Treatment

Patient underwent antrectomy with gastroduodenostomy.

Related reports

Several studies about gastric inflammatory polyp (IFP) have reported its CT findings of enhancement images on portal phase. To the best of our knowledge, no previous report has been described about the dynamic enhanced CT findings of a gastric IFP with delayed enhancement which mimicking malignant tumor such as signet ring carcinoma.

Term explanation

In this case report, the uncommon difficult terms have not been used.

Experiences and lessons

Gastric IFP should be considered in the differential diagnosis if the submucosal layer of the gastric wall is markedly thickened with delayed enhancement and the mucosal layer is preserved without perigastric fat infiltration.

Peer-review

It is a very good case report, with many images that allow an easy reading of the manuscript. It is well written, and the explanation and conclusion is clear.

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