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^[1]Passed away on October 20, 2007

^[2]Passed away on June 11, 2007



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Cytomegalovirus infection after liver transplantation: Current concepts and challenges

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Abstract

Cytomegalovirus (CMV) is a common viral pathogen that influences the outcome of liver transplantation. In addition to the direct effects of CMV syndrome and tissue-invasive diseases, CMV is associated with an increased predisposition to acute and chronic allograft rejection, accelerated hepatitis C recurrence, and other opportunistic infections, as well as reduced overall patient and allograft survival. Risk factors for CMV disease are often interrelated, and include CMV D+/R-serostatus, acute rejection, female gender, age, use of high-dose mycophenolate mofetil and prednisone, and the overall state of immunity. In addition to the role of CMV-specific CD4+ and CD8+ T lymphocytes, there are data to suggest that functionality of the innate immune system contributes to CMV disease pathogenesis. In one study, liver transplant recipients with a specific polymorphism in innate immune molecules known as Toll-like receptors were more likely to develop higher levels of CMV replication and clinical disease. Because of the direct and indirect adverse effects of CMV disease, its prevention, whether through antiviral prophylaxis or preemptive therapy, is an essential component in improving the outcome of liver transplantation. In the majority of transplant centers, antiviral prophylaxis is the preferred strategy over preemptive therapy for the prevention of CMV disease in CMV-seronegative recipients of liver allografts from CMV-seropositive donors (D+/R-). However, the major drawback of antiviral prophylaxis is the occurrence of delayed-onset primary CMV disease. In several prospective and retrospective studies, the incidence of delayed-onset primary CMV disease ranged from 16% to 47% of CMV D+/R- liver transplant recipients.

Current data suggests that delayed-onset CMV disease is associated with increased mortality after liver transplantation. Therefore, optimized strategies for prevention and novel drugs with unique modes of action are needed. Currently, a randomized controlled clinical trial is being performed comparing the efficacy and safety of maribavir, a novel benzimidazole riboside, and oral ganciclovir as prophylaxis against primary CMV disease in liver transplant recipients. The treatment of CMV disease consists mainly of intravenous (IV) ganciclovir, and if feasible, a reduction in the degree of immunosuppression. A recent controlled clinical trial demonstrated that valganciclovir is as effective and safe as IV ganciclovir for the treatment of CMV disease in solid organ (including liver) transplant recipients. In this article, the author reviews the current state and the future perspectives of prevention and treatment of CMV disease after liver transplantation.

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Key words: Cytomegalovirus; Outcome; Hepatitis; Transplantation; Valganciclovir; Maribavir; Prophylaxis; Treatment

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INTRODUCTION

Throughout the four decades that have elapsed since the first successful liver transplantation in 1967, cytomegalovirus (CMV) has remained the single most common viral pathogen influencing the outcome of this procedure. Infection with CMV is not only a very common complication after liver transplantation but it also contributes significantly to the morbidity and mortality, both by direct and indirect mechanisms^[1,2].

CMV is a ubiquitous herpes virus that infects 60%-100% of humans^[1,2], with primary CMV infection occurring most commonly during the first 2 decades of life. If immunocompetent, the infected individuals are mostly asymptomatic or may present with a benign febrile infectious mononucleosis-like illness. However, in individuals with compromised immunity, such as liver transplant recipients, clinical disease with high morbidity may develop and, in some cases, may lead to death^[1,2].

Facilitated by its ability to evade the immune system, infection with CMV results in a state of latency in several host cells^[1,2]. Consequently, these cellular sites of viral latency become reservoirs of reactivation during periods of stress and cytokine release, and serve as vehicles for transmission to susceptible hosts. Both these scenarios are operational in liver transplant recipients, wherein the pharmacologic-induced impairment of immune response to “endogenously reactivated” or “allograft-transmitted” CMV leads to febrile and tissue-invasive diseases^[1,2]. Because of the lack of a pre-existing CMV-specific immunity, CMV-seronegative recipients of liver allografts from CMV-seropositive donors (CMV D+/R-) are at the highest risk of CMV disease and its complications^[3-5].

This article reviews the current concepts and challenges in the management of CMV after liver transplantation. Historical aspects of the disease are discussed to emphasize the remarkable improvements that have been achieved over the past several years. Conversely, ongoing issues of delayed-onset and drug-resistant CMV disease are discussed in detail, to highlight future perspectives in terms of CMV disease prevention and treatment.

CLINICAL IMPACT OF CMV IN LIVER TRANSPLANTATION

Direct CMV effects

The clinical illness caused by CMV commonly manifests as fever, bone marrow suppression, and organ-invasive diseases (Table 1)^[1]. These direct clinical effects are traditionally classified as CMV syndrome (fever with myelosuppression) and tissue-invasive CMV disease, which most often involves the gastrointestinal tract (in the form of CMV gastritis, esophagitis, enteritis, and colitis), although virtually any organ system may be involved^[6]. Infection of the liver (i.e., CMV hepatitis) is especially common in liver transplant recipients (compared to other solid organ transplant recipients), and this may manifest with symptoms indistinguishable from acute allograft rejection^[7]. The availability of sensitive tests for the rapid detection of CMV in the blood may obviate the need for liver biopsy to differentiate the CMV infection from rejection. However, in many cases, a liver biopsy is needed to differentiate or to demonstrate the co-existence of CMV disease and allograft rejection.

In the absence of effective antiviral prophylaxis, the

Table 1 Direct and indirect clinical effects of CMV after solid organ transplantation

Direct effects	Indirect effects
CMV syndrome	Acute allograft rejection
Fever	
Myelosuppression	
Malaise	
Tissue-invasive CMV disease ¹	Chronic allograft rejection
Gastrointestinal disease (colitis, esophagitis, gastritis, enteritis)	Vanishing bile duct syndrome
Hepatitis	Chronic ductopenic rejection
Pneumonitis	Hepatitis C virus recurrence
CNS disease	Allograft hepatitis, fibrosis and allograft failure
Retinitis	Opportunistic and other infections
	Fungal superinfection
	Nocardiosis
	Bacterial superinfection
	Epstein-Barr virus and PTL
	HHV-6 and HHV-7 infections
	Vascular thrombosis
	Mortality
Mortality	

¹Any organ system may be affected by CMV.

Table 2 Estimated incidence of CMV disease during the first 12 mo after liver transplantation

	Use of anti-CMV prophylaxis	
	Yes ¹	No
CMV D+/R-	12%-30%	44%-65%
CMV D+/R+	2.7%	18.2%
CMV D-/R+	3.9%	7.9%
CMV D-/R-	0	0
All patients	4.8%	18%-29%

D: Donor; R: Recipient. ¹Most cases occur as delayed-onset CMV disease. CMV disease occurs rarely during prophylaxis with oral valganciclovir. Data adapted from^[4,5,7].

direct effects of CMV occur most commonly during the first 3 mo after liver transplantation^[6]. Overall, it is estimated that 18%-29% of all liver transplant recipients will develop CMV disease (Table 2)^[4,5,8-10]. However, this incidence varies widely depending upon donor and recipient CMV serologic status; it may be as high as 44%-65% in CMV D+/R-, or as low as 8%-19% in CMV-seropositive liver transplant recipients (CMV R+)^[4,8,10]. The incidence is markedly reduced in liver transplant recipients who receive prophylaxis with 3 mo of valganciclovir and oral ganciclovir. Recent studies have reported CMV disease rates of 12%-30% in high-risk CMV D+/R-, and < 10% in CMV R+ liver transplant recipients who received 3 mo of antiviral prophylaxis^[3,4,8,10-12]. In individuals who received antiviral prophylaxis, CMV disease occurred 3 mo to 6 mo after completing antiviral prophylaxis; hence, the term “delayed-onset (also termed late-onset) CMV disease” (Figure 1)^[3].

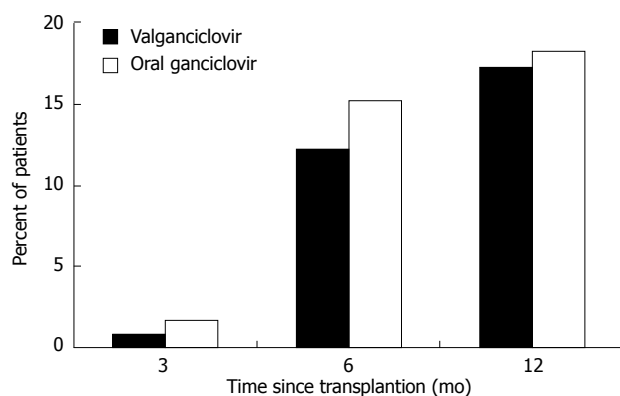


Figure 1 Time to the onset of CMV disease in solid organ transplant recipients who received 3 mo of oral ganciclovir or valganciclovir prophylaxis. Data obtained from the study by Paya *et al*^[6].

Indirect CMV effects

The clinical impact of CMV extends beyond the direct effect of the virus. Numerous indirect outcomes, believed to be mediated by the ability of the virus to modulate the immune system have been reported (Table 1)^[1,2]. CMV is known to be a potent up-regulator of alloantigens, thereby increasing the risk of acute rejection and chronic allograft dysfunction^[13]. CMV is associated with vanishing bile duct syndrome and ductopenic rejection, leading to chronic cholestasis and eventually allograft failure^[14-16]. Several studies have reported a higher incidence of vascular and hepatic artery thrombosis in liver transplant recipients with CMV disease, an effect that is believed to result from CMV infection of the vascular endothelial cells^[17,18]. The immunomodulatory effects of CMV have also been blamed for the higher predisposition to other opportunistic infections including fungi, other viruses, and bacteria such as *Nocardia* sp.^[19,20]. CMV-infected transplant recipients are more likely to develop Epstein-Barr virus-associated post transplant lymphoproliferative disorders (PTLDs), or develop co-infections with other viruses such as human herpes virus (HHV)-6 and HHV-7^[19,21]. A well-described interaction between members of the beta-herpes group of viruses has been described, exemplified by the observation that reactivation of HHV-6 and HHV-7 is associated with an increased predisposition to CMV disease after liver transplantation^[22-24]. In a similar manner, there is a significant association between CMV and hepatitis C virus^[25-30], manifested by an accelerated course of HCV recurrence in patients who develop CMV infection after liver transplantation. In our analysis of 92 HCV-infected liver transplant recipients, there was a four-fold higher risk of allograft failure and mortality in patients with CMV infection and disease^[28,30]. Three years after liver transplantation, 48% patients who developed CMV disease had allograft loss or had died, compared to 35% patients with asymptomatic CMV infection, and 17% in those who did not develop CMV infection^[28,30].

Impact on mortality

CMV infection is an independent predictor of mortality

Table 3 Selected traditional and novel factors associated with the increased risk of CMV disease after liver transplantation

Traditional factors	Recently identified factors
CMV D+/R- > CMV R+	Toll-like receptor gene polymorphism
Allograft rejection	Mannose binding lectin deficiency
High viral replication	Chemokine and cytokine defects (IL-10, MCP-1, CCR5)
Mycophenolate mofetil	Deficiency in CMV-specific CD4+ T cells
Muromonab-CD3	Deficiency in CMV-specific CD8+ T cells
Anti-thymocyte globulin	Expression of immune evasion genes
Alemtuzumab	Programmed cell death 1 expression
HHV-6	
HHV-7	
Renal insufficiency	
Others ¹	

¹Others factors include re-transplantation, volume of blood transfusion, sepsis and factors associated with high tumor necrosis factor- α secretion^[1,4,11,13,21,39-42,77,89-93].

after solid organ transplantation, by mechanisms which may be direct, indirect or immunomodulatory^[19,31,32]. CMV was a major cause of mortality after liver transplantation prior to the availability of intravenous (IV) and oral ganciclovir. Several recent meta-analyses have demonstrated that the use of anti-CMV drugs, either through antiviral prophylaxis or preemptive therapy, have led to significant reduction in the overall mortality after solid organ transplantation^[19,33-35]. However, despite much improvement in outcome, there is emerging data to suggest that even in the contemporary era, with widespread use of antiviral prophylaxis, development of delayed onset CMV disease remains a common problem, and importantly, is associated with significantly increased risk of mortality after liver transplantation^[32]. An analysis of 437 liver transplant recipients demonstrated that CMV disease occurred in 37 patients (8.5%), and its occurrence was independently associated with a 5-fold increased risk of all-cause mortality, and an 11-fold increased risk of infection-related mortality after liver transplantation^[32]. The other significant and independent predictors of mortality in this study included the need for pre-liver transplant hemodialysis, a higher model for end-stage liver disease (MELD) score, and post-transplant occurrence of bacterial and fungal infections^[32].

RISK FACTORS FOR CMV DISEASE AFTER LIVER TRANSPLANTATION

Lack of pre-existing CMV-specific immunity

The most common predisposing factor for the occurrence of CMV disease after liver transplantation is the lack of an effective CMV-specific immunity^[4,19]. As a result, CMV D+/R- are at the highest risk of CMV disease^[4,19], while CMV R+ patients have a modest risk and CMV D-/R- have the lowest risk of CMV disease after liver transplantation (Table 3).

Drug-induced immunodeficiency

The use of highly potent pharmacologic immuno-

suppression severely impairs the ability of liver transplant recipients to mount an effective immune response against reactivating CMV, thereby predisposing to increased risk of CMV disease^[4,19]. The severity of immune dysfunction is particularly intense with lymphocyte-depleting drugs such as muromonab-CD3 (OKT3) and anti-thymocyte globulin^[36,37]. More recently, the use of alemtuzumab has been found to be associated with higher risk of CMV disease^[38]. Drugs used for maintenance immunosuppression have also been associated with CMV disease, particularly high doses of mycophenolate mofetil^[30,39]. It is very likely that immunosuppressive drugs not only predispose to CMV disease, but the net state of combined pharmacologic immunosuppression increases the risk of CMV disease after liver transplantation^[1,2,19].

Defects in innate and CMV-specific cell-mediated immunity

The appreciation of the role of the immune system in controlling CMV led to recent observations that inherent defects in immunity, such as mutations in the innate immunity-associated genes, increased the risk of CMV disease after liver transplantation (Table 3). In a study of 92 liver transplant recipients, a genetic polymorphism in the Toll-like receptor (TLR)-2 gene, which resulted from the substitution of arginine to glutamine at position 753 in the protein-receptor, was significantly associated with a higher degree of CMV replication and a higher incidence of CMV disease^[40]. TLR2 is a pattern recognition receptor expressed in innate immune cells, and its function is to sense the glycoprotein B of CMV, thereby signaling the immune cells to produce antiviral peptides and other cytokines^[40]. Our *in vitro* data suggests that this specific genetic polymorphism causes an impairment of cellular recognition of CMV by TLR2-expressing cells^[40].

Likewise, the CMV-specific T cell compartment is necessary for adequate control of CMV after liver transplantation^[41], although a recent study indicated that CMV-specific T cells may not necessarily predict the risk after liver transplantation^[41]. There are ongoing studies in this field that may further clarify the prognostic role of CMV-specific T cell assays in stratifying CMV disease risk after liver transplantation.

Other immune measures, such as programmed death-1 expression^[42] and immune evasion genes^[43] have also been assessed as prognostic indicators of CMV disease after liver transplantation. In one study, programmed death-1 receptor up-regulation was significantly associated with incipient and overt CMV disease and with CMV viremia^[42].

Allograft rejection

Allograft rejection *per se* is one of the most potent inducers of CMV reactivation, and thus considered a significant risk factor for CMV disease after liver transplantation^[12]. Cytokines released during acute rejection, particularly tumor necrosis factor- α ^[44], are

potent transactivators of latent CMV^[45], as demonstrated in animal models^[46]. Moreover, therapy for allograft rejection with the intensification of immunosuppressive regimen further increases the risk of CMV disease both by enhancing its reactivation and by impairing the ability to generate effective cell-mediated immunity against replicating CMV^[47]. Conversely, CMV induces allostimulation and increases the risk of allograft rejection, thereby creating a bidirectional relationship between CMV and allograft rejection^[13].

Virus-to-virus interactions

Virus-virus interaction may influence the risk of CMV disease after liver transplantation^[21,22,26-30]. Reactivation of HHV-6 has been shown to predispose to an increased incidence of CMV disease after liver transplantation^[21,22,24]. In a study on 247 patients, HHV-6 seroconversion was an independent marker of CMV disease after liver transplantation. Likewise, HCV-infected liver transplant recipients also have a higher incidence of CMV disease^[48], although our data in the era of valganciclovir prophylaxis has refuted this observation^[25].

Degree of viral replication

The risk of CMV disease after liver transplantation is associated, in direct proportion, with the degree of CMV replication, which is partly a function of over-immunosuppression^[8,23,49,50]. In one study, a viral load of 1-2860 CMV copies/10⁶ peripheral blood mononuclear cells (PBMC) increased CMV disease risk by nine-fold, while viral loads > 2860/10⁶ PBMC increased the risk by 50-fold^[8].

Other factors

Other factors associated with CMV disease after transplantation include cold ischemia time, bacterial and fungal infections and sepsis, the amount of blood loss, fulminant hepatic failure as the indication for transplantation, age, female gender, Hispanic race, and renal insufficiency^[2,3,19,51]. It is likely that other factors that have not yet been identified may also influence the risk of CMV disease after liver transplantation.

PREVENTION OF CMV DISEASE AFTER LIVER TRANSPLANTATION

Because of the adverse effects of CMV on transplant outcome, its prevention is key to management of such patients^[19]. Over the years, the pharmacologic agents used for CMV prevention have evolved, from the use of acyclovir^[52] and immunoglobulins^[53] to IV and oral ganciclovir^[4] and more recently, valganciclovir^[5]. There are two major strategies for CMV disease prevention after liver transplantation: (1) preemptive therapy (wherein CMV reactivation is aggressively monitored by sensitive assays and upon detection, antiviral therapy is administered preemptively to prevent its progression to clinical disease); and (2) antiviral prophylaxis (wherein

antiviral drugs such as ganciclovir and valganciclovir are administered to patients at risk of CMV disease after liver transplantation^[19]. Both strategies are highly effective in preventing CMV disease after liver transplantation^[4,5,54-57]. However, antiviral prophylaxis is generally regarded as a more efficient approach and is used by the majority of transplant centers in preventing primary CMV disease in high-risk CMV D+/R- liver transplant recipients^[4,8,54]. Indeed, the current American Society of Transplantation recommendation is to use antiviral prophylaxis in all CMV D+/R- liver (and other solid organ transplant) recipients^[58]. Moreover, primary antiviral prophylaxis has the added benefit of reduction in bacterial and fungal opportunistic infections and mortality^[33,34].

Preemptive therapy

The basic principle of preemptive therapy is to detect the presence of CMV reactivation prior to the onset of clinical symptoms, so that antiviral drugs are administered early in order to halt the progression of asymptomatic infection to full-blown clinical disease^[50,54,55,57,59]. The success of this approach relies on patient compliance with CMV surveillance^[60], availability of highly sensitive CMV assay that predicts the risk of disease^[61], and early administration of antiviral drugs such as IV ganciclovir and oral valganciclovir^[9,55,59]. With the advance in molecular diagnostic microbiology, including the availability of polymerase chain reaction (PCR), it is now possible to employ successfully preemptive therapy in liver transplant recipients (reviewed in^[61]). Several studies have reported the success of IV or oral ganciclovir and valganciclovir in the preemptive treatment of CMV reactivation in liver transplant recipients, including high-risk CMV D+/R- patients^[56,59]. However, some studies have indicated that preemptive therapy may not be completely effective in CMV D+/R- liver transplant recipients since the replication kinetics of CMV in immune-deficient individuals is so rapid^[49] that it may result in clinical illness prior to CMV detection with once a week PCR assay^[8,54]. Indeed, in our clinical experience, nearly 25% of CMV D+/R- liver transplant recipients who developed CMV disease were not identified early by a protocol-based weekly CMV PCR assay^[8,54]. Accordingly, the current guideline from the AST does not recommend preemptive approach in CMV D+/R- liver transplant recipients^[58]. However, this approach is recommended, and is highly effective, in CMV-seropositive liver transplant recipients. Reassuringly, clinical trials have demonstrated the efficacy of preemptive therapy in CMV disease prevention^[54-56,59]. Three meta-analyses that collectively analyzed data from prospective clinical trials confirmed the efficacy and benefits of preemptive therapy in the prevention of CMV disease^[34,35,62]. When conducted properly, preemptive therapy, with the use of oral ganciclovir, IV ganciclovir, or valganciclovir resulted in reduction of CMV disease by about 70%^[34,35,62]. Moreover, preemptive therapy is not associated with late onset CMV disease (unlike with antiviral prophylaxis,

as discussed below)^[55,59]. Currently, valganciclovir is the most commonly used drug for preemptive therapy, and in one study, was demonstrated to be as effective in terms of clinical and virologic response, when compared with IV ganciclovir^[55,59]. In addition, preemptive therapy may be beneficial in reducing the indirect effects of CMV. In one study, the incidence of major opportunistic infections, bacteremia, bacterial infection, HCV recurrence, and rejection were not significantly different between liver transplant patients who received preemptive therapy and those who did not have CMV reactivation^[63].

Antiviral prophylaxis

Several clinical trials have demonstrated that antiviral prophylaxis is highly effective in preventing the direct, and possibly the indirect effects of CMV after liver transplantation^[4,5]. Recent meta-analyses have highlighted the clinical benefits^[34,35,62]. Compared to placebo or no treatment, patients who received antiviral prophylaxis had lower incidence of CMV disease (58%-80% reduction) and CMV infection (about 40% reduction)^[62]. In one meta-analysis, a 25% reduction in the incidence of acute allograft rejection was also observed^[34]. In two studies, a reduction in all-cause mortality was also observed^[34,62], mainly due to a decline in CMV-related death^[62]. A reduction in the incidence of other herpes viruses, bacterial, and protozoal infections was also observed^[62]. Indeed, a survey of several transplant centers showed a general preference for antiviral prophylaxis over preemptive therapy in the prevention of CMV disease in CMV D+/R- and R+ liver transplant recipients.

Acyclovir prophylaxis

The use of acyclovir as anti-CMV prophylaxis after liver transplantation has been supplanted by ganciclovir (and valganciclovir) because of the superior efficacy of the latter drugs in CMV disease prevention. In a study on 143 liver transplant recipients, CMV infection developed in 61% patients who received 3 mo of high-dose oral acyclovir compared to 24% patients who received 14 d of IV ganciclovir followed by 3 mo of acyclovir ($P < 0.001$)^[64]. In a second study, 57% and 23% patients in the acyclovir group compared to 37% and 11% patients in the ganciclovir-acyclovir group developed CMV infection and disease, respectively^[52]. In a third randomized controlled trial on 250 liver transplant recipients, CMV infection and disease occurred in 38% and 10% of patients in the acyclovir group, respectively, compared to 5% and 1% in the ganciclovir group, respectively^[65].

Ganciclovir prophylaxis

The current data indicates that ganciclovir-based regimen is more effective (compared to acyclovir and immunoglobulins) in reducing the incidence of CMV after liver transplantation. In one study, the administration of IV ganciclovir for 90-100 d reduced the incidence of CMV disease in CMV D+/R- liver

transplant recipients to 5.4% (compared to 40% in patients who received < 7 wk of prophylaxis)^[65]. The major drawback to IV ganciclovir was the need for long-term IV access and the risk of thrombosis, phlebitis, and line-associated infections^[37,66]. Subsequently, oral ganciclovir became available, and in a landmark randomized trial that compared the drug with placebo, oral ganciclovir for 98 d reduced significantly the 6-mo incidence of CMV infection (51.5% *vs* 24.5%; $P < 0.001$), and CMV disease (19% *vs* 5%; $P < 0.001$) in liver transplant recipients^[4], including CMV D+/R- patients (44% *vs* 15%, $P = 0.02$) and patients who received antilymphocyte antibodies (33% *vs* 5%; $P = 0.002$)^[4]. Among CMV R+ liver transplant recipients, oral ganciclovir for 12 wk reduced the incidence of CMV disease to 1% (compared to 7% in patients who received acyclovir)^[67]. These studies were in support of the United States FDA approval of oral ganciclovir prophylaxis for the prevention of CMV disease in liver transplant recipients. Oral ganciclovir, however, is poorly absorbed, and its oral administration results in low systemic ganciclovir levels^[68]. This factor has been implicated in the emergence of ganciclovir-resistant CMV in certain clinical settings^[69,70], such as high-risk CMV D+/R- patients, and those receiving potent immunosuppressive regimens.

Valganciclovir prophylaxis

Valganciclovir, a valine ester of ganciclovir, which results in enhanced absorption, resulting in systemic drug levels that are comparable to IV ganciclovir^[68,71]. Pharmacokinetic studies indicate that a 900 mg dose of valganciclovir achieves a similar daily area under the concentration time curve (AUC_{24}) as an IV dose of 5 mg/kg of ganciclovir^[68]. The role of valganciclovir in the prevention of CMV disease after liver transplantation was evaluated in a multicenter randomized non-inferiority clinical trial that compared it with oral ganciclovir in a cohort of 364 CMV D+/R- solid organ transplant (including liver) recipients (Figure 1)^[5]. Overall, the 6-mo incidence of CMV disease was 12% and 15% in the valganciclovir and oral ganciclovir groups, respectively^[5]. Follow-up at one year, demonstrated that the incidence of protocol-defined CMV disease in all patients was 17.2% and 18.4% with valganciclovir and oral ganciclovir, respectively^[5] (Notably, the incidence of investigator-determined CMV disease cases was about 28% and 30%, respectively).

However, in 177 liver transplant recipients who participated in the clinical trial, the incidence of CMV disease was 19% in the valganciclovir group as opposed to only 12% in the ganciclovir group^[5]. There was also a higher incidence of tissue-invasive CMV disease in the valganciclovir group. While the clinical trial was not designed to determine differences between the transplanted organs, these results raised skepticism about the efficacy of valganciclovir prophylaxis after liver transplantation. As a result of these findings, valganciclovir prophylaxis did not gain approval from the US-FDA for prophylaxis against CMV disease after

liver transplantation (valganciclovir received approval for prevention of CMV disease in heart, kidney, and pancreas recipients). Although not FDA-approved for prophylaxis in liver transplant recipients, valganciclovir is the most widely used drug for the prevention of CMV disease after liver transplantation^[72].

The efficacy of valganciclovir (and oral ganciclovir) prophylaxis is undermined by the emergence of late-onset CMV disease (Figure 1). In a retrospective study on 203 liver transplant recipients who received valganciclovir 900 mg daily for 3 to 6 mo, the overall incidence of CMV disease was 14%^[73]. The incidence varied among the different CMV serogroups (16% in D+/R+ group; 7% in D-/R+ group; and 26% in D+/R-group)^[73]. These findings illustrate that the burden of delayed-onset CMV disease remains high particularly in the CMV D+/R- group^[5]. In our analysis of 67 CMV D+/R- liver transplant recipients who received 3 mo of oral ganciclovir and valganciclovir prophylaxis, the two year incidence of CMV disease was 29%^[3]. The incidence of delayed-onset CMV disease was not significantly different between patients who received oral ganciclovir or valganciclovir (22% *vs* 28%; $P = 0.63$)^[3].

Maribavir prophylaxis (investigational)

The search for anti-CMV strategies continues to evolve with the recent entry of maribavir into clinical trials. Maribavir, a novel benzimidazole riboside compound that inhibits viral DNA assembly and egress of viral capsids^[74], is now undergoing clinical trials for the prevention of primary CMV disease after liver transplantation^[75,76]. Because it has a unique mechanism of action that is distinct from ganciclovir, foscarnet, and cidofovir (all of which act to inhibit CMV DNA polymerase), maribavir is expected to expand the therapeutic armamentarium against CMV^[75]. So far, it does not show cross-resistance with the currently available drugs. Therefore, it has a good potential as an alternative drug for the treatment of ganciclovir-resistant CMV. In addition, maribavir provides a more favorable toxicity profile compared to foscarnet and cidofovir, both of which are highly nephrotoxic. In preliminary studies conducted in allogeneic bone marrow transplant recipients, maribavir was found to be safe and did not have myelosuppressive effects. In terms of efficacy, when compared with placebo, maribavir showed significant reduction in CMV viremia^[76]. The ongoing comparative multicenter trial of maribavir and oral ganciclovir in liver transplant recipients will likely complete enrollment in 2009. In this multi-center international randomized trial, the incidence of CMV disease will be compared between patients randomized to oral maribavir, and the currently approved standard oral ganciclovir.

The challenge of delayed- and late- onset CMV disease

With the success of a 3-mo anti-CMV prophylaxis program (in terms of the almost complete elimination of CMV disease in individuals who are actively taking antiviral drugs), the challenge of delayed- and late-onset CMV disease has emerged. Indeed, in many

high-risk CMV D+/R- individuals, the use of antiviral prophylaxis has only delayed the onset of CMV disease to 3-6 mo after liver transplantation^[3-5,12]. In one of these retrospective studies, CMV disease occurred in 14 of 54 (26%) CMV D+/R- liver transplant recipients who received valganciclovir for at least 3 mo^[73]. Our clinical data suggests that, while no breakthrough CMV disease occurred during the 3 mo of oral ganciclovir or valganciclovir prophylaxis, 29% of CMV D+/R- liver transplant recipients developed delayed-onset primary CMV disease^[3]. Thus, one out of every four CMV D+/R- liver transplant recipients will develop CMV disease after cessation of antiviral prophylaxis^[3]. Delayed-onset CMV disease commonly presents as CMV syndrome, with fever and bone marrow suppression^[3]. In less than half of the patients, CMV manifested as tissue-invasive disease, and frequently affected the gastrointestinal tract^[3]. Factors such as age^[3], female gender^[3,77], renal dysfunction^[77], and allograft rejection^[12] predisposed to the development of delayed-onset primary CMV disease^[3,12,77,78]. Delayed-onset CMV disease appears to be clinically less severe, although it is associated with significant mortality after liver transplantation^[32]. Therefore, a better method for CMV prevention is needed among CMV D+/R- liver transplant recipients.

Currently, there is an ongoing effort (in kidney transplant recipients only) to assess the efficacy and safety of 3 mo *vs* 6 mo of valganciclovir prophylaxis. Foreshadowing what may be expected from this trial, a recent single center study on 68 CMV D+/R- kidney transplant recipients demonstrated a significantly lower incidence of CMV disease in patients who received 24 wk compared to 12 wk of oral ganciclovir prophylaxis (7% *vs* 31%, respectively)^[79]. If this practice is proven safe and effective, it may eventually be adopted in the liver transplant field. There are concerns regarding ganciclovir resistance, drug toxicity, and cost with such a prolonged prophylactic approach. In addition, the long-term drug toxicity of ganciclovir-based regimen is not known. In animal studies, ganciclovir has been shown to be mutagenic, teratogenic, carcinogenic, and has caused aspermatogenesis, although the clinical relevance of these findings in humans is unclear^[68].

Another strategy that is gaining interest is an aggressive effort to minimize immunosuppression, including the use of prednisone-free regimens. In one Kidney and Pancreas Transplant Program, the incidence of CMV disease was markedly reduced in patients receiving a steroid-free immunosuppressive regimen^[80]. Many liver transplant programs (including ours) have adapted this approach, and have minimized immunosuppression gradually so that patients are maintained on tacrolimus monotherapy beyond the 4th mo after liver transplantation. In a retrospective analysis, we observed a higher incidence of CMV disease among transplant recipients who were still receiving mycophenolate mofetil and prednisone at the time they discontinue antiviral prophylaxis. The major consequence of this approach, however, is the risk of allograft rejection when the level of immunosuppression

is reduced to levels lower than necessary for the prevention of allo-stimulation^[13].

TREATMENT OF CMV DISEASE AFTER LIVER TRANSPLANTATION

The current recommendation for antiviral treatment of CMV disease after liver transplantation is IV ganciclovir^[58,66,81]. Equally important is the reduction in the degree of pharmacologic immunosuppression^[19]. Oral ganciclovir should not be used for the treatment of active CMV disease because of its poor bioavailability^[19]. Valganciclovir, a prodrug of ganciclovir that provides high systemic ganciclovir concentrations^[71], has now made it possible for oral treatment of CMV disease^[68,81]. Indeed, in AIDS patients, valganciclovir is approved as induction and maintenance treatment of CMV retinitis^[82]. There is good clinical data to support the use of valganciclovir for the treatment of CMV after solid organ transplantation^[81]. Viral kinetic studies showed comparable viral decay between IV ganciclovir and valganciclovir^[50]. In a recent study, 321 solid organ (including liver) transplant recipients with non-severe CMV disease were randomized to valganciclovir or IV ganciclovir for a fixed 21-d course, followed by valganciclovir maintenance treatment for 4 wk; the proportion of patients with viral eradication at 21 and 49 d were comparable in the IV ganciclovir and valganciclovir groups (Figure 2)^[81]. The overall time to viral eradication was 21 d with valganciclovir and 19 d with IV ganciclovir^[81]. The calculated viral decay was 11.5 d with valganciclovir and 10.4 d with IV ganciclovir^[81]. Likewise, clinical resolution was not different between the two groups. It was noted that patients enrolled in this trial were mostly CMV-seropositive, the majority were kidney recipients, and patients with severe CMV disease were excluded. Despite these limitations, this pivotal trial now supports the use of valganciclovir for oral treatment of CMV disease, at least in selected transplant patients^[81]. In many instances, valganciclovir is used as a step-down treatment when the clinical symptoms have resolved after an initial induction treatment with IV ganciclovir.

The duration of treatment of CMV disease should be individualized^[58,83]. The persistence of the virus at the end of therapy (by polymerase chain reaction [PCR] or pp65 antigenemia) is associated with a higher risk of clinical relapse^[84]. It is now generally accepted that multiple (at least two) weekly negative CMV PCR results should be obtained before antiviral therapy is discontinued. Although this may be true for non-tissue invasive CMV syndromes, the utility of such an approach may not necessarily apply to tissue-invasive disease, which may manifest as "compartmentalized disease"^[19].

The challenge of treating compartmentalized CMV disease

Compartmentalized CMV disease refers to clinical

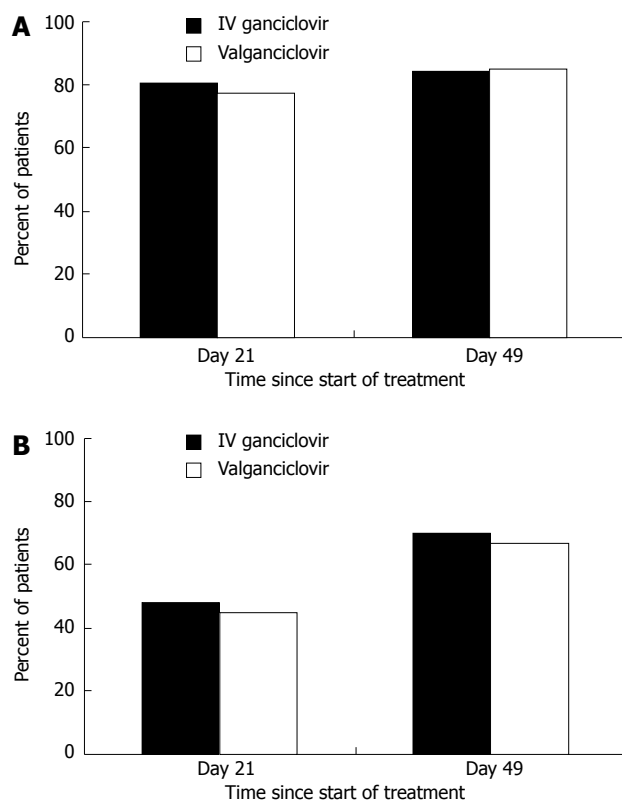


Figure 2 The proportion of solid organ transplant patients with resolution of clinical symptoms (A) and viremia eradication (B) at day 21 and 49 following the start of valganciclovir or IV ganciclovir treatment of CMV disease. Data obtained from the study by Asberg *et al*^[81].

syndromes wherein the virus is detected in the affected tissues but is minimally detectable or undetectable in the blood^[19]. In the current era, gastrointestinal CMV disease (in the form of gastritis, esophagitis, enteritis, colitis) constitutes the vast majority of tissue-invasive patients^[3,19], and in a number of cases, this type of CMV disease is “compartmentalized.” Such a clinical presentation is reminiscent of CMV retinitis, a very rare manifestation of tissue-invasive CMV disease after transplantation, that is often not accompanied by viremia^[82,85]. This dilemma brings to the forefront the limitation of viral load monitoring in assessing duration of treatment. In our clinical practice, it is not uncommon to have negative blood PCR assay even when there is histologic evidence of tissue invasion. Accordingly, it has become a more common practice to perform colonoscopy or upper endoscopy to document clearance of gastrointestinal CMV disease prior to discontinuation of therapy. Our anecdotal experience however indicates that this may not be necessary in mild to moderate disease as long as sufficient therapy is provided.

The challenge of treating ganciclovir-resistant CMV disease

Ganciclovir-resistant CMV is now emerging as an important complication of prolonged antiviral drug use after transplantation^[2,19,70]. Currently, ganciclovir-resistant CMV is very rarely seen in liver transplant recipients (it is more common after kidney-pancreas and

lung transplantation). Unlike lung and kidney-pancreas transplant recipients who have rates as high as 9% and 13%, respectively, the estimated incidence of ganciclovir resistant CMV after liver transplantation is < 0.5%^[70,86]. Several studies have identified risk factors for ganciclovir-resistant CMV^[2,19,70], including CMV D+/R- status, high levels of viral replication, potent immunosuppressive therapy, and suboptimal ganciclovir levels. The vast majority of drug-resistant cases involve the selection of viral strains with UL97 (kinase) mutation^[2,19,70,75,87]. UL97 mutation generally confers resistance to ganciclovir, although in some cases, a concomitant UL54 mutation (CMV DNA polymerase) is also observed, in which case, cross-resistance with cidofovir and/or foscarnet is likely. As noted, no cross-resistance has been observed with the investigational drug, maribavir.

Drug-resistant CMV is associated with significant morbidity and mortality, and there is a very limited number of antiviral drugs (which are often toxic) available for treatment^[86]. Drug-resistant CMV should be suspected when viral load or antigenemia rises or does not decline to undetectable levels despite IV ganciclovir treatment. The diagnosis is confirmed by genetic analysis to demonstrate mutational changes in UL97 and UL54 genes encoding for kinase and polymerase, respectively^[70,86]. In our retrospective study of 225 CMV D+/R- solid organ transplant recipients who received 3 mo of valganciclovir prophylaxis, CMV disease occurred in 65 patients (29%), including four (8%) caused by drug-resistant CMV, judged by the failure of the viral load to decline to undetectable levels while on IV ganciclovir treatment^[70,88]. In our cohort, one liver transplant recipient was clinically suspected to have ganciclovir-resistant strain, although the genotypic assay failed to document any mutations^[88]. The treatment of ganciclovir-resistant CMV should be guided by genotypic analysis. In patients where foscarnet or cidofovir was used, nephrotoxicity was a major adverse effect^[88]. Other potential drugs for the treatment of multi-drug resistant CMV include the off-label use of immunoglobulins and leflunomide, although data supporting their use are only anecdotal^[19]. The potential clinical utility of maribavir in the treatment of resistant CMV is highly anticipated^[74-76,87].

CONCLUSION

Remarkable advances in molecular diagnostics and therapeutics has led to marked reduction in the incidence and severity of CMV disease after liver transplantation, and a parallel decline in the associated morbidity and mortality. However, despite these improvements, CMV remains a common infectious complication and continues to negatively influence the outcome of liver transplantation. In addition to viral factors and pharmacologic immunosuppression, the role of innate and adaptive immune deficiencies is being recognized in the pathogenesis of CMV disease after liver transplantation. Such novel findings should provide additional avenues and opportunities for

improving our management strategies. Prevention of CMV with antiviral prophylaxis and preemptive therapy is effective, although a well-controlled trial assessing these two strategies in a head-to-head comparison is yet to be conducted after liver transplantation. Currently, valganciclovir prophylaxis is the most common approach for the prevention of CMV disease in CMV D+/R- and R+ liver transplant recipients. The availability of predictive diagnostic tests has paved the way for the successful use of preemptive therapy in preventing the progression of CMV reactivation to clinical disease even in high-risk liver transplant patients. IV ganciclovir remains the standard of treatment for established CMV disease, although valganciclovir has now been shown to be equally effective in the treatment of mild to moderate CMV diseases. The duration of treatment should be individualized, depending upon clinical and laboratory parameters such as the decline of CMV load in the blood as measured by rapid and sensitive molecular testing. In this context, it is generally recommended that treatment should be continued until all evidence of active infection, such as positive CMV viral load, has resolved. Ganciclovir-resistant CMV and compartmentalized tissue-invasive disease (most commonly with gastrointestinal CMV disease) are emerging challenges to the management of CMV after liver transplantation. These, together with the common occurrence of late-onset CMV disease in high-risk patients, should serve as catalysts to the ongoing search for the optimal preventive strategy for CMV disease after liver transplantation.

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Cytokine orchestration in post-operative peritoneal adhesion formation

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Abstract

Peritoneal adhesions are a near inevitable occurrence after laparotomy and a major cause of both patient and physician misery. To date, clinical attempts at their amelioration have concentrated on manipulating the physical factors that affect their development despite a wealth of experimental data elucidating the molecular mechanisms that underlie their initiation, development and maturation. However, the advent of targeted, specific anti-cytokine agents as directed therapy for inflammatory and neoplastic conditions raises the prospect of a new era for anti-adhesion strategies. To harness this potential will require considerable cross-disciplinary collaboration and that surgeon-scientists propel themselves to the forefront of this emerging field.

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INTRODUCTION

Post-operative peritoneal adhesion formation remains a considerable source of patient and physician frustration and a significant burden on hospital resources^[1]. As the commonest cause of small bowel obstruction in patients who have previously undergone laparotomy, adhesions account for 40% of all cases of intestinal obstruction and 60%-70% of those affecting the small bowel. After a first such clinical episode, 53% of patients will go on to develop a second relapse, and 83% of these will have chronic symptoms^[2]. Some 14% of those who manifest overt adhesive intestinal obstruction do so within 2 years of their initial surgery, with 2.6% requiring operative adhesiolysis for its relief^[3]. Furthermore, approximately 20% of patients developing adhesional bowel obstruction do so at a remove of more than ten years after their index operation^[4]. Post-operative adhesions are also a common cofactor in female infertility in those with prior laparotomy^[5,6] and they add markedly to the technical complexity of any repeat abdominal operation. By doing so, they give rise to considerable surgeon frustration^[7] and a heightened risk of patient morbidity^[8].

For all these reasons, this iatrogenic complication weighs heavily on the balance books of health care providers. Indeed, in overall costs, the financial cost due to adhesion-related morbidity approximates the expenditure required for the surgical management of gastric or rectal cancer^[9] and this is then further compounded by the cost of medicolegal claims and settlements. Finally, the considerable number of bed-days consumed by the sequelae and treatment of post-operative adhesions (indeed in Finland, adhesion-related admissions exceeds the number of bed-days appropriated to varicose vein surgery) also reinforces the urgency for developing effective means of adhesion abrogation.

Unfortunately, however, clinical strategies and therapies aimed at controlling or alleviating adhesion formation have been largely inadequate in their address of both ongoing human suffering^[10] and economic cost^[11]. To date these attempts have mostly concentrated on employing physical means to align^[12-14] or separate^[15] adjacent loops of bowel in the early post-operative period (so that any configuration of interloop bands is either organised or hindered respectively) or have focused on manipulating peritoneal fibrinolytic mechanisms^[16-18].

CYTOKINE ORCHESTRATION IN POST-OPERATIVE ADHESION FORMATION

Adhesions however represent a form of secondary wound healing. Therefore the mesothelial tissue response to injury (occurring either directly due to handling and dissection or indirectly due to desiccation, cooling or relative ischaemia at sites both adjacent to and distant from the actual operative site) is initiated locally and thence both propagated and orchestrated by cytokine signaling. Although systemic^[19] and genetic elements^[20] may also influence the severity of the cascade and factors such as bacterial contamination can potentiate it^[21], interruption or manipulation of key cellular processes early in the response cascade would seem likely to markedly diminish all downstream events including the ultimate fibrotic endpoint. Furthermore, the increasing sophistication of anti-cytokine therapies now allows single components of complex cellular processes to be specifically targeted. In addition, potentially efficacious agents have already been proved both safe and useful in the management of anti-neoplastic^[22] and anti-inflammatory conditions^[23]. Therefore a new era in the approach to adhesion amelioration may be in the offing.

SPECIFIC TARGETTING OF SELECTED CYTOKINES

There has of course been a vast array of cytokines and chemokines implicated in the initiation, development and maturation of abdominal adhesions after laparotomy (Table 1) and therefore it may initially appear forbidding to try and narrow the therapeutic target most likely to lead to unopposed benefit. Tumor necrosis factor was one of the earliest cytokines investigated and certainly seems to represent one important factor. However its recent elucidation as a key mediator of the bacterial response to infection seems to mitigate against using monoclonal antibodies (already commercially available) to abrogate this cytokine early after intestinal operation^[24]. Equally, the variability of action depending on the relative proportions of its isoforms and the central role it plays in wound healing would also seem to deter use of directed therapy against transforming growth factor-beta. Of the remaining candidate targets the majority only really have a slender evidence base to support their selection from out of the general post-operative molecular milieu. The one exception, at present, would seem to be vascular endothelial growth factor (VEGF).

Although this important signaling protein is best known as a potent angiogenic cytokine (and indeed may be proposed as having a role in the process of adhesion growth through the induction of new blood vessels into areas of operative tissue injury^[25]), VEGF is now also well established as being directly involved in restorative tissue processes, including early inflammatory responses, as well as wound repair and remodeling *via* effecting fibroblast function^[26]. Furthermore, the central role of VEGF in facilitating increased vascular permeability

(essential for the early proinflammatory response to injury) as well as the subsequent deposition of the fibrin-rich matrix necessary for subsequent cellular migration and proliferation^[27,28] would seem to make it a prime putative agent in the formation of peritoneal adhesions. It is not surprising therefore that VEGF has been consistently positively implicated (albeit non-selectively) in this process^[29]. The realization that peritoneal mast cells both constitutively and inducibly express this cytokine^[30,31] further suggests an intriguing link given that these cells are known also to be central to adhesion formation^[32]. However, it may well be that rather than through direct secretion, mast cells effect the threshold concentration of this cytokine by exciting the egress of neutrophils and monocytes from the circulation into the peritoneum and that it is these cells that instead then contribute most to regional VEGF levels.

Regardless of its exact cellular origin, VEGF seems to represent an ideal target as its levels correlate with adhesion formation in animal models with its regulation (either positively^[19] or negatively^[32]) affecting the degree to which they form after peritoneal operations. The clinical success and safety of VEGF neutralization by a specific monoclonal antibody in the treatment of malignant diseases^[33] adds further impetus to the need to try its pharmacological manipulation as an anti-adhesion strategy particularly as selective therapeutic targeting of the cytokine does not seem to disrupt operative wound healing in a clinically important fashion^[34].

DETERMINATION OF CLINICAL EFFICACY

Clinical evidence of efficacy of anti-adhesion therapies is notoriously difficult to attain as second look-laparotomy to assess distribution and intensity of peritoneal reaction is not ethically justifiable (although may be possible in the case of certain gynecological procedures^[35]). Additionally, the mere presence of adhesions, even if extensive, does not necessarily correlate with the incidence and severity of subsequent symptomatic episodes and long-term follow-up is required to determine the full-extent of the problems arising. These challenges are not however insurmountable as have been shown by those who advance the cause of bioactive substances^[36,37] and the difficulties that would be encountered in establishing a progressing and adequately powered multi coated blinded study would be markedly outweighed by the huge benefit to patients of many differing specialties. With regard to monoclonal antibody therapies in particular, there now exists the opportunity to piggy-back on the human safety testing performed on this class of drug in alternative settings. While pursuit of molecular mechanisms for adhesion amelioration will undoubtedly still be expensive^[38], the cost incurred by the management of adhesion-related morbidity^[39,40] economically justifies considerable investment in any potential means of their attenuation.

CONCLUSION

There have long been a multitude of groups proposing

Table 1 Overview of literature to date regarding cytokine orchestration in postoperative adhesion formation. Included in the list are cytokines, chemokines, and proteases as well as trigger enzymes

Cytokine ^{Ref}	Mechanism investigated	<i>In vitro/vivo</i>	Species	Experimental model	Effect on adhesion formation
Heparin-binding growth factor ^[41]	Macrophage and neutrophil omental migration	<i>In vivo</i>	Mouse	(1) Partial hepatectomy (2) Omental adherence	Exacerbated by Midkine- omental inflammation reduced
HGF ^[42]	Mesothelial cell proliferation and migration	Both	Rat	Cecal abrasion	Exacerbated by local HGF gene transfer
IFN- γ , HGF ^[43]	Natural killer T cell activity	Both	Mouse	Cecal cauterization	Attenuated by HGF
IL-1 ^[44]	Nonspecific inflammation	<i>In vivo</i>	Rat	Cecal abrasion	Exacerbated by IL-1
IL-1, TNF ^[45]	Proinflammatory markers	<i>In vivo</i>	Human	Adhesion samples	IL-1 & TNF- α associated with adhesion
IL-1, IL-6, TNF- α ^[46]	Cellular mediation	<i>In vitro</i>	Human	Peritoneal fluid sampling	Adhesions associated with IL-6 and IL-1
IL-10 ^[47]	Natural antiinflammatory	<i>In vivo</i>	Mouse	Peritoneal injury	Attenuated by IL-10 but no effect with IL-10 mAb. No associated with IL-10 levels
IL-10 ^[48]	Immunosuppression	<i>In vivo</i>	Mouse	Peritoneal injury	Attenuated by IL-10
IL-1b, TNF- α , TGF- β 1, IL-10, IFN γ , GM-CSF ^[49]	Inflammatory	<i>In vitro</i>	Human	Peritoneal fluid sampling	Only IFN- γ and TGF- β 1 associated with adhesion formation. No association found with other cytokines.
IL-6 ^[50]	Early proinflammatory effects	<i>In vivo</i>	Rat	Cecal abrasion with C ₂ H ₅ OH	Exacerbated by IL-6, attenuated by monoclonal Ab to IL-6
PAF ^[51]	Early inflammatory mediators	<i>In vivo</i>	Rat	Uterine horn abrasion	Adhesions and IL-6 levels attenuated by Lexipafant (PAF antagonist)
Substance P ^[52]	Substance P mediation	<i>In vivo</i>	Rat	Peritoneal ischaemic buttons	Substance P and TGF- β 1 as well as ICAM-1 and VCAM-1 increased
TGF ^[53]	TGF isoforms	<i>In vivo</i>	Mouse	Serosal abrasion and apposition	Exacerbated by TGF- β 3, attenuated by combined TGF- β 1 and TGF- β 2 mAb
TGF- β ^[54]	TGF- β regulation of extracellular matrix	<i>In vitro</i>	Human	Human fibroblast culture	Dichloroacetic acid inhibited fibronectin and collagen type III expression
TGF- β ^[55]	Chemoattraction	<i>In vitro</i>	Rat	Cecal abrasion	TGF- β mRNA increased by trauma
TGF- β ^[56]	Mast cells	<i>In vivo</i>	Hamster	Uterine horn abrasion	Exacerbated by chymase inhibitor
TGF- β ^[57]	Chemoattraction	<i>In vivo</i>	Rat	Uterine horn abrasion	Exacerbated by TGF- β
TGF- β ^[58]	Mast cells	<i>In vitro</i>	Human	Cell culture	TGF- β and tryptase increased collagen
TGF- β ^[59]	Peritoneal repair	<i>In vivo</i>	Rat	Uterine horn abrasion	No antiadhesion effect of anti-TGF mAb
TGF- β ^[60]	Immunosuppression	<i>In vivo</i>	Rat	Small bowel transplant	Adhesions attenuated by tacrolimus
TGF- β ^[61]	Mast cells	<i>In vivo</i>	Rat	Uterus scraping	TGF- β increased by trauma, adhesions attenuated by chymase inhibition
TGF- β ^[62]	Cellular effects of Tisseel	<i>In vitro</i>	Human	Cell culture	Fibroblasts TGF- β reduced
TGF-b, MMP-9, TIMP-1 ^[63]	Matrix factors	<i>In vivo</i>	Human	Sampled peritoneal fluid	Adhesion assoc with reduced MMP-9 but elevated MMP-9/TIMP-1 ratio
TGF- β /MDF ^[64]	Carboxymethylcellulose sponge	<i>In vivo</i>	Rat	Cecal denudation & apposition	Effect of sponge independent to cytokine release (barrier function)
TGF- β 1 ^[65]	Chemoattraction	<i>In vitro</i>	Human	Cell culture	TGF- β 1 increased in scar tissue
TGF- β 1 ^[66]	Extracellular matrix	<i>In vivo</i>	Mouse	Cecal abrasion	Exacerbated by haploid insufficiency
TGF- β 1 ^[67]	Fibrinolysis	<i>In vitro</i>	Human	Biopsy sampling	Attenuated by TGF- β 1 overexpression
TGF- β 1 ^[68]	Peritonitis	<i>In vivo</i>	Rat	Cecal ligation and puncture	Peritonitis upregulates TGF- β 1 expression
TGF- β 1 ^[69]	Mitogenicity of macrophages & fibroblasts	<i>In vivo</i>	Rat	Small Bowel transection and re-anastomosis	Adhesions and TGF-1 levels attenuated by ACE inhibition
TGF- β 1, MMP1&2, TPA, TIMP-1 ^[70]	Cellular effects of seprafilm	<i>In vitro</i>	Human	Human fibroblast & mesothelial cell culture	No cytokine effect induced by Seprafilm (barrier effect important)
TGF- β 1, TGF- β 2 ^[71]	Basal expression	<i>In vitro</i>	Human	Biopsy sampling	Sit-specific TGF- β 1 & TGF- β 3 expression
TGF- β 1 ^[72]	Cellular effects of chengtong	<i>In vivo</i>	Rat/rabbit	Cecal abrasion	TGF- β reduced in rats
TNF, IL-1, IL-6 ^[73]	Effects of gloves and powders	<i>In vivo</i>	Rat	Cecal abrasion	Adhesions increased by glove powder
TNF- α ^[74]	Proinflammatory effects of TNF- α	<i>In vivo</i>	Rat	Cecal abrasion	Adhesion formation attenuated by infliximab but no histological effect
TNF- α , IL-1 ^[75]	Proinflammatory markers	<i>In vivo</i>	Rat	Cecal abrasion or small bowel resection	TNF- α appears a good biological marker for adhesion formation
TNF- α , IL-1 ^[76]	Immunosuppression	<i>In vivo</i>	Rat	Cecal abrasion	Adhesion formation attenuated by mAbs to IL1 and IL-1/TNF- α
TNF- α , IL-6 ^[77]	Proinflammatory mediators	<i>In vitro</i>	Mouse	Murine macrophages	Adhesion formation attenuated by hyaluronic acid and dexamethasone
TNF- α , MMP ^[78]	Mesothelium reaction to peritoneal injury	<i>In vivo</i>	Rat	Peritoneal wounding	No effect of MMP & TACE inhibition, TNF- α may not be adhesiogenic
TNF- α , TGF- β 1 ^[79]	PROACT to injured peritoneum	<i>In vivo</i>	Human	Tissue sampling	TNF- α and TGF- β reduced by heating
VEGF ^[80]	Angiogenesis	<i>In vivo</i>	Rat	Uterus-peritoneal scrub	Associated by angiogenesis
VEGF ^[29,32]	Vascular permeability	<i>In vivo</i>	Mouse	Peritoneal injury	Adhesions attenuated by Antiserum and monoclonal antibody
VEGF, basic-FGF ^[25]	Fibrovascular band formation	<i>In vivo</i>	Human	Adhesion samples	VEGF in endothelial cells associated with adhesion formation

VEGF, IL-6 ^[21]	Bacterial Translocation	Both	Mouse	Caecal abrasion & suture	Adhesions attenuated by rBPI
VEGF, PlGF ^[81]	Pneumoperitoneum	<i>In vivo</i>	Mouse	Lap. uterine horn model	Exacerbated by VEGF and CO ₂
CCL 1-CCR 8 ^[83]	Specific recruitment of peritoneal macrophages	Both	Mouse	Peritoneal ischaemic button & colitis-associated peritoneal adhesions	Unaffected by CCR8 gene deficiency and antiCCL1-neutralizing antibody
CD 28 T cell costimulatory pathway ^[84]	CD28 T cell costimulatory pathway/Inhibitor programmed death-1 pathway	Both	Mouse	Caecal abrasion	Exacerbated by CD28 T Cell costimulatory pathway but unaffected by death-1 pathway
Interferon-inducible protein-10 ^[85]	Regulates influxing neutrophils, monocytes and lymphocytes	<i>In vivo</i>	Mouse	Peritoneal side wall injury	
Broad spectrum of chemokines ^[86]	Broad spectrum chemokine inhibitor NR58-3.14.3	<i>In vivo</i>	Mouse	Peritoneal traumatization	Adhesions significantly attenuated
MCP-1 ^[87]	Fibroblast and mononuclear cell chemotaxis	<i>In vivo</i>	Mouse	Peritoneal injury	Attenuated by MCP-1 antibody
MCP-1 ^[88]	Fibroblast and mononuclear cell chemotaxis	<i>In vivo</i>	Human	Cell culture	
MCP-1 ^[89]	Fibroblast and mononuclear cell chemotaxis	<i>In vivo</i>	Human	Cell culture	
T cells, IL-17, CXCL1 ^[90]	CD4+ T cells	<i>In vivo</i>	Mouse	Caecal abrasion	Unaffected by anti-IL-17 antibodies

HGF: Hepatocyte growth factor; IFN- γ : Interferon-gamma; IL: Interleukin; TNF- α : Tumour necrosis factor-alpha; TGF- β : Transforming growth factor-beta; GM-CSF: Granulocyte macrophage colony stimulating factor; PAF: Platelet activating factor; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase; MDF: Macrophage deactivating factor; TPA: Tissue plasminogen activator; VEGF: Vascular endothelial growth factor; FGF: Fibroblast growth factor; PlGF: Placental growth factor; MCP: Monocyte chemotactic protein.

novel, potential therapies for the attenuation of adhesion formation at a preclinical level- the onus now though is on leading surgeon-scientists to corral their endeavour and progress their preclinical expertise into the clinical setting. For a start, the most likely candidate cytokine must be agreed (in our mind VEGF would seem the most apposite) and the most appropriate means of affecting its activity (whether directly^[32] or indirectly^[21]) selected. Furthermore industry interest will need to be stimulated for its support for Phase II and III trials as well as for the subsequent manufacture and marketing processes is crucial. Above all, though it must be realized that the timing for a concerted attempt to prove that molecular manipulation of post-operative peritoneal formation has never been better.

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Narrow-band imaging optical chromocolonoscopy: Advantages and limitations

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in vivo visualization of vascular structures, but further study assessing reproducibility and effectiveness in the colorectum is ongoing at various medical centers.

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Abstract

Narrow-band imaging (NBI) is an innovative optical technology that modifies the center wavelength and bandwidth of an endoscope's light into narrow-band illumination of 415 ± 30 nm. NBI markedly improves capillary pattern contrast and is an *in vivo* method for visualizing microvessel morphological changes in superficial neoplastic lesions. The scientific basis for NBI is that short wavelength light falls within the hemoglobin absorption band, thereby facilitating clearer visualization of vascular structures. Several studies have reported advantages and limitations of NBI colonoscopy in the colorectum. One difficulty in evaluating results, however, has been non-standardization of NBI systems (Sequential and non-sequential). Utilization of NBI technology has been increasing worldwide, but accurate pit pattern analysis and sufficient skill in magnifying colonoscopy are basic fundamentals required for proficiency in NBI diagnosis of colorectal lesions. Modern optical technology without proper image interpretation wastes resources, confuses untrained endoscopists and delays inter-institutional validation studies. Training in the principles of "optical image-enhanced endoscopy" is needed to close the gap between technological advancements and their clinical usefulness. Currently available evidence indicates that NBI constitutes an effective and reliable alternative to chromocolonoscopy for

INTRODUCTION

In 1971, Folkman proposed that all tumor growth was angiogenesis-dependent. This was the foundation for the development of angiogenic research and helped to stimulate investigation that is now being pursued by scientists in many different fields worldwide^[1]. New blood vessel creation favors a transition from hyperplasia to neoplasia (i.e., the passage from a state of cellular multiplication to a state of uncontrolled proliferation characteristic of tumor cells)^[2].

An *in vivo* means for visualizing angiogenesis or microvessel morphological changes in superficial neoplasms would constitute a promising method for the diagnosis of early gastrointestinal tumors. Narrow-band imaging (NBI) is an innovative optical technology developed in Japan that modifies the center wavelength and bandwidth of an endoscope's light into a narrow-band illumination of 415 ± 30 nm. By utilizing this narrow spectrum, contrast in the capillary pattern of the superficial layer is markedly improved^[3], thereby facilitating clearer visualization of vascular structures during gastrointestinal endoscopy^[4].

The first clinical study of the NBI system for the diagnosis of gastrointestinal tumors was reported by Sano *et al*^[5] in 2001. Their promising observations resulted in the first pilot colorectal study in which the NBI system demonstrated better vascular pattern

visualization than conventional colonoscopy in the diagnosis of colorectal polyps^[6]. These early studies opened the way for subsequently using NBI in the diagnosis of pre-malignant and malignant lesions of the hypo-pharynx, esophagus and stomach^[4,7,8].

This review focuses on the current advantages and limitations of using the NBI system in the diagnosis of colorectal lesions.

SCIENTIFIC BASIS FOR NBI

Video endoscopes use white light from a xenon source for illumination. In order to understand the reflectance spectrum of any tissue, both the scattering process and absorption must be taken into account. Based on the Monte Carlo simulation, several investigations into the mechanism of scattering from tissue structures have determined that the penetration depth of the light depends on the wavelength. The depth of penetration into the gastrointestinal tract mucosa is superficial for the blue band, intermediate for the green band and deep for the red band (penetration depth range: 0.15 to 0.30 mm). As a result, NBI systems use optical filters for green and blue sequential illumination and narrow the bandwidth of spectral transmittance^[9,10] (Figure 1).

The scientific basis for the NBI system is that light with a short wavelength falls within the hemoglobin absorption band, so that blood vessels may be more clearly seen due to sufficient contrast^[6].

IMAGE RECONSTRUCTION FROM REFLECTED LIGHT

Two different types of NBI systems are used to reconstruct images from the reflected light. The non-sequential system (Exera II), also referred to as the “color chip system”, uses a color charge coupled device (CCD) in which pixels are selectively assigned to specific wavelength ranges. The CCD captures the full range of the white light and transfers it in a single step to the processor in order to reconstruct natural color on the video monitor (Figure 2).

In contrast, the sequential system (Lucera Spectrum) uses a monochrome CCD in which pixels are not selectively attributed to specific colors, but transferred sequentially in the RGB bands to the processor. A rotating RGB interference filter is interposed after the white light source and the mucosa is illuminated alternately in each of the three RGB bands^[11] (Figure 3).

Although the concept and basic design is the same for both the NBI sequential and non-sequential systems, a difference in color images exists due to differences in the color spectral characteristics of the RGB rotary filters used in the Lucera Spectrum and the color CCD used in the Exera II. There is considerable potential for further development, however, by improving NBI technology in the non-sequential endoscopic video system.

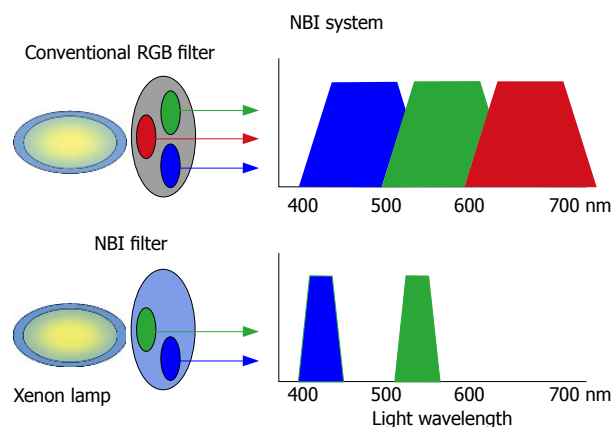


Figure 1 NBI system. Different from the conventional RGB filter, the NBI filter consists of two narrow bands (415 ± 30 nm and 540 ± 30 nm, respectively) that make it possible to observe clearly superficial vascular patterns for clinical evaluation.

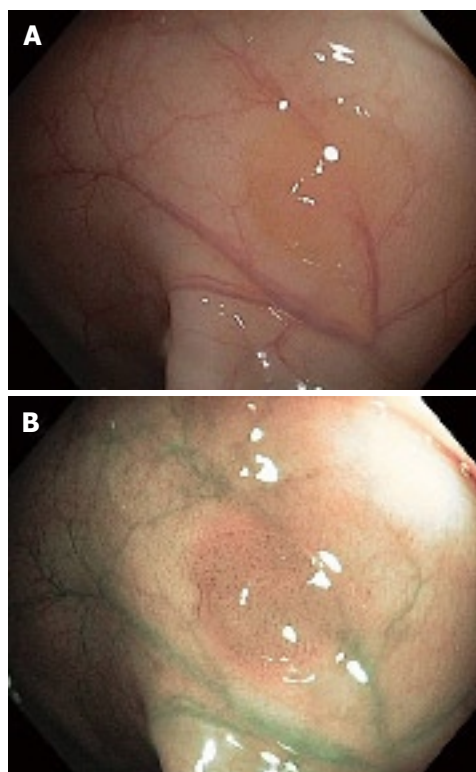


Figure 2 NBI colonoscopy image with non-sequential system. **A:** Conventional view of an Is polyp, 12 mm in diameter located in the sigmoid colon; **B:** NBI view clearly showing the superficial meshed vascular pattern on the polyp's surface indicating an adenomatous polyp.

ARE YIELDS OF SMALL AND FLAT ADENOMAS HIGHER WITH NBI?

An interesting Japanese study involving 48 patients in which conventional white light colonoscopy was first performed followed later by blind NBI colonoscopy on the same patients found that the total number of neoplastic lesions detected by NBI was significantly higher than the total number of neoplastic lesions detected using conventional colonoscopy ($P = 0.02$). Based on macroscopic appearance, location and tumor

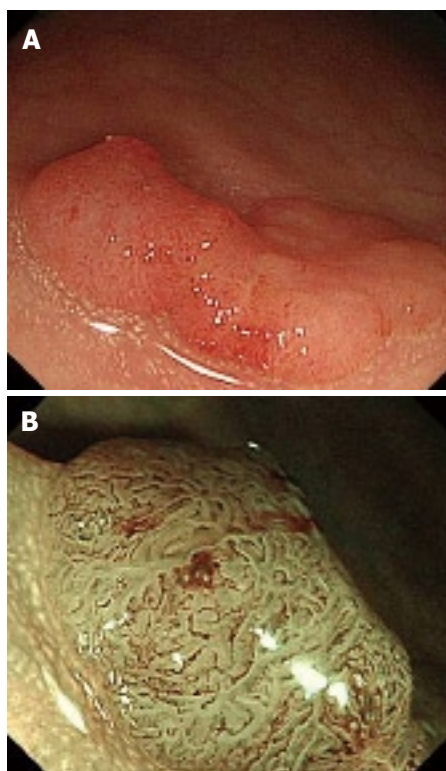


Figure 3 NBI colonoscopy image with sequential system. **A:** Conventional view of an IIa polyp, 20 mm in diameter located in the rectum; **B:** Meshed capillary vessels are clearly seen using magnifying NBI as dark brown areas diagnosing an intramucosal cancer.

size, flat lesions < 5 mm located in the right colon in particular were more frequently diagnosed using NBI^[12].

Although no Western study has as yet validated those Japanese results, a recent report indicated that adenomas were detected more frequently in the NBI group (23%) than in the control group (17%), but the difference was not statistically significant ($P = 0.129$)^[13]. In contrast, it has also been recently reported that NBI did not result in better detection of adenomas. In that particular study, a colonoscopist with a known high detection rate using white light colonoscopy conducted patient examinations with high-definition colonoscopes using either white light or NBI^[14].

The fact that differences still exist between Japan and Western countries demonstrates that prospective studies are needed to determine which of these early reports are valid.

NBI FOR NON-NEOPLASTIC AND NEOPLASTIC LESIONS

For lesions < 10 mm, it is generally accepted that hyperplastic polyps and other non-neoplastic colorectal lesions do not require endoscopic treatment because they are benign and have no malignant potential^[15,16]. In contrast, adenomatous polyps should be removed to prevent progression of the adenoma-carcinoma sequence^[17].

Magnified chromocolonoscopy (MCC) has been

presented as the best means for *in vivo* selective management of colorectal polyps^[18,19] and it is suggested that colorectal polyps should not be treated only on the basis of polyp size, but also with respect to the underlying histological characteristics observed during MCC^[20]. The NBI system has been proposed for optical image-enhanced endoscopy because it features a simple one-touch button for changing from white light to NBI and does not require indigo carmine dye spraying.

An early study of an NBI prototype used for differentiating non-neoplastic from neoplastic lesions in 34 patients with 43 lesions reported better visualization of the mucosal vascular network and lesion compared to conventional endoscopy. Chromocolonoscopy and NBI both had a sensitivity of 100% and a specificity of 75%^[6]. Thereafter, the effectiveness of conventional colonoscopy, chromoendoscopy and the NBI system in distinguishing between non-neoplastic and neoplastic colonic polyps was assessed in 78 patients with 110 lesions. No significant difference existed between the NBI system and chromoendoscopy, but the sensitivity, specificity and accuracy of conventional colonoscopy were significantly lower (82.9%, 80.0% and 81.8%, respectively) compared to both chromoendoscopy and the NBI system (95.7%, 87.5% and 92.7%, respectively)^[21].

More recently, a classification of colorectal polyps based on the presence or absence of superficial meshed capillary vessels and their diameter, observed under NBI (CP type I–III) was proposed in Japan by Sano *et al*^[22]. Although a promising and exciting alternative to differentiate the nature of colorectal polyps, Western prospective studies, however, are needed for its standardization worldwide.

NBI FOR INVASIVE AND NON-INVASIVE COLORECTAL CANCER

There is growing evidence to support the theory that lesions with submucosal (sm) invasion < 1000 μm (sm1) without lympho-vascular invasion or a poorly differentiated component do not involve lymph node metastases^[23]. In Japan, analysis of the pit pattern types proposed by Kudo *et al*^[24] has been proven effective in predicting the level of sm invasion. In practice, however, limitations have been reported using the V_1 pit pattern to discriminate between mucosal (m), slight submucosal (sm1) and, deep submucosal (sm2) or deeper invasion^[25]. The invasive pattern proposed by Fujii *et al*^[26–28] (distorted and irregular crypts and a demarcated area) has also been reported to be effective in predicting sm2.

One promising area for NBI is in the accurate estimation of invasive depth for early colorectal cancers. Hirata *et al*^[29] analyzed 148 colorectal lesions and recently reported a high degree of correspondence between pit pattern analysis by NBI and chromoendoscopy although the correspondence between MCC and NBI in evaluating the V_1 pit pattern of 48 early carcinomas

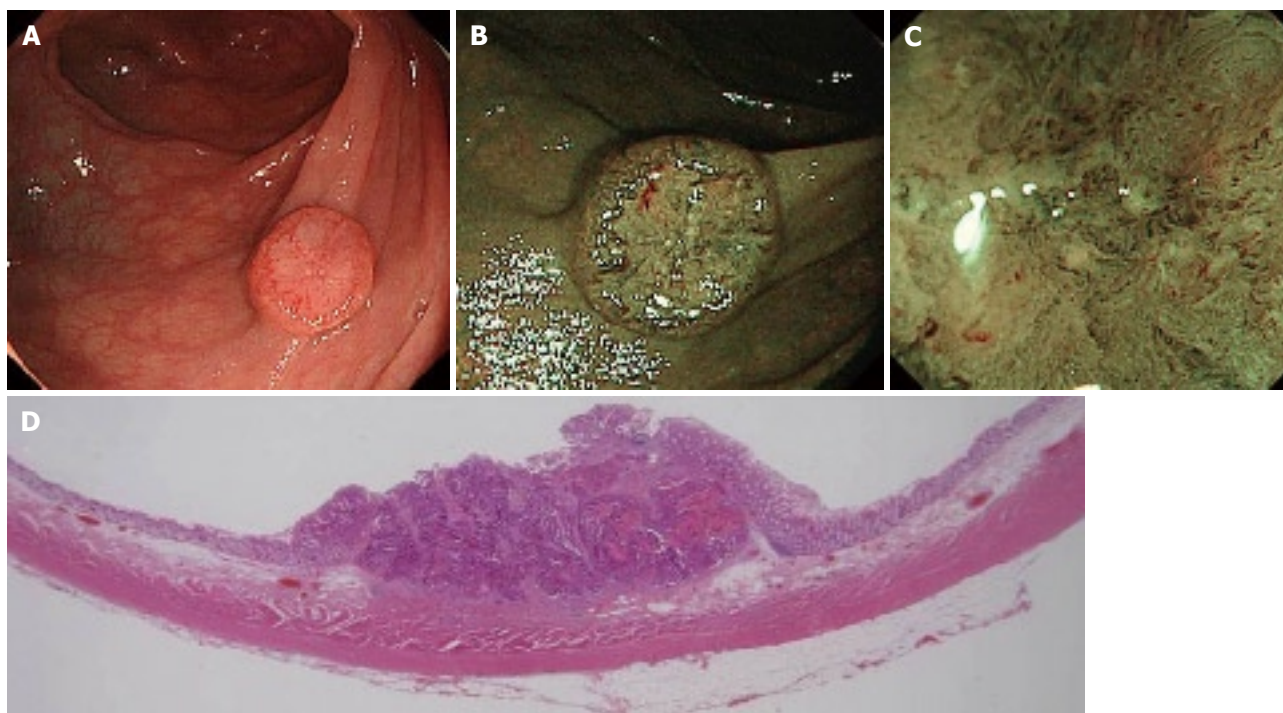


Figure 4 NBI image of colorectal cancer. **A:** Conventional view of an IIa + IIc lesion, 12 mm in size, located in the transverse colon; **B:** NBI view shows a well demarcated area and meshed capillary vessels clearly visible characterized by thick diameter, branching and curtail irregularity; **C:** Magnifying NBI view additionally shows the presence of a nearly avascular or loose microvascular area due to histological desmoplastic changes in the stromal tissue, suggesting deep submucosal invasion; **D:** Histopathological analysis revealed an adenocarcinoma invading deeply into the submucosa (2500 μ m) with lymphovascular invasion.

was only 78%. Diagnosis using the type V pit pattern was possible by also evaluating various capillary features including vessel diameter, irregularity and the capillary network observed during NBI and not by relying solely on the pit pattern.

Two other promising studies on predicting the depth of invasion of early colorectal cancer by analyzing the microvascular architecture were published recently. Using NBI with magnification, Fukuzawa *et al*^[30] observed several microvascular architecture characteristics in 61 early colorectal lesions (m-sm1: 37; sm2-3: 24). Univariate analysis showed that wide caliber, irregular caliber, tortuosity, irregularity, short length and non-dense arrangement were significantly more frequent in sm2-3 lesions compared to m-sm1 lesions ($P < 0.001$). Multivariate analysis, however, revealed that irregularity and non-dense arrangement were the remaining independent factors^[30] (Figure 4). Horimatsu *et al*^[31] analyzed the presence of “meshed brown capillary vessels” in 27 colorectal lesions (m-sm1: 12; sm2: 15) also using NBI colonoscopy with magnification. The overall diagnostic accuracy, sensitivity, and specificity of microvessel density and the lack of uniformity in microvessel diameters for distinguishing between sm1 and sm2 lesions was 82.4% (14/17), 93.3% (14/15) and 75.0% (9/12), respectively^[31] (Figure 4).

DETECTION OF DYSPLASTIC AREAS IN ULCERATIVE COLITIS

Although patients with longstanding ulcerative

colitis are at increased risk of developing colorectal cancer, endoscopic detection of early neoplasia is difficult because these lesions can be subtle and even macroscopically invisible at times. A laborious protocol has been proposed involving not only target biopsies from suspicious lesions, but also two to four random biopsies taken every 10 cm of the colon^[32]. MCC has emerged as the best method currently available for identifying dysplastic lesions in an inflammatory bowel disease setting^[33,34].

In terms of NBI research, the limitations of the first NBI prototype were recently shown in a prospective randomized crossover study of 42 patients with longstanding ulcerative colitis. In that study, the sensitivity of NBI for the detection of neoplasia was merely comparable to conventional colonoscopy although a larger number of suspicious lesions were found during NBI colonoscopy^[35]. A more positive report on the effectiveness of a third generation NBI prototype plus magnification indicated that just as NBI reveals fine superficial blood vessels whose diameters and densities are increased in neoplastic lesions compared with normal mucosa, dysplastic lesions observed using NBI also have a darker capillary vascular pattern compared with normal mucosa^[36].

WILL CONVENTIONAL CHROMOCOLONOSCOPY BE REPLACED BY NBI?

It is still too early to answer this question. In Japan,

chromocolonoscopy has demonstrated its effectiveness in the differentiation between adenomatous and hyperplastic polyps and is a promising method for distinguishing superficial from deep submucosal cancers, but it is regarded as an inconvenient and difficult procedure in Western countries^[37]. Indigo carmine dye spraying is inexpensive and differs in practice from the NBI system in that it does not target superficial vascular patterns, but instead accentuates lesion contours and highlights the pit pattern of colonic crypts^[25]. It is interesting to note that indigo carmine dye spraying is not recommended before an NBI examination because it might obscure blood vessel visualization.

In contrast, NBI even without magnification when using the non-sequential system provides accurate definition of vascular vessels throughout the entire colonic mucosa and more clearly defines the borders of a lesion without the necessity of using dye spraying. The recently developed NBI system requires an expensive new processor, however, so the cost-benefit issue requires further analysis^[38]. In addition, the diagnostic accuracy of NBI is affected by the learning curve associated with this new methodology and extra time may be needed to perform the examination.

USELESS TECHNOLOGY IN UNQUALIFIED HANDS

The acquisition and use of NBI technology is increasing in many countries, but it should be emphasized that accurate analysis of the pit pattern types and familiarity with MCC are basic fundamentals necessary to become proficient in NBI diagnosis of colorectal lesions. Modern optical technology without proper image interpretation wastes valuable resources, can cause confusion for inadequately trained endoscopists and may result in the delay of inter-institutional validation studies. Training general endoscopists in the principles and applications of optical image-enhanced endoscopy as practiced in Japan (i.e., stereomicroscopy, conventional chromoendoscopy, magnifying endoscopy and pit pattern analysis)^[20,24-26] in approved centers by qualified experts will be required to narrow and, hopefully, close the existing gap between the latest advancements in optical technology and their clinical usefulness.

CONCLUSION

Several studies have previously reported on the advantages and limitations of NBI optical image-enhanced colonoscopy in the diagnosis of colorectal diseases. One difficulty in evaluating the results, however, has been non-standardization of the NBI systems and prototypes used in the research. Despite this shortcoming, there seems to be considerable potential for further development by improving NBI technology in the non-sequential endoscopic video system by modifying the characteristics of the interference filters.

In practice, the latest technological advancements

incorporated into third generation NBI prototypes appear to offer a clear advantage over conventional chromocolonoscopy. Additional validation studies are needed, however, to confirm the effectiveness of NBI for screening colonoscopy, identification of adenomatous polyps, determining depth of invasion of early colorectal cancers, evaluating free margins after endoscopic resection and detection of dysplastic lesion in an inflammatory bowel disease setting.

A number of other questions remain unsolved that deserve additional examinations including whether NBI is less time-consuming, its cost effectiveness, whether magnification is absolutely required and whether the NBI system should completely replace chromocolonoscopy. Further studies assessing these issues are ongoing at various medical centers worldwide.

At the present time, NBI constitutes an effective and reliable alternative to chromocolonoscopy for *in vivo* visualization of vascular structures. Due to widespread incorporation of NBI technology outside Japan, however, there is an increasing need to train general endoscopists in the basic principles and applications of advanced optical image-enhanced endoscopy.

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Low intensity ultrasound-induced apoptosis in human gastric carcinoma cells

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Abstract

AIM: To investigate the low intensity ultrasound (US)-induced apoptosis in human gastric carcinoma cells and its potential mechanism and to suggest a new therapeutic approach to gastric carcinoma.

METHODS: Human SGC-7901 gastric carcinoma cells were cultured *in vitro* and irradiated by low intensity US for 10 min at different intensities with different incubation times after irradiation. Morphologic changes were examined under microscope with trypan blue staining and then the percentage of early apoptotic cells was detected by flow cytometry (FCM) with double staining of fluorescein isothiocyanate (FITC)-Annexin V/propidium iodide (PI). Two-dimensional electrophoresis (2DE) was used to get the protein profile and some proteins differently expressed after US irradiation were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Functional analysis was performed to investigate the mechanism of US-induced cell apoptosis.

RESULTS: The percentage of apoptotic cells increased about 10% after US irradiation (12.0 W/cm², 12 h culture). The percentage of early apoptosis and secondary necrosis in the US-irradiated cells increased with the increased US intensity. Moreover, apoptotic cells increased with the increased culture time after US irradiation and reached its maximum at about 12 h.

Several new proteins appeared after US irradiation and were up or down regulated more than 2 times. Some heat shock proteins (HSPs) were found to be associated with the signal process simulating the apoptosis of cells.

CONCLUSION: Low intensity US could induce apoptosis in human gastric carcinoma cells. US-induced apoptosis is related to US intensity/culture time. US-induced apoptosis may be caspases-dependent and endoplasmic reticulum (ER) stress-triggered apoptosis may also contribute to it. Proteomic experimental system is useful in finding the protein alteration in carcinoma cells after US irradiation, helping to develop a new cancer therapy.

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Key words: SGC-7901 human gastric carcinoma cells; Low intensity ultrasound; Apoptosis; Caspases-dependent; Proteomics

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INTRODUCTION

Gastric carcinoma is the second commonest cause of cancer-related deaths worldwide. Although surgical removal of the stomach is the only curative treatment in clinical practice, more studies revealed that chemotherapy and radiation therapy given after surgery could improve the chance of a cure for many patients. To improve the survival rate, new therapeutic methods should be developed.

In non-surgical cancer treatment, induction of

apoptosis is a preferred mode of killing cancer cells^[1,2]. Several investigators have reported that US irradiation could induce apoptosis in human leukemia cell lines^[3-7], including K562, HL-60, Nalm-6 and U937. It was also reported that apoptosis of some solid carcinoma cells, such as human ovarian carcinomas cells^[8], SMMC-7721 cells (data not shown) and Walker 256 carcinosarcoma cells^[9], are triggered by US irradiation. Different from high intensity focused ultrasound (HIFU) which can thermally ablate tissues *via* hyperthermia in different carcinomas^[10-12], low intensity ultrasound (US) defined as therapeutic US, with a relatively lower intensity than HIFU, has a great potential in apoptosis therapy for cancer and can be relatively easily applied^[8,13]. So it is suggested that low intensity US-induced apoptosis in tumor cells could probably offer a new approach to elimination of cancer^[4]. To our knowledge, US-induced apoptosis in gastric carcinoma has not yet been reported. Compared with human leukemia cell lines, low intensity US-induced apoptosis in solid carcinoma cells or tissues is still in the process of investigation. The mechanism has also not yet been elucidated.

The aim of this study was to prove low-intensity US-induced apoptosis in human SGC-7901 gastric carcinoma cells and investigate the effects of US intensity and culture time after US irradiation on US-induced apoptosis. Comparative proteomic analysis was performed to examine the alteration in protein profile of gastric carcinoma cells induced by US irradiation. The potential molecular mechanism was suggested *via* the protein functional analysis.

MATERIALS AND METHODS

Chemicals and materials

Fluorescein isothiocyanate (FITC)-Annexin V/propidium iodide (PI) kit was purchased from Jingmei Biotechnology Company, China. Immobilized pH gradient (IPG) strips (pH3-10, nonlinear, 13 cm) and IPG buffer (pH3-10, nonlinear) were purchased from Pharmacia, Amersham Biotech. Dithiothreitol (DTT), PMSF and CHAPS were purchased from Sigma Company (USA). All the buffers were made using high purity MilliQ water. Elite ESP flow cytometer was purchased from Coulter Electronics Hialeah, FL. IPGphor isoelectric focusing equipment, Hoefer SE 600 vertical chambers, electrophoresis apparatus, Image Master 2D Elite 4.01 software were purchased from Pharmacia, Amersham Biotech. Voyager-DE MALDI-TOF MS was a product from Applied Biosystems (USA).

SGC-7901 cell line cultures

SGC-7901 human gastric carcinoma cells (Center of Laboratory Animals of the Forth Military Medical University) were cultured in RPMI 1640 culture medium in a water-saturated atmosphere with 50 mL/L CO₂ at 37°C.

US irradiation set

The US irradiation system is shown in Figure 1. It consists

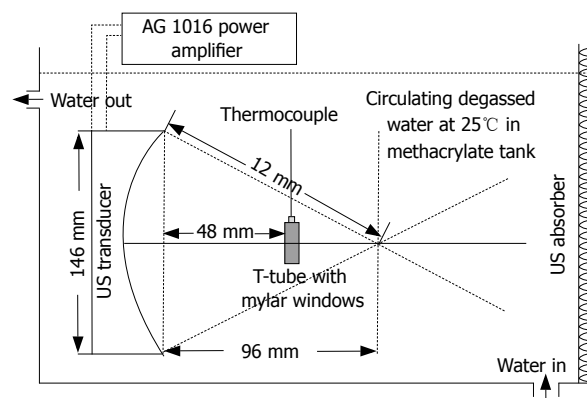


Figure 1 US irradiation system.

of a AWG 2021 arbitrary waveform generator (Sony Tektronix Co., Tokyo, Japan), a AG 1016 power amplifier (T&C Power conversion, Inc., Rochester, NY, USA) and a transducer designed as a semispherical ring with an aperture 146 mm and a radius of curvature 120 mm (Imasonic, Besancon, France), a 750 mm × 750 mm × 500 mm polymethyl methacrylate tank filled with degassed water with 10-cm high water above the transducer. The central frequency is 1.2 MHz. Temperature of water in the tank was controlled at 25°C by circulating. Cell samples were carried in a specially designed inverted “T” tube as previously described^[6,9].

US treatment and sample collection

The cell samples were divided into ten groups and each treatment was given in three replicates. The first group was used as the control that imitated the whole process with no irradiation. The 2-5 groups were irradiated for 10 min at the intensity of 3.0 W/cm², 6.0 W/cm², 9.0 W/cm² and 12.0 W/cm² (I_{SPTA}), respectively, and then incubated at 37°C in a humidified air containing 50 mL/L CO₂. Apoptosis detection and microscopy observation were performed in each sample. Remained cells were washed 3 times with cold PBS, centrifuged at 1500 × *g* for 10 min to get cell pellets, then frozen quickly in liquid nitrogen and stored in a -80°C ultra low temperature freezer (Legaci™ Refrigeration system, REVCOTechnologies) for subsequent 2D PAGE analysis. The whole process was performed in sterile condition.

To investigate the relationship between culture time after US irradiation and cell apoptosis, the 6-10 groups were irradiated at the intensity of 12.0 W/cm² and incubated at 37°C in a humidified air containing 50 mL/L CO₂ for 0, 6, 12, 18 and 24 h, respectively. The following morphologic observation and apoptosis detection were performed as described above.

Morphological observation and flow cytometry (FCM) examination

The morphologic changes in apoptotic and necrosis cells could be examined under microscope and detected by analysis of a light scatter signal by FCM^[14].

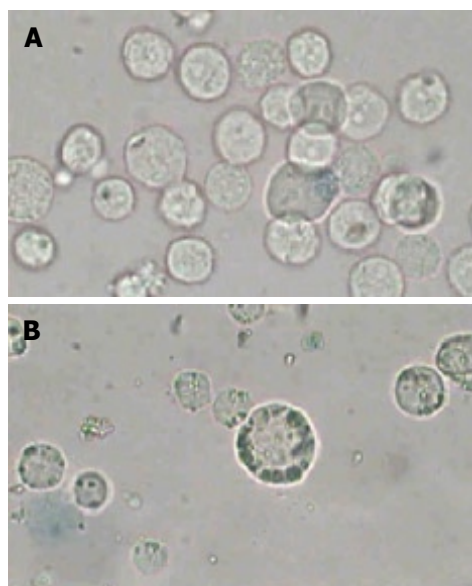


Figure 2 Morphologic characteristics of SGC-7901 cells before and after US irradiation.

SGC-7901 cells were stained with trypan blue and then observed under microscope (Olympus BX40) to detect their structure change after US irradiation. Furthermore, the cells were analyzed with FCM to detect phosphatidylserine (PS) exposure on the cell membrane as a typical marker of early apoptosis.

Two-dimensional electrophoresis (2DE) analysis

Proteins were extracted from control and irradiated cells, respectively, with the freeze-thaw lysis method. Then, the protein solution (containing 200 μ g proteins) was loaded to perform isoelectric focusing and second dimension separation as previously described^[15]. Gels were silver stained.

2D gel images of control and irradiated cells were acquired using a Sharp JX-330 scanner, compared and matched *via* Image Master 2D Elite software. New protein spots and proteins expressing 2 times higher or lower were selected for the following identification based on MALDI-TOF-MS.

Identification of proteins using MALDI-TOF-MS

The selected protein spots were excised from the gel and in-gel digestion with trypsin was performed. The extracted enzymolyzed peptides were identified by peptide mass fingerprinting based on MALDI-TOF-MS (Voyager-DETM), analyzed using data explore, and matched in ProFound-peptide mapping and SWISS-PROT.

RESULTS

Morphologic observation

The morphologic observations showed that the distinct morphologic characteristics of apoptotic cells, such as condensation of nucleus, chromatin margination (Figure 2B) were found after the cells were irradiated at 12.0 W/cm² for 10 min and incubated for 12 h

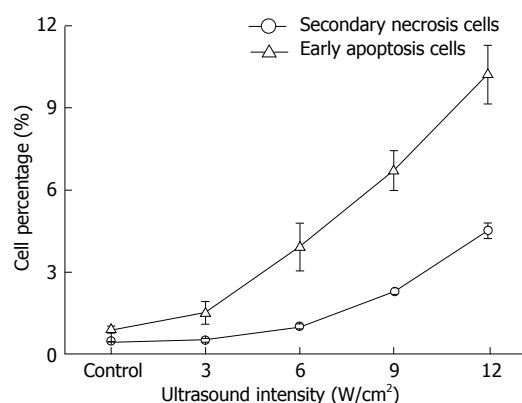


Figure 3 Percentage of early apoptotic cells and secondary necrosis cells in control and US-irradiated SGC-7901 cells at different US intensities.

after irradiation. Simultaneously, the characteristics of apoptotic cells were not observed in the control samples incubated at 37°C in a humidified air containing 50 mL/L CO₂ (Figure 2A). However, the morphologic observation could not unambiguously give the quantity information about the proportion of cells in different physiological states, such as viable cells, apoptotic cells and secondary necrotic cells. So a further analysis was performed for the detection of functional characteristics such as exclusion of PI or plasma membrane integrity or such dyes as Annexin V-FITC.

Apoptosis assay

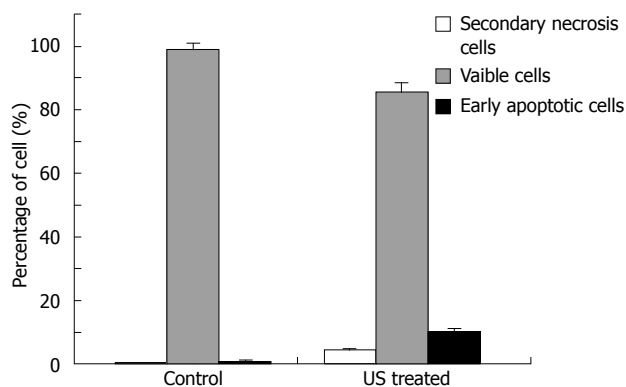
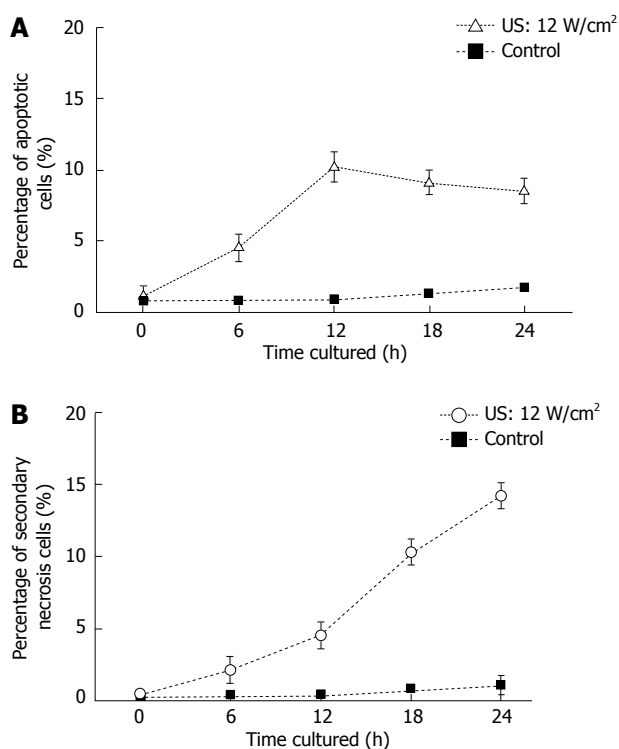
FCM with double staining of FITC-Annexin V/PI was performed to detect PS expression on the cell membrane as an end point of early apoptosis^[16], showing the exact percentage of cells in different styles, including viable cells (Annexin V/PI⁻), early apoptotic cells (Annexin V⁺/PI⁻), and secondary necrosis (Annexin V⁺/PI⁺).

The relationship between cell apoptosis and US intensity/culture time after US irradiation was investigated. The results show that the percentage of early apoptosis and secondary necrosis in the US irradiated cells increased with the increased US intensity (Figure 3). In the mean time, the percentage of viable cells decreased after US irradiation. The percentage of early apoptosis (incubated for 12 h after irradiation at the intensity of 12.0 W/cm² (I_{SPTA}) for 10 min) increased more than 10% compared with the control cells cultured at 37°C in a humidified air containing 50 mL/L CO₂ (Figure 4), suggesting that apoptosis of cells can be induced by US.

US induced early apoptosis (Figure 5A) and secondary necrosis (Figure 5B) as a function of culture time after US irradiation, are determined by FCM with double staining of FITC-Annexin V/PI. Human SGC-7901 gastric carcinoma cells were irradiated by US at the intensity of 12.0 W/cm² for 10 min in this study. Apoptotic cells increased with the increased culture time after US irradiation and reached its maximum at 12 h. Then, the percentage of apoptotic cells gradually decreased. However, the secondary necrosis cells kept increasing. The percentage of viable cells kept decreasing after US irradiation.

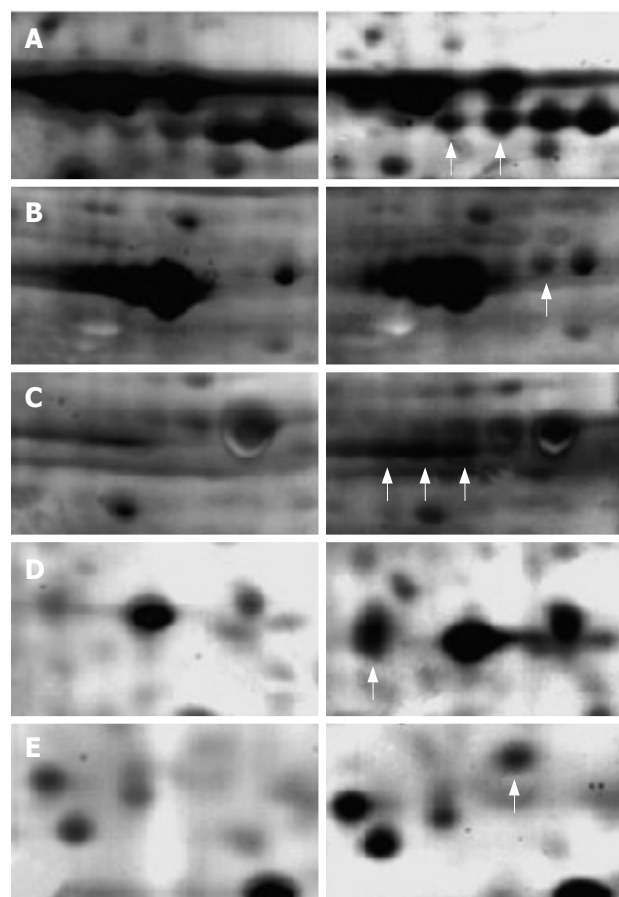
Table 1 Identified information about proteins related to US-induced apoptosis

Swiss-Prot No.	Protein name	Theo. pI/Mw	Exp. pI/Mw	Protein function
P11021	GRP78, Bip	5.2/71.0	5.1/74.4	ER-stress associated
P38646	HSP70 (Heat shock 70 kDa protein)	5.9/73.7	5.8/72.1	Activation of pre-caspase 3
P10809	CH60 (60 kDa HSP)	5.7/61.2	5.5/58.9	Tumor suppressor and pro-apoptotic protein
P27797	Calreticulin precursor	4.3/48.2	4.5/52.8	ER-stress triggered apoptosis
P35232	Prohibitin	5.6/29.9	5.4/31.3	Inhibit the tumor cell growth

**Figure 4** Percentage of viable cells, early apoptotic cells and secondary necrosis cells in the control and US-irradiated SGC-7901 cells.**Figure 5** US-induced early apoptosis (A) and secondary necrosis (B) as a function of culture time after US irradiation, determined by FCM.

2DE analysis

A total of 1137 protein spots were successfully separated from the human gastric carcinoma SGC-7901 cells, 798 of which had a good match with the proteins in the 2D map of US irradiated cells. The unmatched protein spots amounted to 339. Some of the matched proteins had obviously different expressing characteristics (more than 2 times), indicating that they are obviously up-regulated

**Figure 6** Magnified features of protein spots with different expressing characteristics after US irradiation. Arrow points to the differently expressed protein spots. A: Bip; B: HSP70; C: CH60; D: Calreticulin; E: Prohibitin.

or down-regulated after US irradiation. The protein expression between control cells (left) and US irradiated cells (right) is also shown in Figure 6.

Identification of proteins by MALDI-TOF-MS

Proteins were identified by peptide mass fingerprinting based on MALDI-TOF-MS. The identified information about the proteins shown in Figure 6 is summarized in Table 1. The expression of these proteins increased more than 2 times after US irradiation. However, 4 proteins were unmatched although with good spectra. Further identification of them is needed *via* the tandem MS/MS.

DISCUSSION

In the present study, we first demonstrated that low intensity US could induce early apoptosis and secondary

necrosis in gastric carcinoma cells. US irradiation could trigger apoptosis in both carcinoma and normal cells. However, a series of investigations showed that malignant cells are much more susceptible to US exposure and more prone to being killed than normal cells^[17,18]. Lagneaux *et al.*^[4] also demonstrated that carcinoma cells are sensitive to ultrasonic treatment with a high percentage of apoptotic cells observed 5 h after treatment, whereas ultrasonic treatment has no effect on normal cells isolated from leukemic patients. This characteristic of carcinoma cells gives the chance to develop US irradiation as a new therapeutic approach to cancer treatment. Furthermore, during the US irradiation, focused US oriented by targeted multi-functional US contrast agents (UCA), could aim at the targeted region, decreasing side-effects on normal tissues around it. In addition, Gene regulation of target cells may be utilized in modifying cellular response to US treatment, thus increasing the sensitivity of diseased cells while making normal cells resistant to the side effects^[19]. Therefore, US-induced apoptosis, as a novel cancer therapy, is of practical importance.

Low intensity US-induced apoptosis in gastric carcinoma cells may be an early event that increased with time in this study. The culture time for apoptotic cells to reach their maximum apoptosis is 12 h. Another study on US-induced apoptosis of human hepatocarcinoma cells performed in our laboratory (data not shown) showed that the culture time is 6 h after treatment. This discrepancy may be due to the different sensitivities of carcinoma cells to apoptosis-inducing factors. Similar results have also been reported by other investigators^[5,20], suggesting that the sensitivity of carcinoma cells to US irradiation is related to the time when apoptotic cells reach their maximum.

Apoptosis is always a special cellular response to stress stimuli, such as exposure to chemotherapeutic drugs, oxidative stress, free radicals, X-rays, ultraviolet radiation and shear stress. US irradiation belongs to the physical stress factor. Ashush *et al.*^[3] first reported that apoptosis is induced by high-intensity pulsed US in human leukemia cell lines, Lagneaux and Meulenaer demonstrated that low intensity US could also trigger apoptosis in human leukemia cell lines and low intensity US-induced apoptosis in tumor cells could probably offer a new approach to the elimination of cancer^[4]. Feril *et al.*^[6] also pointed out that US without increasing its temperature and time can increase hyperthermia-induced apoptosis. It was reported that early apoptosis and necrosis induced by US are mainly caused by inertial cavitation^[5-7]. During US cavitation, collapsing bubbles could create drastic conditions in an extremely short time, with a temperature of 2000 to 5000 K and a pressure up to 1800 atm inside it. Shear forces, jets and shock waves are also produced outside the bubbles. The inertial cavitation also induces free radical formation *via* the thermal dissociation of water into $\cdot\text{OH}$ and $\cdot\text{H}$ radicals. Any kind of these stress factors from US cavitation might cause apoptosis of tumor

cells. Apoptosis may be triggered as a response to cell membrane and DNA damage induced by inertial cavitation or *via* the action of residual hydrogen peroxide^[21,22]. It is further proved when introduction of dissolved gases or UCA, OptisonTM and YM454 to the system enhanced the US-induced cell killing *in vitro*^[5,7]. Moreover, intracellular ROS generated from mitochondria rather than from extracellular ROS (directly produced by inertial cavitation in the medium), are involved in the regulation of apoptosis induced by US^[23]. However, Lagneaux and Meulenaer claimed that US-induced apoptosis is probably related to the oxidative stress^[4].

To investigate the molecular mechanism of US-induced apoptosis in carcinoma cells, comparative proteomic analysis is a novel method that was introduced in this study. Because the biologic processes are directly executed by proteins that are dynamically modified and processed at multiple levels during or after sonication, the alteration in protein profile can reflect the status of differentiation and physiological conditions of cells. Protein functional analysis can also help reveal the molecular signal processes triggered by US irradiation in tumor cells or tissues.

Apoptosis is well-known to require the activation of caspases, a group of enzymes involved in apoptotic cascade events^[24]. Apoptotic stimulus can activate apoptosis-related proteins to enter mitochondria, then induce mitochondria membrane to form pores to release molecules into cytosol, such as cytochrome C (CytC). The released molecules can activate caspase-9, which cleaves procaspase-3 to caspase-3, finally inducing apoptosis^[25,26]. The activity of different caspases was not detected in this study, but some proteins, such as CH60, related to the activation of caspases were up-regulated in the cells after sonication. CH60 is regarded as a protective protein induced by the response process of mammalian cells under stress, which promotes apoptosis by helping maturation of precaspase-3^[27,28]. Precaspase-3 is the key enzyme involved in the caspases-dependent apoptotic process, indicating that the US-induced apoptosis may be caspases-dependent. It is known that members of the heat shock protein (HSP) family function at multiple points in the apoptotic signal pathway. Sometimes, due to their cytoprotective role, they inhibit the apoptotic response by inhibiting the key steps in the apoptotic process. However, in other times, they serve as chaperones of a key signaling protein or directly promote apoptosis. Vykhodtseva reported that focused US sonication close to the thermal threshold exposures could induce apoptosis, suggesting that the mechanism leading to apoptosis is the production of HSPs^[29]. In this study, CH60, HSP70 and Bip were found belonging to the HSP family. They participate in the apoptosis induced *via* different ways. Bip and calreticulin participate in the press-induced reactions on the endoplasmic reticulum (ER), both of them are pivotal molecules mediating ER-initiated apoptosis involved in ER stress. Prohibitin is an antiproliferative

protein and functions as a tumor suppressor. Commonly, it is involved in blocking DNA synthesis and negative regulation of cell proliferation. In US-induced apoptosis of tumor cells, the increased expression of prohibitin would help inhibit the growth of tumor cells.

In summary, low intensity US can induce apoptosis in human gastric carcinoma cells. The percentage of early apoptosis and secondary necrosis in the US-irradiated cells are increased with increased US intensity; Moreover, apoptotic cells increase with increased culture time after US irradiation and reach their maximum at about 12 h. Group of HSPs participates in the US-induced apoptosis. US-induced apoptosis is caspases-dependent, and ER stress-triggered apoptosis may also contribute to it. Moreover, experimental system can also be introduced into the research of bio-effects caused by other physical and chemical factors on tumor cells, which would be significant in investigating tumor cell apoptotic mechanism and cancer therapy.

COMMENTS

Background

Low intensity ultrasound (US) irradiation could trigger apoptosis in carcinoma cells, and therefore has distinct potential as a technique for cancer therapy. To investigate US-induced apoptosis in different carcinoma cells is significant to better understand its mechanism and develop a new therapeutic approach.

Research frontiers

The present study demonstrated low intensity US-induced apoptosis in gastric carcinoma cells. Based on protein functional analysis, the molecular mechanism of US-induced apoptosis is suggested.

Innovations and breakthroughs

Some previous studies have proved that US can induce apoptosis in human leukemia cells and solid carcinoma cells. The present study first demonstrated that low intensity US could induce early apoptosis and secondary necrosis in gastric carcinoma cells. The effects of US intensity and culture time after treatment on US-induced apoptosis are revealed. The potential molecular mechanism of US-induced apoptosis is suggested via comparative proteomic analysis.

Applications

The present study would help to make better analysis of US-induced apoptosis in gastric carcinoma cells, and promote new therapeutic schemes. Further investigation of its mechanism would supply more useful information about US-induced apoptosis in solid carcinoma cells.

Peer review

The authors demonstrated that low intensity US could induce early apoptosis and secondary necrosis in gastric cell line SGC-7901. As suggested by the authors, low intensity US irradiation may help to develop new cancer therapies. Therefore, this study may provide important information about gastric cancer.

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COLORECTAL CANCER

Prognostic significance of BMP and activin membrane-bound inhibitor in colorectal cancer

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Abstract

AIM: To investigate the clinical significance of BMP and activin membrane-bound inhibitor (BAMBI) which is a pseudoreceptor of transforming growth factor-beta (TGF- β) type I receptors and acts as a negative regulator of TGF- β signaling and expression aberrantly elevated in colorectal cancers (CRCs). We studied BAMBI expression in CRCs.

METHODS: We studied BAMBI expression in 183 surgically resected CRCs by immunochemical and immunoblotting analyses using a generated monoclonal anti-BAMBI antibody. Commercially available anti- β -catenin and anti-p53 antibodies were also applied for immunochemical analyses as a comparison control.

RESULTS: Immunohistochemical analysis revealed that BAMBI expression was observed in 148 (80.8%), and strong BAMBI expression was observed in 46% of the CRCs. Strong BAMBI expression was positively correlated with histological type, depth of invasion, lymph node metastases, and tumor node metastasis (TNM) stage ($P < 0.05$). Clear associations were found between BAMBI and β -catenin ($P = 0.035$) and p53 ($P = 0.049$) expression. In curatively resected CRC, 5-year recurrence-free survival was 51.9% ($P = 0.037$) for strong BAMBI expression compared to 79.8% for weak BAMBI expression. In the Cox's multivariate analysis, lymph node metastases (RR 6.685; $P < 0.001$) and depth of invasion (RR 14.0; $P = 0.013$) were significant indicators for recurrence, and strong BAMBI expression (RR 2.26; $P = 0.057$) tended to be significant.

CONCLUSION: BAMBI was linked to a potentially aggressive tumor phenotype and predicted tumor recurrence and cancer-related death in CRC. BAMBI expression might be applicable in the routine clinical setting of CRC.

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Key words: BMP and activin membrane-bound inhibitor; Colorectal cancer; Transforming growth factor-beta signal; Prognosis; Wnt signal

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INTRODUCTION

Activation of the Wnt/ β -catenin pathway is a critical process in the malignant transformation of colonic

epithelium^[1-5]. Wnt signaling promotes the stabilization and accumulation of β -catenin, which in turn interacts with the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and activates the transcription of downstream genes such as *c-Myc*, *cyclin D1*, and *Axin2*^[6-10]. Constitutive activation of β -catenin-TCF-mediated transcription is believed to be a critical step in the tumorigenesis of various cancers^[11-13]. Conversely, the transforming growth factor-beta (TGF- β) pathway participates in tumor suppressor activities such as growth inhibition and apoptosis, and this negative regulation is lost during colorectal carcinogenesis, with mutations in the type II receptor, SMAD2, and SMAD4^[14-17]. TGF- β also mediates tumor-promoting effects such as growth stimulation, as well as increases in motility, invasion, and metastasis through either differential effects on tumor and stromal cells or a fundamental alteration in the TGF- β responsiveness of the tumor cells themselves^[18-20].

The TGF- β and Wnt/ β -catenin signaling pathways cross talk to regulate tumor biology^[20]. Axin, a negative regulator of the Wnt pathway, activates TGF- β signaling through Smad3 binding^[21], while TCF/LEF interacts directly with Smad3 to regulate the expression of their target genes^[22]. It is also known that β -catenin and TCF/LEF both interact directly with Smad4 to regulate target genes during development^[23].

BMP and activin membrane-bound inhibitor (BAMBI) is a transmembrane glycoprotein induced by BMP signaling^[24] that is related to TGF- β -type I receptors, but lacks an intracellular kinase domain^[25]. BAMBI inhibits TGF- β signaling by forming a heterodimer with TGF- β -type II receptors^[25]. Previously, we found that both Wnt/ β -catenin signaling and TGF- β signaling activate transcription of *BAMBI* and that *BAMBI* expression is aberrantly elevated in most colorectal cancers (CRCs)^[26]. To analyze the clinical significance of BAMBI, we studied its expression in CRC using immunohistochemical staining. We show that BAMBI overexpression is correlated with aggressive tumor phenotypes and predicts tumor recurrence and cancer-related death in CRC. BAMBI may be usable as a target for diagnostic and antibody medicine.

MATERIALS AND METHODS

Materials

Colorectal tumor tissues were obtained from 183 consecutive patients who underwent to surgical resection between January 1995 and July 2006 at Gunma University Hospital. All of the patients underwent to radical colorectal resection intended to obtain clear pathological margins and regional lymphadenectomy, even in Stage IV. The clinicopathological features of the patients are shown in Table 1. The subjects were 115 males and 68 females with a mean age of 66 years (range 27-95). Of these, 113 were colon and 70 were rectal cancers. The tumor tissues were staged pathologically according to the American Joint Committee on Cancer

Table 1 Baseline characteristics and clinicopathological classification

Characteristic	Category	Data	
Age	Mean (yr)	66	
	Range	27-95	
Gender	Male	n = 115	62.8%
	Female	n = 68	37.2%
Location ¹	Right side	61	33.3
	Left side	52	28.4
	Rectum	70	38.3
Tumor size (mm)	Mean	45.1	
	Range	10-110	
	≤ 39	74	40.4
	40-59	62	33.9
	≥ 60	47	25.7
Depth of invasion ²	T0	7	3.8
	T1	18	9.9
	T2	22	12.0
	T3	129	70.5
	T4	7	3.8
Histology ³	G1	51	27.9
	G2	116	63.4
	G3	16	8.7
TNM stage	I	40	21.8
	II	51	27.9
	III	53	29.0
	IV	39	21.3

¹The colon was divided into the right and left sides at the splenic flexure; ²According to the TNM classification; ³G1: Well-differentiated adenocarcinoma; G2: Moderately differentiated adenocarcinoma; G3: Poorly differentiated adenocarcinoma.

Tumor-Node-Metastasis (TNM) classification. The tumors were categorized according to the World Health Organization (WHO) classification as well differentiated (G1; 51 cases, 27.9%), moderate (G2; 116 cases, 63.4%), and poor (G3; 16 cases, 8.7%).

Anti-BAMBI antibodies were generated by immunizing mice with a fragment comprising either amino acids 45-147 or 177-241. Known methods were used to prepare the anti-BAMBI antibody, collect antibody-producing cells, obtain cell fusion, select and clone hybridomas, collect and purify monoclonal antibodies^[26]. Twenty-six mouse monoclonal anti-BAMBI antibodies were generated at the first screening. To check the utility of immunostaining for paraffin sections, these monoclonal antibodies were further screened using various immunochemistry methods, using formalin-fixed and paraffin-embedded colon cancers. Finally, one monoclonal antibody was selected and its specificity was confirmed in an absorbed test using excess GST-BAMBI protein and in the immunoblotting analysis^[26].

Methods

Frozen samples (25 CRCs and 5 tubular adenomas were picked at random) kept at -80°C were thawed, cut into small pieces, and homogenized in SDS lysis buffer (Sigma-Aldrich, St. Louis, MO). The homogenate was then centrifuged at 10000 g for 15 min at 4°C, and the protein concentration of the supernatant was estimated using the BCA protein assay kit (Pierce, Rockford, IL).

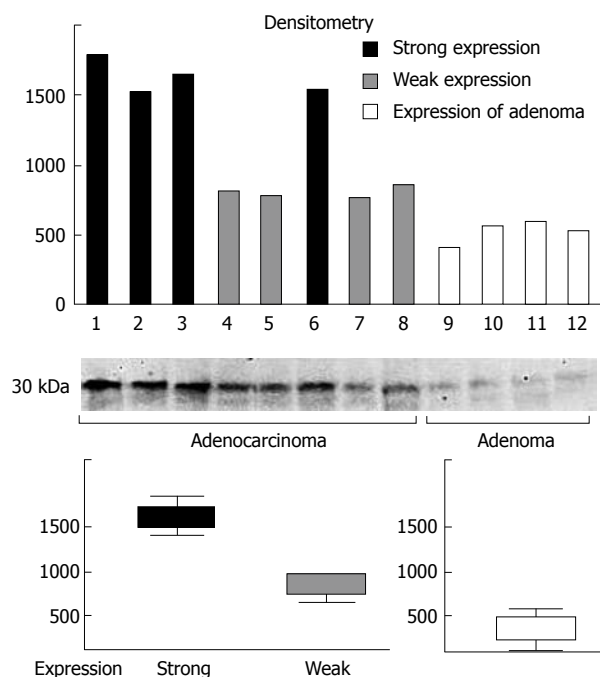


Figure 1 Immunoblotting analysis using anti-BAMBI monoclonal antibody detected BAMBI as a 30 kDa protein in all of the CRC and tubular adenoma specimens. No.1 to 3 and 6: Strong expression; No. 4, 5, 7 and 8: Weak expression. The levels of BAMBI expression in CRCs were higher than those in tubular adenomas (lanes No. 9 to 12). The quantitative analysis using densitometry of the bands of BAMBI protein revealed that the band intensity in the weak and strong expression groups in CRCs were about two and three times higher than that in tubular adenomas, respectively.

Twenty micrograms of protein from each supernatant was electrophoresed on 7.5% SDS polyacrylamide gel under reducing conditions and electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After treatment with blocking solution, the membrane was incubated with anti-BAMBI antibody (dilution 1:1000) overnight at 4°C. The membrane was then treated with goat anti-mouse IgG conjugated with horseradish peroxidase (Dako, Carpinteria, CA, dilution 1:200 000) for 1 h at room temperature, and the enhanced chemiluminescence kit (ECL Advance detection kit, Amersham, Piscataway, NJ) was used for development according to the manufacturer's instructions. The luminescence image was captured and digitized using Lumi Analyst 3.0v (Boehringer Mannheim GmbH, Germany), and the integrated densities of each band were quantified using densitometry software (ImageJ 1.37v, National Institutes of Health, Bethesda, MD). To examine the equality of protein loading, the total amount of protein loaded in each lane was examined by staining with Coomassie blue.

For the immunohistochemical study, 3 μ m thick sections were cut from formalin-fixed, paraffin-embedded tissue blocks containing representative tumor histology. The sections were de-waxed in xylene, rehydrated with graded ethanol, and subjected to an antigen retrieval step. For antigen retrieval, the slides for β -catenin and p53 were placed in 0.01 mol/L citrate buffer, 20% ZnSO₄ solution, and heated in a microwave processor (Energy Beam Science, East Granby, CT) at

95°C for 20 min. They were then cooled and rinsed in 0.01 mol/L phosphate-buffered saline (PBS), pH 7.4. The antigen retrieval was not performed on the immunohistochemistry for BAMBI. The slides were then reduced with nonspecific antibody binding by incubating the sections with 10% normal serum in PBS for 30 min at room temperature. After decanting the excess serum, the sections were incubated overnight at 4°C with each monoclonal antibody for BAMBI (1:2000 dilution), β -catenin (1:1600 dilution), and p53 (1:50 dilution) in humidified chambers. After washing thoroughly with PBS, the sections were set in a Ventana Nexus Automated Stainer (Ventana Medical Systems, Tucson, AZ) for immunostaining. The automated protocol was performed according to the manufacturer's instructions, which were based on the labeled streptavidin-biotin method and used the I-VIEW DAB universal kit (Ventana Medical Systems); the kit included a universal biotinylated IgG secondary antibody (anti-mouse), avidin horseradish peroxidase, DAB, 0.03% H₂O₂, and 0.5% CuSO₄. After immunostaining, the sections were lightly counterstained with hematoxylin, and then mounted in a carousel inside the staining module and run to completion. To standardize and confirm the immunohistochemical evaluation of BAMBI, 26 samples of colon carcinoma with tubular adenoma were collected and were tentatively subjected to immunohistochemistry using paraffin sections. Immunohistochemical staining intensity was categorized blindly by two pathologists (T.N, S.S). Most tubular adenomas showed weak immunoreactivity compared to the cancer component.

Therefore, diffuse BAMBI immunoreactivity, which was observed in the cytoplasm and cell membrane, but not in the nucleus, was evaluated as weak expression if the positivity was similar to that of tubular adenomas. If the BAMBI immunoreactivity was far stronger than that of tubular adenomas, it was evaluated as strong expression (Figures 1 and 2). No BAMBI immunoreactivity was evaluated as negative.

The intensity of nuclear β -catenin immunostaining was graded semiquantitatively into four categories: negative, weakly positive, moderately positive, and strongly positive^[27]. This evaluation was grouped into two categories: low expression (negative and weakly positive) and high expression (moderately to strongly positive). To evaluate the p53 immunostaining, the nuclear immunoreactivity for p53 was counted in five random tumor areas at high magnification ($\times 400$), and the average percentage of these positive cells was calculated. The p53 immunoreactivity was evaluated as negative or positive expression if the percentage of nuclear positivity was less than or more than 70%, respectively^[28].

Statistical analysis was performed using SPSS 11.5 software (SPSS Japan, Tokyo, Japan). The Mann-Whitney U-test, Kruskal-Wallis test, and Spearman's coefficient of rank correlation were used to examine the correlation of BAMBI with other protein and pathological factors. Recurrence-free and overall survival curves were generated using the Kaplan-Meier method,

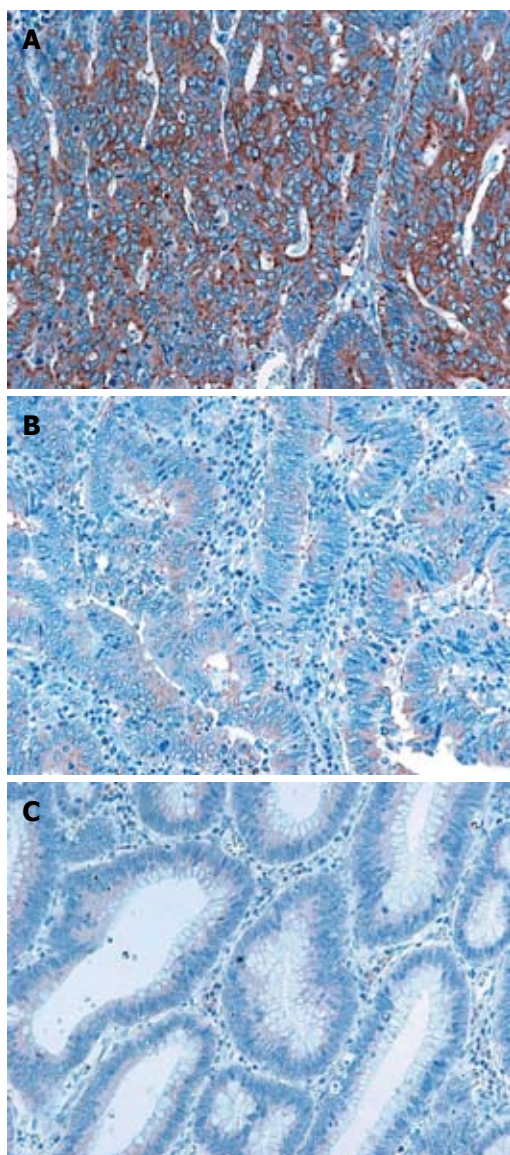


Figure 2 Examples of the immunostaining of BAMBI at strong expression (A), weak expression (B) levels, and expression of adenoma (C) (x 200). The immunohistochemical expression level correctly reflected the expression level of BAMBI protein in CRC tissues.

and the log-rank test was used to compare the curves. Deaths without recurrence were included as censored observations. The relative risk and confidence limits were estimated for each variable using the Cox univariate model, using the most suitable prognostic category as the referent group. A multivariate Cox proportional hazard model was also developed using stepwise regression (forward selection) with predictive variables. The limits for entry and removal were both $P < 0.5$. Statistical significance was set at $P < 0.05$.

RESULTS

Immunoblotting analysis

Immunoblotting analysis using anti-BAMBI monoclonal antibody detected BAMBI as a 30-kDa protein in all of the CRC and tubular adenoma specimens. The levels of BAMBI expression in CRCs were higher than those

in tubular adenomas (Figure 1). By contrast, no specific band was detected in normal colonic epithelium (data not shown). The quantitative analysis of the bands revealed that the band intensity was divided into two groups: strong expression and weak expression was three and two times higher than that of tubular adenoma, respectively (Figure 1). Furthermore, CRC tissues with strong expression in immunoblotting were strongly positive for BAMBI immunohistochemistry, whereas other CRC tissues with weak expression revealed weak immunoreactivity to BAMBI (Figure 2). These results indicate that the immunohistochemical expression level correctly reflected the level of BAMBI protein expressed in CRC tissues.

Immunohistochemically, no BAMBI expression was detected in normal colonic epithelium. In contrast, diffuse BAMBI immunoreactivity was observed in the cytoplasm and cell membrane of tubular adenoma and CRC cells (Figure 3). We observed BAMBI immunoreactivity in 148 (80.8%) of 183 CRC tissues, and weak and strong BAMBI expression was observed in 80 (54.1%) and 68 (45.9%) CRCs, respectively.

Nuclear β -catenin immunostaining was not observed in normal colonic mucosa. Nuclear β -catenin immunoreactivity was observed in 154 (84.1%) of 183 CRCs. Nuclear accumulation of p53 was observed in 74 (40.4%) of 183 CRCs (data not shown).

Relationship between BAMBI expression and clinicopathological factors

Strong immunohistochemical expression of BAMBI was positively correlated with histological type ($P = 0.023$), depth of invasion ($P = 0.021$), the presence or absence of lymph node metastases ($P = 0.035$), and TNM staging ($P < 0.001$), whereas no correlations were found with gender, age, location, or tumor size (data not shown).

Correlation between BAMBI and β -catenin/p53 expression

We evaluated the correlation of immunohistochemical BAMBI expression with immunohistochemical β -catenin and p53 expression. Strong expression of BAMBI was significantly associated with β -catenin ($P = 0.035$) and p53 ($P = 0.049$) expression (Table 2).

Recurrence-free and overall survival according to BAMBI expression

The Stage IV patients who underwent to macroscopic resection of metastasis with no residual disease were excluded from the recurrence-free and overall survival groups and multivariate analysis. The patients who died from non-cancer-related causes were excluded from the recurrence-free survival and overall survival groups. In all, 157 of 183 patients were analyzed with a mean follow-up period of 33.7 mo (range, 2-137). In the background analysis, histological type ($P = 0.043$) and TNM staging ($P = 0.035$) were significant in strong BAMBI expression compared to weak BAMBI expression. The tumor depth ($P = 0.054$) tended to be

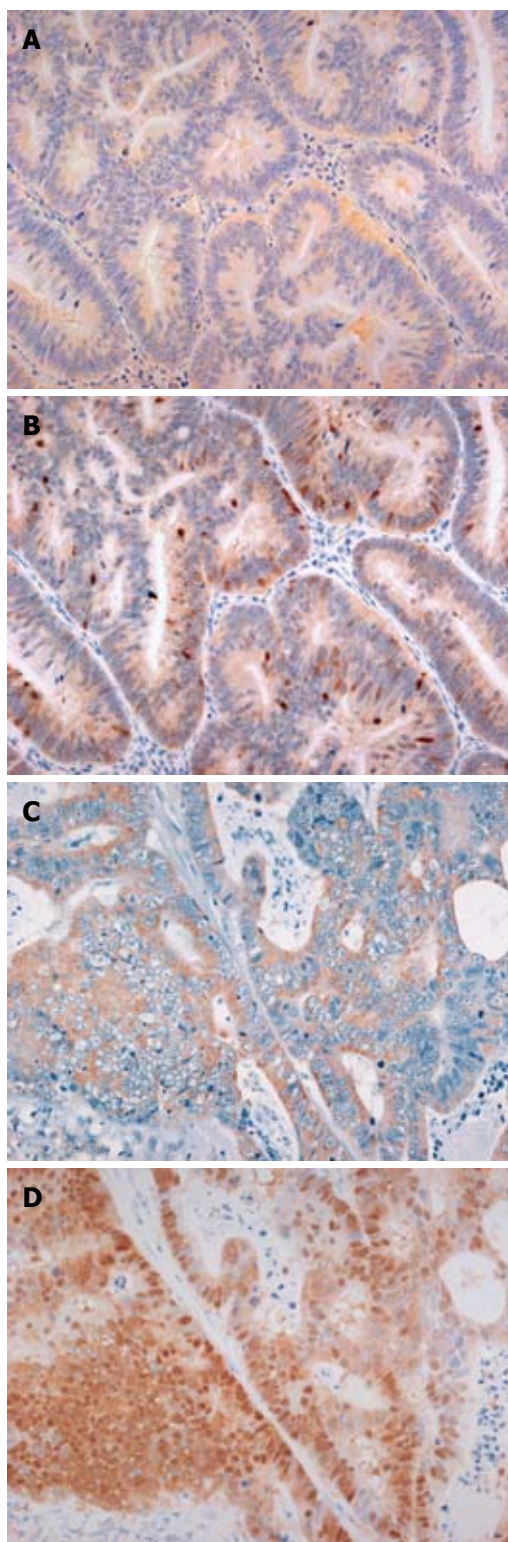


Figure 3 BAMBI and β -catenin expression in adenoma and cancer (x 200). Adjacent sections of an adenoma showing weak BAMBI expression in the cytoplasm and cell membrane (A) and weak nuclear localization of β -catenin (B); adjacent sections of a tumor showing strong BAMBI expression in the cytoplasm and cell membrane (C) and strong nuclear localization of β -catenin (D).

significant in strong BAMBI expression (Table 3). The cancer recurred in 30 patients, and 20 patients died of cancer-related causes.

No significant differences were observed in the recurrence-free and overall survival at each stage

Table 2 Correlation between BAMBI expression and β -catenin/p53 expression

			BAMBI expression ¹		<i>P</i> ²
			Weak (<i>n</i>)	Strong (<i>n</i>)	
β -catenin ³	Low	Negative	8	6	0.035
		Weak	19	10	
	High	Moderate	24	14	
p53 ³	Negative	Strong	29	38	0.049
			55	36	
	Positive		25	3	
			80 (54.1%)	68 (45.9%)	

¹BAMBI expression was scored as described in the Methods; ²Mann-Whitney test; *P* < 0.05 was considered statistically significant; ³ β -Catenin/p53 expression were scored as described in the Methods.

Table 3 BAMBI expression in human colorectal carcinomas after curative surgery: Correlations with clinicopathological parameters

		BAMBI expression				<i>P</i> ¹
		Weak		Strong		
		<i>n</i>	%	<i>n</i>	%	
Gender	Male	41	26.1	11	7.1	NS
	Female	69	43.9	36	22.9	
Age	27-59	60	38.2	24	15.4	NS
	60-95	50	31.8	23	14.6	
Location	Right side	37	23.6	12	7.6	NS
	Left side	27	17.2	16	10.2	
Tumor size (mm)	Rectum	46	29.3	19	12.1	NS
	≤ 39	50	31.8	19	12.1	
	40-59	35	22.3	15	9.6	
	≥ 60	25	15.9	13	8.3	
Depth of invasion	Tis, T1, T2	38	24.2	9	5.7	0.054
	T3, T4	72	45.9	38	24.2	
Histology	G1	36	22.9	9	5.7	0.043
	G2	67	42.7	32	20.4	
	G3	7	4.5	6	3.8	
LN metastases ²	No	59	37.6	22	14.0	NS
	Yes	51	32.5	25	15.9	
TNM stage	I	33	21.0	7	4.5	0.035
	II	35	22.3	16	10.2	
	III	35	22.3	18	11.4	
	IV	7	4.5	6	3.8	
Metastatic site						
Liver	No	2	28.6	5	83.3	NS
	Yes	5	71.4	1	16.7	
Lung	No	6	85.7	6	100	NS
	Yes	1	14.3	0	0	
Distant lymph nodes	No	7	100	6	100	NS
	Yes	0	0	0	0	

¹Kruskal-Wallis or Mann-Whitney test; *P* < 0.05 was considered statistically significant; ²Lymph node (LN) metastases. NS: Not significant.

according to the BAMBI expression level. However, the 5-year recurrence-free survival rate differed significantly (*P* = 0.037); it was 51.9% with strong BAMBI expression compared to 79.8% for weak BAMBI expression (Figure 4A). The 5-year overall survival rate was 61.0% for strong BAMBI expression and 75.0% for weak BAMBI expression (*P* = 0.495). No significant differences were detected in recurrence-free survival according to β -catenin (low *vs* high: 77.2% *vs* 65.9%, *P* = 0.109) and p53 (negative *vs* positive: 75.8% *vs* 61.1%, *P* =

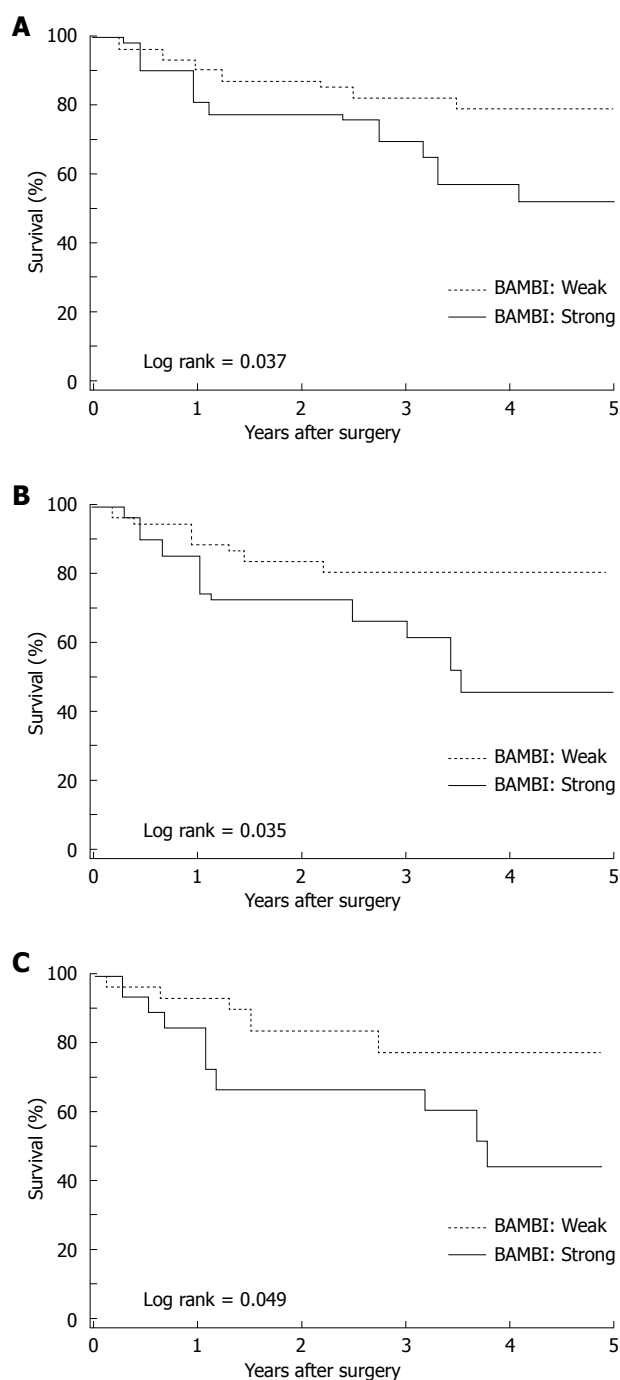


Figure 4 **A:** The 5-year recurrence-free survival rate differed significantly ($P = 0.037$) and was 51.9% for strong BAMBI expression compared to 79.8% for weak BAMBI expression; **B:** The impact of the coexpression of BAMBI and β -catenin on the recurrence-free survival with high β -catenin expression, the 5-year recurrence-free survival was 46.9% for strong BAMBI expression compared to 80.8% for weak BAMBI expression ($P = 0.035$); **C:** The impact of the coexpression of BAMBI and p53 on the recurrence-free survival with positive p53 expression, the 5-year recurrence-free survival rate was 43.4% for strong BAMBI expression compared to 78.2% for weak BAMBI expression ($P = 0.049$).

0.417) expression.

Furthermore, we evaluated the impact of the coexpression of BAMBI, β -catenin, and p53 on recurrence-free survival. With high β -catenin expression, the 5-year recurrence-free survival was 46.9% for strong BAMBI expression compared to 80.8% for

weak BAMBI expression ($P = 0.035$; Figure 4B). Weak β -catenin expression did not affect the survival rate according to the BAMBI expression level (weak *vs* strong: 75.0% *vs* 79.2%, $P = 0.6294$). With positive p53 expression, the 5-year recurrence-free survival rate was 43.4% for the strong BAMBI expression group compared to 78.2% for the weak BAMBI expression group ($P = 0.049$; Figure 4C). With negative p53 expression, the 5-year recurrence-free survival rate was 62.4% with strong BAMBI expression *versus* 80.8% for weak BAMBI expression ($P = 0.582$).

Cox's proportional hazards regression models

The relative risk and confidence limits for recurrence-free survival were estimated for each variable using the Cox univariate model. TNM staging (relative risk 4.63; 95% CI 2.84-7.56; $P < 0.001$), presence or absence of lymph node metastases (relative risk 8.937; 95% CI 3.10-25.77; $P < 0.001$), depth of invasion (relative risk 14.77; 95% CI 2.01-108.55; $P = 0.008$), and strong BAMBI expression (relative risk 2.109; 95% CI 1.029-4.323; $P = 0.041$) were significant. In the multivariate Cox proportional hazard model, TNM stage (relative risk 4.35; 95% CI 2.65-7.14; $P < 0.001$) was the only significant indicator for recurrence. According to Spearman's coefficient of rank correlation, BAMBI expression was significantly correlated with TNM staging ($P = 0.038$) and the nuclear expression of β -catenin ($P = 0.01$). In the model excluding TNM stage and the nuclear expression of β -catenin, lymph node metastases (relative risk 6.685; 95% CI 2.24-19.93; $P < 0.001$), depth of invasion (relative risk 14.01; 95% CI 1.74-112.69; $P = 0.013$), and strong BAMBI expression (relative risk 2.26; 95% CI 0.95-5.38; $P = 0.057$) were significant indicators of recurrence (Table 4).

DISCUSSION

BAMBI was expressed in 80% of CRC tumors, and strong BAMBI expression was observed in 46% of CRC tumors. The region with BAMBI expression matched the region of β -catenin expression, and the expression of BAMBI and β -catenin were correlated, consistent with the fact that β -catenin is responsible for the aberrant expression of BAMBI^[26]. In addition, the association of BAMBI expression and p53 expression is consistent with a previous report that p53 induces Siah-1, which mediates β -catenin degradation^[29]. The overexpression of BAMBI inhibits the response of tumor cells to TGF- β signaling, and interferes with TGF- β -mediated growth arrest *in vitro*^[25,26]. These results indicate that BAMBI expression is one of the critical early genetic events in CRC tumorigenesis.

Strong BAMBI expression was more frequently associated with deep tumor penetration, lymph node metastases, and advanced TNM stage. Patients with strong BAMBI expression had shorter recurrence-free and overall survival, and strong BAMBI expression was an independent factor predicting a lower recurrence-free survival in the multivariate analysis. The nuclear

Table 4 Relative risks of prognosis according to proportional-hazards regression models

Characteristics	Category	Univariate analysis			Multivariate analysis		
		Hazard ratio	P	95% CI	Hazard ratio	P	95% CI
Depth of invasion	Tis, T1, T2/T3, T4	14.773	0.008	2.011-108.548	14.008	0.013	1.741-112.694
LN metastases	Yes/No	8.937	< 0.001	3.100-25.768	6.685	< 0.001	2.242-19.927
TNM stage	0/ I / II / III / IV	4.633	< 0.001	2.838-7.563			
BAMBI	Weak/Strong	2.109	0.041	1.029-4.324	2.265	0.057	0.952-5.385
β -Catenin	Low/High	1.944	0.108	0.864-4.377			
p53	Negative/Positive	1.702	0.147	0.830-3.489	0.937	0.871	0.427-2.057

accumulation of β -catenin contributes to the transactivation of target genes, which encode regulators of differentiation and effectors supporting invasion and metastasis (CD44, laminin-52, matrix metalloproteinase MMP-7 and MT1-MMP) and angiogenesis (vascular endothelial growth factor), among others^[5]. In fact, the overexpression of nuclear β -catenin is reported to be an independent predictor of short patient survival time^[30-33]. Inconsistent with these reports, we showed that nuclear β -catenin accumulation alone does not affect survival and is not a prognostic factor in the univariate and multivariate analyses. However, we found that patients with both strong BAMBI expression and the overexpression of β -catenin have poorer survival compared to those without β -catenin expression. Smad activation or expression is lost in approximately 10% of CRC occurrences, and these patients have a poor prognosis because of its association with advanced disease and the presence of lymph node metastases at diagnosis^[34]. BAMBI expression allows tumor cells to escape from TGF- β signaling and activate oncogenic processes such as growth stimulation, increases in motility, and invasion. Therefore, BAMBI plays an important role in the invasiveness and metastatic potential of colon cancers through cross-talk with the Wnt and TGF- β signaling pathways.

Although the available data on the correlation of p53 status and the prognosis of colorectal cancers are controversial^[35], we found that p53 expression status did not influence the recurrence-free and overall survival and was not a prognostic factor in the univariate and multivariate analyses. Nevertheless, patients with both strong BAMBI expression and p53 overexpression had lower survival compared to those without p53 expression. We speculate that target genes induced by BAMBI overexpression may complement or cooperate with those induced by p53 inactivation and thereby contribute to poorer survival.

Although CRC prognosis is stage and grade dependent, cancers with similar clinicopathological features may have major differences in outcome. A large proportion of patients with Stage II disease and some with Stage III disease can be cured by surgery alone and do not derive any benefit from adjuvant therapy^[36]. Therefore, the identification of robust molecular prognostic markers to supplement conventional pathological staging systems is highly desirable. Several molecular markers are promising prognostic predictors for the outcome in CRC, including deleted in colorectal

cancer (DCC), microsatellite instability (MSI), and loss of heterozygosity at 18q^[37,38]. CRC patients with strong BAMBI expression had more aggressive disease and their recurrence-free survival was markedly shorter. In contrast, patients with weak BAMBI expression had less aggressive disease and significantly longer recurrence-free survival. Strong BAMBI expression was tending to be an independent molecular predictor for survival secondary to lymph node metastasis and depth of tumor in the Cox hazard model. Therefore, strong BAMBI expression might be applicable in clinical practice to predict recurrence in addition to lymph node metastasis and depth of tumor.

In clinical scenarios, targeting the TGF- β signaling pathway for chemoprevention and the treatment of human cancers should decrease or abrogate TGF- β signaling, particularly for advanced or metastatic disease^[20]. Given that the TGF- β signaling pathway has a important role in tumor-induced immunosuppression^[39], inhibitors of this pathway may be used to improve natural immunosurveillance of tumor cells or to enhance the effectiveness of active or passive immunotherapy strategies. Our findings suggest that the development of a new active monoclonal anti-BAMBI antibody may offer a great improvement in survival of CRC patients and might also serve as a diagnostic tool for CRC prognosis.

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COMMENTS

Background

In generally, BMP and activin membrane-bound inhibitor (BAMBI) expression is aberrantly elevated in most colorectal cancers (CRCs). However, few studies are reported on BAMBI expression in colorectal tissue. To analyze the clinical significance of BAMBI, authors studied its expression in CRC using immunohistochemical staining. They show that BAMBI overexpression is correlated with aggressive tumor phenotypes and predicts tumor recurrence and cancer-related death in CRC. This study was to map BAMBI expression in colorectal tissue and analyze the relationship between BAMBI expression and CRC prognosis.

Research frontiers

To analyze the clinical significance of BAMBI, authors studied its expression in CRC using immunohistochemical staining. They show that BAMBI overexpression is correlated with aggressive tumor phenotypes and predicts tumor recurrence and cancer-related death in CRC. BAMBI may be usable as a

target for diagnostic and antibody medicine.

Innovations and breakthroughs

The results of this study show that BAMBI expression plays a role in the pathogenesis of colorectal cancer.

Applications

The expression level of BAMBI plays an important role in the pathogenesis of colorectal cancer. The development of a new active monoclonal anti-BAMBI antibody may offer a great improvement in survival of CRC patients and might also serve as a diagnostic tool for CRC prognosis.

Peer review

This is an extremely well written and researched paper. It discovers yet another marker of prognosis for CRC.

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Irritable bowel syndrome subtypes differ in body awareness, psychological symptoms and biochemical stress markers

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CONCLUSION: IBS subtypes showed different profiles in body awareness, somatic and psychological symptoms and in biochemical variables. D-IBS differed compared to the other groups by lowered body awareness, less psychological symptoms and a higher sense of coherence and elevated C-peptide values. C-IBS and A-IBS subtypes suffered more from depression and anxiety, associated with a lower quality of life. These differences may be important and will be taken into account in our treatment of these patients.

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Key words: Irritable bowel syndrome subtypes; Physiotherapy; Body awareness; Stress; Biochemistry

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Abstract

AIM: To elucidate the differences in somatic, psychological and biochemical pattern between the subtypes of irritable bowel syndrome (IBS).

METHODS: Eighty IBS patients, 30 diarrhoea predominant (D-IBS), 16 constipation predominant (C-IBS) and 34 alternating IBS (A-IBS) underwent physiotherapeutic examinations for dysfunctions in body movements and awareness and were compared to an apparently healthy control group (AHC). All groups answered questionnaires for gastrointestinal and psychological symptoms. Biochemical variables were analysed in blood.

RESULTS: The D-IBS group showed less body awareness, less psychological symptoms, a more normal sense of coherence and psychosocial rating as well as higher C-peptide values. C-IBS had a higher degree of body dysfunction and psychological symptoms, as well as the lowest sense of coherence compared to controls and D-IBS. They also demonstrated the most elevated prolactin levels. A-IBS had the lowest degree of body disturbance, deteriorated quality of life and affected biochemical pattern. All subtypes had higher pain scores compared to controls. In addition they all had significantly increased triglycerides and elevated morning cortisol levels, however, without statistical significance compared with the controls.

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INTRODUCTION

Irritable bowel syndrome (IBS) is considered to be the most common of all gastrointestinal dysfunctions^[1-4]. Patients with IBS are suffering from a variety of gastrointestinal complaints, as well as associated symptoms like headache and dysuria. Furthermore, there is also a strong connection to fibromyalgia, chronic fatigue syndrome, anxiety, and depression^[5,6].

The understanding of IBS and especially the interaction between the central and enteric nervous systems has grown considerably over the last years^[7]. There are several studies demonstrating abnormalities in the autonomic nervous system^[8,9], whereas the expression of different biochemical parameters has been studied with somewhat diverging results^[10-14].

The division of IBS into different subgroups is based on the fact that these patients behave in dissimilar ways. According to the Rome II criteria, building on stool and defecation patterns, IBS can be divided into diarrhoea

predominant (D-IBS), constipation predominant (C-IBS) and alternating (A-IBS) subtypes^[15,16]. Although, lately questioned, the Rome II criteria are widely used in clinical practice^[17].

When comparing the various subtypes of IBS, Whitehead *et al*^[18] did not find any disparity in colonic motility and psychological testing. In contrast, other authors have found differences in gender, abdominal discomfort/pain and psychological comorbidity between the IBS subtypes^[19-22]. Disparities in endocrine factors between the subtypes of IBS have not been extensively studied. However, Elsenbruch *et al*^[23] found a significant increase in postprandial saliva cortisol in D-IBS patients, not evident in C-IBS patients and controls. Jonsson and Theorell^[24] found that plasma cortisol correlated negatively with diarrhoea symptoms and lower prolactin values were seen in patients with functional dyspepsia.

In earlier studies, we have shown that IBS could be associated with deviated tension in the body^[13,25]. A physiotherapeutic approach to adjust and reduce pathological tension in the body is by the use of Body Awareness Therapy (BAT). This method is devoted to take care of, and improve, the patient's ability to become aware of his or her own capability by the use of self recruited resources to recapture a normal balance in the body^[26-30]. We found that 12 wk of treatment with BAT gave symptom relief of both gastrointestinal and psychological symptoms^[25]. However, patients with C-IBS were more relieved than the D-IBS and alternating types of IBS. In a second trial we treated the patients for 24 wk and satisfying effects were obtained for the entire group of IBS patients^[13].

The aim of the present study was to elucidate the differences in somatic and psychological symptoms, as well as biochemical stress markers in the IBS subtypes. The hypothesis was that these subtypes present with dissimilar symptomatic expressions.

MATERIALS AND METHODS

Study population

All patients with IBS as diagnosed by gastroenterologists, GI surgeons or GPs referred for Body Awareness Therapy at the Unit for Functional Gastroenterology, participated in the study. Patients with an acute psychiatric disease and patients not understanding the Swedish language were excluded from the study.

IBS patients, 73 women and 7 men (age, 21-65 years), with a BMI 23.3 ± 3.7 participated in the study. According to the Rome II criteria patients were divided into 3 groups: D-IBS ($n = 30$, 24 women and 6 men), C-IBS ($n = 16$, 15 women and 1 man) and A-IBS suffering from combined symptoms ($n = 34$, all women). Fifty-six IBS patients had suffered from their gastrointestinal symptoms for more than 5 years (for D-IBS 67%, for C-IBS 88% and for A-IBS 65%). There were no differences in BMI between the subtypes.

A healthy control group consisting of 18 women and 3 men (age, 21-61 years) had a BMI of 22.3 ± 2.2 . They

were free of gastrointestinal symptoms and without ongoing pharmacological treatment.

Study design

The groups of IBS test patients and the AHC group underwent complete physiotherapeutic examinations in accordance to the Body Awareness Scale (BAS). They also filled in the questionnaires GIS, SCL90, SOC, PRS, and pain drawing. Blood samples were taken from an antecubital vein. The ethics committee of the University of Göteborg approved the study. All subjects gave their written consent before acceptance of inclusion into the study.

Body examinations

BAS test is based on one hand observations by the physiotherapist of dysfunctions in defined items of basic movements (BASobs) and was carried out during video recording. In addition, standardized questions in order to measure the patients' own opinion concerning their body awareness (BASself) was performed. The variables were ranging from 0-6 where a higher score represented more symptoms^[31,32].

Questionnaires

A modified form of Gastro Intestinal Symptom questionnaire (GIS) was used^[33]. This survey evaluates 35 general gastrointestinal symptoms. A total score and scoring of specified symptoms were used. The test utilizes a seven-graded scale (0-6). A higher score means increased gastrointestinal complaints.

The Symptom Checking List questionnaire (SCL90) is a self-rating scale evaluating symptomatic behaviour of psychological state using questions related to everyday life^[34]. The questionnaire includes 90 questions. The answers score in a five-graded scale (0-4) and allow subdivision into different items. A higher score reflected more symptoms.

The Sense of Coherence Scale (SOC) measures the degree to which individuals find the world around them comprehensible and manageable and thus represents a measurement of coping skills^[35]. The questionnaire includes 29 questions. The answers score in a seven-graded scale (0-6) and allow subdivision into different items. A high SOC score is linked to successful coping with factors that induce stress and is consequently reflecting a higher health level/quality of life.

Psychosocial rating scale (PRS) (slightly modified from Headley Court psychosocial rating scale) has 33 items and score from 0-4 ranging from 'very severe problems' to 'no problems'^[36].

The distribution of pain was visualized on a pain-map, figuring the human body with a front and backside. When calculating the results, the body was divided into 45 sections^[37]. Points were given for every section where pain was marked. The points were summed up to a score.

Biochemistry in blood

Venous blood samples were taken under fasting condi-

Table 1 Body awareness scale (BAS-H)

Category	AHC (<i>n</i> = 21)		D-IBS (<i>n</i> = 30)		C-IBS (<i>n</i> = 16)		A-IBS (<i>n</i> = 34)	
	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)
BASobs								
Total	1.5	2 (2)	2.6	3 (3) ^c	2.7	3 (3) ^c	2.2	2 (4) ^{c,f,i}
Grounding	1.5	2 (2)	2.6	2 (2) ^c	2.9	3 (2) ^c	1.9	2 (2) ^{b,f,i}
Mid-line	2.1	2 (2)	3.6	4 (3) ^c	3.4	3 (2) ^c	3.0	3 (2) ^{c,d}
Centring	2.1	2 (1)	3.3	3 (2) ^c	3.4	4 (2) ^c	2.8	3 (2) ^{c,e,g}
Flow	1.5	1 (2)	2.5	3 (4) ^c	2.7	3 (3) ^c	2.4	2 (4) ^c
Respiration	1.4	1 (2)	3.9	4 (2) ^c	3.9	4 (2) ^c	3.6	4 (1) ^c
Boundaries	0.5	0 (0)	1.1	0 (2) ^c	1.1	0 (2) ^b	1.0	0 (2) ^b
BASself								
Total	1.6	2 (2)	1.8	2 (3) ^a	2.7	2 (4) ^{c,f}	2.3	2 (4) ^{c,f,h}
Grounding	0.8	0 (2)	1.0	0 (2)	1.8	2 (3) ^{c,e}	1.8	2 (4) ^{c,f}
Mid-line	1.6	2 (2)	1.8	2 (4)	3.2	4 (5) ^{c,f}	2.4	2 (4) ^{b,e,g}
Centring	1.3	0 (2)	1.3	1 (2)	2.2	2 (4) ^{a,d}	1.4	1 (2)
Flow	2.1	2 (4)	2.8	3 (5) ^a	3.8	4 (4) ^{c,e}	3.5	4 (4) ^{c,d}
Respiration	1.0	0 (2)	1.6	2 (2)	2.4	2 (3) ^{c,d}	2.9	4 (3) ^{c,f}
Boundaries	1.7	2 (3)	2.0	2 (3)	2.6	2 (4) ^{c,e}	2.0	2 (4) ^g

Results from BASobs and BASself examination score from AHC group and D-IBS, C-IBS and A-IBS patients. The results are shown in total and as items categorised. The higher score, the more symptoms. ^a*P* < 0.05, ^b*P* < 0.01, ^c*P* < 0.001 *vs* AHC; ^d*P* < 0.05, ^e*P* < 0.01, ^f*P* < 0.001 *vs* D-IBS; ^g*P* < 0.05, ^h*P* < 0.01, ⁱ*P* < 0.001, C-IBS *vs* A-IBS. M: Mean; Md: Median; IQR: Inter quartile range.

tions from an antecubital vein in the morning for analysis of C-peptide, triglycerides, prolactin and cortisol (at 8:00 and 13:00).

Statistical analysis

This study consisted of ordinal data (BAS and questionnaires) and quantitative data (biochemical parameters). A high value for BAS, GIS, SCL90, PRS, and pain drawing meant more symptoms. A high SOC value reflected a higher degree of sense of coherence. Median (Md), inter-quartile range (IQR), mean (M), standard deviation (SD) and percentage were used for presentation of data. Although median and IQR were optimal for ordinal data, means were presented as well for better visualization. Mann Whitney *U* test was used for ordinal and quantitative data^[38].

RESULTS

Study population

There was an obvious gender difference between the IBS subtypes with 20% men in the D-IBS, 6.25% in the C-IBS and 0% men in the A-IBS subgroup.

Physiotherapeutic data

BAS: All subtypes of IBS patients scored higher in the BASobs than the controls. Comparing the subtypes, the A-IBS group showed less body disturbances compared to the other two groups (Table 1). In BASself the D-IBS group expressed lower score compared to the other two groups, with levels similar to the AHC group.

Questionnaires

Gastrointestinal symptoms: All subtypes scored higher than the control group. There was no difference between the subtypes in total score. The A-IBS group

scored higher for constipation and flatulence and less for diarrhoea and motility compared to the D-IBS group. The C-IBS group scored more constipation, less motility and less diarrhoea compared to the D-IBS group (Table 2). Thus, the patients scored in accordance with their own subtype.

Psychological symptoms: The C-IBS and the A-IBS group scored higher psychological symptoms compared to the D-IBS group and all groups scored higher than the controls. Also, the C-IBS group scored more symptoms compared to the A-IBS group, especially for sensitivity, phobic anxiety, psychoticism and somatisation (Table 3).

SOC: The SOC questionnaire revealed that there were significant differences between the subtypes compared to the controls. The A-IBS group and the C-IBS group scored lower sense of coherence than the D-IBS group, which differed from healthy controls only in the total score and present time and external conditions (Table 4).

PRS: All subtypes showed lower psychosocial rating compared to the control group (Table 5). Besides, both C-IBS and A-IBS showed lower psychosocial rating/quality of life compared to the D-IBS group.

Pain: The subgroups of IBS showed higher scores of pain presented on the body drawings compared to the healthy controls. However, there were no differences in pain score for the different locations between the subtypes (Table 6).

Biochemical analysis: The D-IBS group differed from the other two subtypes and the AHC with significantly higher C-peptide values (Table 7). The C-IBS patients

Table 2 Gastrointestinal scale (GIS)

Category	AHC (n = 21)		D-IBS (n = 30)		C-IBS (n = 16)		A-IBS (n = 34)	
	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)
Total	0.4	0 (1)	1.9	1 (3) ^c	1.7	1 (3) ^c	2.0	1 (3) ^c
Pain	0.5	0 (1)	1.9	2 (2) ^c	2.0	2 (2) ^c	1.9	2 (2) ^c
Flatulence	0.5	0 (0)	2.4	2 (3) ^c	2.5	3 (3) ^c	2.9	3 (4) ^{c,e}
Nausea	0.5	0 (1)	1.5	0 (3) ^a	1.3	1 (2)	1.4	0 (2) ^a
Constipation	0.4	0 (1)	0.8	1 (1) ^a	3.8	4 (2) ^{c,f}	2.9	2 (3) ^{c,f}
Diarrhoea	0.5	0 (1)	3.2	3 (3) ^c	0.5	0 (1) ^f	2.2	2 (2) ^{c,f}
Motility	0.7	0 (1)	3.8	4 (3) ^c	2.6	2 (3) ^{c,e}	3.0	3 (3) ^{c,d}
Miscellaneous	0.3	0 (0)	1.2	1 (1) ^c	1.1	0 (1) ^c	1.2	1 (2) ^c

GIS score from AHC group, D-IBS, C-IBS, and A-IBS patients. The results are shown in total and as items categorised. The higher score, the more symptoms. ^a $P < 0.05$, ^c $P < 0.001$ vs AHC; ^d $P < 0.05$, ^e $P < 0.01$, ^f $P < 0.001$ vs D-IBS. M: Mean; Md: Median; IQR: Inter quartile range.

Table 3 Psychological symptoms (SCL-90)

Category	AHC (n = 21)		D-IBS (n = 30)		C-IBS (n = 16)		A-IBS (n = 34)	
	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)
Total	0.3	0 (0)	0.8	0 (1) ^c	1.3	1 (2) ^{c,f}	1.1	1 (2) ^{c,f,i}
Obsessive-comp	0.4	0 (1)	1.0	1 (2) ^c	1.5	1 (2) ^{c,f}	1.3	1 (3) ^{c,e}
Sensitivity	0.3	0 (0)	0.6	0 (1) ^c	1.3	1 (2) ^{c,f}	0.9	0 (2) ^{c,f,h}
Depression	0.4	0 (1)	1.1	1 (2) ^c	1.6	2 (2) ^{c,f}	1.5	1 (3) ^{c,f}
Anxiety	0.4	0 (0)	1.0	0 (2) ^c	1.3	1 (2) ^{c,e}	1.2	1 (2) ^{c,d}
Hostility	0.2	0 (0)	0.3	0 (0) ^b	0.8	0 (1) ^{c,f}	0.7	0 (1) ^{c,f}
Phobic anxiety	0.4	0 (0)	0.4	0 (0)	0.7	0 (1) ^{c,f}	0.5	0 (0) ^{c,g}
Paranoid ideation	0.2	0 (0)	0.4	0 (1) ^b	1.2	1 (2) ^{c,f}	0.7	0 (1) ^{c,d,i}
Psychoticism	0.1	0 (0)	0.3	0 (0) ^c	0.7	0 (1) ^{c,f}	0.4	0 (1) ^{c,d,i}
Somatisation	0.2	0 (0)	1.3	1 (2) ^c	1.9	2 (2) ^{c,f}	1.5	1 (3) ^{c,d,i}

SCL 90 score from AHC group, D-IBS, C-IBS and A-IBS patients. The results are shown in total and as items categorised. The higher score, the more symptoms. ^b $P < 0.01$, ^c $P < 0.001$ vs AHC; ^d $P < 0.05$, ^e $P < 0.01$, ^f $P < 0.001$ vs D-IBS; ^g $P < 0.05$, ^h $P < 0.01$, ⁱ $P < 0.001$, C-IBS vs A-IBS. M: Mean; Md: Median; IQR: Inter quartile range.

Table 4 Sense of coherence (SOC)

Category	AHC (n = 21)		D-IBS (n = 30)		C-IBS (n = 16)		A-IBS (n = 34)	
	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)
Total	4.2	4 (2)	3.8	4 (3) ^b	3.1	3 (3) ^{c,f}	3.4	4 (3) ^{c,f,h}
Comprehensibility	3.7	4 (2)	3.3	3 (3)	2.5	3 (3) ^{c,f}	3.1	3 (3) ^{c,i}
Manageability	4.4	5 (3)	4.0	4 (3)	3.3	3.5 (3) ^{c,f}	3.6	4 (3) ^{c,h}
Meaningfulness	4.6	5 (1)	4.2	5 (3)	3.8	4 (2) ^{c,d}	3.7	4 (2) ^{c,f}
Present time	4.5	5 (1)	4.0	5 (3) ^a	3.3	3 (3) ^{c,f}	3.5	4 (3) ^{c,f}
External conditions	4.1	4 (2)	3.7	4 (3) ^a	2.9	3 (4) ^{c,f}	3.4	3 (3) ^{c,h}

Sense of coherence score from the AHC group and C-IBS, D-IBS and A-IBS patients. The results are shown in total and as items categorised. The higher score, the better SOC. ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ vs AHC; ^d $P < 0.05$, ^e $P < 0.001$ vs D-IBS; ^h $P < 0.01$, ⁱ $P < 0.001$, C-IBS vs A-IBS. M: Mean; Md: Median; IQR: Inter quartile range.

Table 5 Psychosocial rating scale (PRS)

Category	AHC (n = 21)		D-IBS (n = 30)		C-IBS (n = 16)		A-IBS (n = 34)	
	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)
PRS	3.75	4 (0)	3.33	4 (1) ^c	3.17	4 (1) ^{c,e}	3.12	4 (2) ^{c,f}

Psychosocial rating scale (PRS) presented as M, Md (IQR) from the AHC group and D-IBS, C-IBS and A-IBS patients. A higher score indicates a better psychosocial rating/quality of life. ^c $P < 0.001$ vs AHC; ^e $P < 0.01$, ^f $P < 0.001$ vs D-IBS. M: Mean; Md: Median; IQR: Inter quartile range.

expressed higher prolactin values both compared to the controls and the D-IBS subtype. Concerning the morning cortisol measurement the subtypes showed

higher values compared to the controls, while the mid day cortisol levels were only slightly raised. However, the differences in cortisol values did not attain statistical

Table 6 Pain

Category	AHC (<i>n</i> = 21)		D-IBS (<i>n</i> = 30)		C-IBS (<i>n</i> = 16)		A-IBS (<i>n</i> = 34)	
	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)	M	Md (IQR)
Total	4.2	4 (4)	12.4	11 (12) ^b	14.1	11 (13) ^b	13.3	11 (12) ^c
Abdominal	0.3	0 (0)	1.5	2 (1) ^b	2.4	2 (1) ^c	1.9	2 (1) ^c
Rest of body	3.9	4 (6)	10.1	9 (10) ^a	13.1	14 (14) ^a	11.4	9 (11) ^b

Pain score presented as M, Md (IQR) experienced as drawings from the AHC group and D-IBS, C-IBS, and A-IBS patients. The higher score the more symptoms. ^a*P* < 0.05, ^b*P* < 0.01, ^c*P* < 0.001, vs AHC. M: Mean; Md: Median; IQR: Inter quartile range.

Table 7 Biochemical levels (mean ± SD, %)

Category	AHC (<i>n</i> = 21)		D-IBS (<i>n</i> = 30)		C-IBS (<i>n</i> = 16)		A-IBS (<i>n</i> = 34)	
C-peptide	0.46 ± 0.14	10/10	0.62 ± 0.28 ^a	40/3	0.55 ± 0.21	25/6	0.57 ± 0.24	35/12
Triglyceride	0.7 ± 0.3	10/0	1.3 ± 0.6 ^c	57/3	1.1 ± 0.5 ^b	38/0	1.2 ± 0.7 ^b	38/0
Prolactin	264 ± 118	5/10	232 ± 94	7/23	374 ± 178 ^{a,c}	31/0	301 ± 148	24/6
Cortisol 8	491 ± 144	10/14	539 ± 239	10/6	591 ± 274	31/19	596 ± 315	44/23
Cortisol 13	316 ± 65	10/14	334 ± 157	27/30	353 ± 118	31/19	333 ± 160	10/14

Biochemical status presented as mean ± SD and percent (%) above/below ± 1SD for the AHC group and D-IBS, C-IBS, and A-IBS patients. Cortisol 8 and 13 equals cortisol level at 8 am, and at 1 pm, respectively. ^a*P* < 0.05, ^b*P* < 0.01, ^c*P* < 0.001, vs AHC; ^a*P* < 0.01, vs D-IBS.

significance. All subgroups showed higher triglyceride levels than controls.

Looking at the variation above or below one standard deviation all subtypes had a larger variation compared to the controls. There was also a difference in the patterns of standard deviation, e.g. the prolactin values were, for the D-IBS group mostly below one standard deviation while for the C-IBS and A-IBS group the values were mostly above one standard deviation.

DISCUSSION

In the present study our IBS population compared to healthy controls, showed overall a higher degree of dysfunctions in basic movements and awareness, as well as more psychological and gastrointestinal symptoms. They also scored a lower sense of coherence and increased pain. In addition the IBS patients had higher and more edged values of biochemical parameters.

When dividing the patients into subgroups according to their stool and defecatory patterns, i.e. D-IBS, C-IBS and alternating type of IBS the following characteristics were identified.

The D-IBS group showed a disturbed body movement pattern on BASObs in the same magnitude as the other two groups. However, on self-estimation (BAS-self) they rated themselves as having less dysfunction reflecting a lower sense of body awareness compared to the other two groups. They had the same amount of gastrointestinal, but less psychological symptoms compared to C-IBS and A-IBS. The D-IBS patients scored a nearly normal degree of sense of coherence and thus a good quality of life, also reflected in a slightly less distorted psychosocial rating scale compared to the other subgroups. However, they expressed a high pain score similar to the other subtypes. They also had a higher C-peptide value, not being so prominent in C-IBS and

A-IBS. All subgroups also showed higher triglyceride values compared to controls.

The C-IBS and A-IBS groups exhibited to some extent similar patterns. However, the A-IBS patients revealed less body disturbance than the C-IBS. On self-estimation (BAS-self) both groups rated themselves at the same level as the physiotherapist. Both subtypes suffered from more gastrointestinal and psychological symptoms, than the AHC group. However, the C-IBS patients had more psychological symptoms than the D-IBS and A-IBS groups. Both groups displayed a lowered sense of coherence, and thus a lower quality of life, also outlined in the psychosocial rating scale. Furthermore, they were afflicted with high pain scores compared to the controls. When looking at the biochemistry the C-IBS patients had elevated prolactin values compared to the other groups.

The outcome of the gastrointestinal scale (GIS) reflecting the same symptom patterns as the subtype is supportive of an initially correct subtyping of the patients prior to referral. Actually, GIS could be used as a tool to subtype the IBS patients.

From the present study it seems as the D-IBS patients differed from the other two groups. They were not aware of their dysfunctional body awareness not realising their depreciated state of health, thus coping with preserved quality of life. These patients had less psychological symptoms, and higher C-peptide values. Thus, the increased C-peptide value could be secondary to hyperinsulinemia, reflecting an altered adrenergic drive. Although, sympathetic activation normally inhibits bowel motility, one could speculate whether this tentative adrenergic abnormality may be one component of the enteric neuropathy seen in D-IBS. Overall, they revealed themselves as ambitious persons, with a higher proportion of men compared to the other subtypes and many of them in the midst of their professional careers.

The higher C-peptide and triglyceride levels may be part of a metabolic syndrome, which is known to correlate with psychosocial stress^[39]. Prolactin may be important in the process of coping with stress and traumatic experience^[40] and Sivik *et al*^[41] reported active soldiers to have lower prolactin values. Sondergaard *et al*^[42] have shown a strong correlation between prolactin and alexithymia specially the item 'difficulty to identifying feelings'. The D-IBS group in our study had both lowered prolactin values and lower body awareness.

These results are in accordance with the outcome of a study by Aggarwal *et al*^[8]. When studying predominant symptoms in IBS and the correlation with autonomic nervous system abnormalities they found that the D-IBS subgroup was associated with adrenergic nervous system malfunctions. They also found that C-IBS patients were more psychologically distressed, with higher degree of depression and anxiety. Also, the C-IBS patients were found to have vagal cholinergic dysfunction in that study. This may also be in line with our results of higher prolactin values for the C-IBS group, which may correspond to increased vagal tone^[43], as well as higher SCL90 scores. The C-IBS and A-IBS patients are characterised by their psychological symptoms of anxiety and depression. Emotional strain is correlated to increased levels of prolactin and this could be one of the reasons for the prolactin increase in the C-IBS group^[44].

Also in agreement with our results, Elsenbruch *et al*^[12] found in a study on postprandial autonomic and cortisol responses that D-IBS patients elicited an enhanced sympathetic drive as measured by heart rate variability compared to the C-IBS patients and controls. The D-IBS had significantly higher postprandial saliva cortisol levels, but the cortisol values at baseline were equal for these groups, which is in conformity with our results on cortisol levels. Although morning fasting cortisol levels were increased equally for all subtypes, the differences compared to controls did not turn out as statistically significant. There was also a considerable spread of the values above and below one standard deviation compared to controls. These findings were also partly true for the mid day cortisol levels.

Although the sample size of the present study is fairly modest in this context, the recruitment of subjects was from patients presenting with fairly advanced disease, with several years history of symptoms. However, since they are referred from different types of care providers they can be regarded as representing a general population of IBS patients. Thus, our results can probably be generalised for a larger IBS population.

IBS is described as a gastrointestinal functional disorder, which onset and course is affected by psychological factors. Asahina *et al*^[45] suggest that treatment of psychological factors should also be considered when dealing with IBS. Moser^[46] points out that functional gastrointestinal disorders are the most frequent clinical conditions seen in practice and suggests that integrated psychosomatic care should be provided i.e. the patient's psychosocial status and the demand for additional psychological care should be assessed and offered^[47]. This

is supported by the results of randomised controlled studies having shown that psychotherapy is superior to conventional therapy^[46]. This is also in line with the results from our studies with physical, psychological and biochemical examinations and treatment of the 'whole person' with body awareness therapy. Jones *et al*^[48] showed that IBS patients had lower quality of life and less interpersonal support and greater reliance on passive coping strategies. IBS patients show in our study lower quality of life and lower body awareness which could be connected to passive coping strategies. The disparities seen in our study of the subtypes of IBS are in agreement with these studies mentioned and may be different expressions of the functional gastrointestinal disorder. With the knowledge gained from the present study body awareness therapy could be adjusted to the different manifestations encountered in the subtypes of IBS.

In conclusion, this study has shown that the D-IBS patients, with a higher proportion of men, scored less body awareness, less psychological symptoms, better sense of coherence and showed higher C-peptide values, possibly indicating an adrenergic drive representing unconscious mental stress. The C-IBS and A-IBS patients expressed higher body awareness, more depression and anxiety with impaired sense of coherence. The raised prolactin levels in C-IBS patients may reflect an increased vagal tone and emotional strain. The importance of the differences seen between the IBS subtypes in the present study and its implications for future treatment of IBS will have to be elucidated in further investigations.

COMMENTS

Background

Irritable Bowel Syndrome (IBS) is the most common of all gastrointestinal disorders, affecting around 15% of the population at least in the Western societies. The division of IBS into subgroups is based on the fact that these patients behave in dissimilar ways. IBS subgroups building on stool and defecation patterns can be divided into diarrhoea predominant (D-IBS), constipation predominant (C-IBS) and alternating (A-IBS) subtypes.

Research frontiers

The understanding of IBS and especially the interaction between the central and enteric nervous systems has grown considerably over the last years. Therefore, in recent year's research has focused more and more on the psychosomatic (body and mind) aspect of the disease. IBS patients are therefore examined more comprehensively with gastrointestinal symptoms, psychological symptoms, biochemical stress markers, quality of life and body awareness. Thus, psychosomatic remedies like hypnotherapy, psychotherapy and body awareness therapy have been applied.

Innovations and breakthroughs

Subgroups of IBS as shown in this study differ in body awareness, quality of life, psychological symptoms and biochemical stress markers.

Applications

Treatment like Body Awareness Therapy (BAT) which is a physiotherapeutic approach, to adjust and reduce pathological tension in the body may in the future also be applied and streamlined for these subgroups in order to get a more optimal treatment.

Peer review

This manuscript reports results of a study of differences in body awareness, pain scores, psychological symptoms and blood levels for prolactin, triglycerides and morning cortisol between healthy controls and patient diagnosed by Rome criteria with either D-IBS, C-IBS and A-IBS. The manuscript is well constructed and written.

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Ultrastructural changes in hepatocytes after taurine treatment in CCl₄ induced liver injury

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INTRODUCTION

Liver cirrhosis is the terminal stage of various chronic liver diseases. Even mild but continuous injury in the liver soon results in excessive production of extracellular matrix components^[1]. Deposition in the space of Disse causes capillarization of sinusoids and alterations in liver functions. This fibrotic stage ultimately progresses to cirrhosis, which is characterized by nodule formation and corruption of liver architecture.

Overproduction and accumulation of extracellular matrix proteins in the liver start usually after chronic hepatocyte injury that initiates a series of complicated cell-to-cell and cell-to-matrix interactions, eventually leading to activation of hepatic stellate cells, which are the main producers of excessive collagen during cirrhosis process^[2]. Since hepatocyte injury seems to be the first and the main fibrogenic stimulus in the liver, healing of these cells could be a desirable goal in preventing progression of fibrosis.

Taurine, 2-aminoethanesulphonic acid, is an essential β -amino acid. It is present at high concentrations in many tissues. It plays important roles in numerous physiological functions including conjugation with bile acids, modulation of calcium levels, and maintenance of osmolarity, antioxidation and stabilization of membranes^[3]. It was reported to have beneficial effects in various physiological and pathological conditions^[4-7] by mainly diminishing production of reactive oxygen species (ROS). It also can prevent DNA damage at physiological concentrations^[8]. Taurine has also hepatoprotective effects such as inhibition of extracellular matrix accumulation in experimental liver fibrosis^[9,10] and improvement of liver function tests in fatty liver disease of children^[11]. Hepatoprotective feature of taurine is attributed to its inhibitory activity on generation of ROS, which are

Abstract

AIM: To search the organelle based changes in hepatocytes after taurine treatment in experimental liver fibrosis induced by CCl₄ administration.

METHODS: Thirty rats were divided into two groups. Group 1 ($n = 15$) was injected with CCl₄ plus taurine and Group 2 ($n = 15$) with CCl₄ plus saline for 12 wk. At the end of 12th wk, mitochondria, rough and smooth endoplasmic reticulum, and nuclei of hepatocytes were evaluated using a scoring system. The results were compared with histopathological findings, as well.

RESULTS: Taurine treatment reduced fibrosis scores significantly as compared to placebo. Organelle injury scores decreased significantly with taurine treatment. Ultrastructural and histopathological scores in both groups were in strong correlation ($r = 0.931$ for CCl₄ plus taurine and $r = 0.899$ for CCl₄ plus saline group).

CONCLUSION: Organelle based transmission electron microscopy findings can reflect successfully histological results as well as tissue healing in hepatocytes from hepatotoxin-induced liver fibrosis.

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Key words: Taurine; Liver fibrosis; Hepatocyte; Ultrastructure

known to play an important role in hepatic injury both *in vitro* and *in vivo*^[12,13].

The effects of acute oxidative stress on the ultrastructure of sinusoidal endothelium, space of Disse, hepatocytes and Kupffer cells in perfused rat liver have been studied previously by Cogger *et al*^[14]. They successfully demonstrated the alterations in the mitochondria of injured hepatocytes. Recently, we have shown beneficial effects of taurine on histopathology and oxidative stress parameters in a rat model of CCl₄-induced liver fibrosis^[15,16] where remarkable histopathological improvement in taurine treated animals subjected to hepatotoxin was observed, and this was associated with oxidative stress reduction and hepatocellular apoptosis. In this work we report on the changes in the chronic setting, with more focusing on the multiple organelle based alterations after administration of CCl₄ that causes hepatic injury primarily *via* increasing ROS production in the liver^[17]. We also studied the correlation of ultrastructural changes with histopathological findings.

MATERIALS AND METHODS

The study was approved by the Institutional Animal Use and Care Committee of the Gulhane School of Medicine, Ankara, Turkey, and was performed in accordance with the National Institute of Health guidelines for the care and handling of animals. The animals were fed with free access to standard rat food and water, and housed in metabolic cages one in one at controlled temperature and 12-h light/dark cycles before and during the experiment. Electron microscopic examinations were performed at the Department of Anatomy, Hacettepe University Medical Faculty, Ankara.

Animals and treatment strategies

Thirty male Sprague Dawley rats weighing 250-400 g were randomly divided into two groups. Group 1 ($n = 15$) was injected with CCl₄ (0.2 mL/100 g twice weekly; S.C) plus taurine (1000 mg/kg per day; I.P), and Group 2 ($n = 15$) with CCl₄ plus saline for 12 wk. At the end of 12th wk all rats were killed under anesthesia and the livers were excised. Adequate numbers of specimens from right and left lobes of each liver were collected for transmission electron microscopy and histopathological examination.

Light microscopy analysis

For light microscopy, tissue sections were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin, examined and scored by two pathologists who were blinded to the treatment protocol. Degree of necrosis, inflammation, fat accumulation, and fibrosis was scored as 0: Absent; 1: Slight; 2: Moderate; and 3: Severe as described elsewhere, with small modifications^[18]. Histopathological evaluation was performed twice in four sections per slide from all animals in each group.

Table 1 Ultrastructural scoring system used in experimental liver fibrosis^[19-21]

Ultrastructure	Assessment	Score
Mitochondria	Normal	0
	Prominent cristae	1
	Edematous mitochondrion	2
	Collection of amorphous material	3
rER	Normal	0
	Dilatation	1
	Irregular lamellar organization	2
	Presence of focal breaks	3
sER	Normal	0
	Dilatation	1
	Vacuolization	2
	Presence of large degenerated areas, myelin figures	3
Nucleus	Normal	0
	Irregular chromatin distribution (margination, clumping)	1
	Increased heterochromatin	2
	Degenerated nucleus	3

rER: Rough endoplasmic reticulum; sER: Smooth endoplasmic reticulum.

Ultrastructural analysis

The specimens were fixed in 2.5% glutaraldehyde for 24 h and subsequently washed in phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide in phosphate buffer (pH 7.4) and dehydrated in increasing concentrations of alcohol. Afterwards, the tissues were washed with propylene oxide and embedded in epoxy-resin embedding media. Semi-thin sections about 3 mm in thickness were cut with a glass knife on a LKB Nova ultramicrotome (Sweden). These sections were stained with methylene blue and examined by a Nikon Optiphot (Japan) light microscope. Ultrathin sections were collected on copper grids, stained with uranyl acetate and lead citrate, and finally examined under a Jeol JEM 1200 Ex (Japan) transmission electron microscope. Twenty cells from each specimen were examined. Mitochondria, nuclei, rough endoplasmic reticulum (rER) and smooth endoplasmic reticulum (sER) of hepatocytes were evaluated by using a previously described scoring system (Table 1)^[19-21]. Twenty nuclei, 50 mitochondria, 20 rERs and 20 sERs were examined for each animal.

Statistical analysis

Results are expressed as mean \pm SEM. Mann-Whitney *U* test for histopathologic scores and Student's *t*-test for ultrastructure scores were used to analyze significance of differences between groups. Correlation of histopathological and ultrastructural scores was assessed with Pearson correlation procedure. The differences were accepted as statistically significant when $P < 0.05$.

RESULTS

Two animals in Group 1 and four in Group 2 died before the end of experiment.

Light microscopy

CCl₄ treatment produced hepatic necrosis, inflammation, fatty accumulation, and fibrosis by the 12th wk. Light

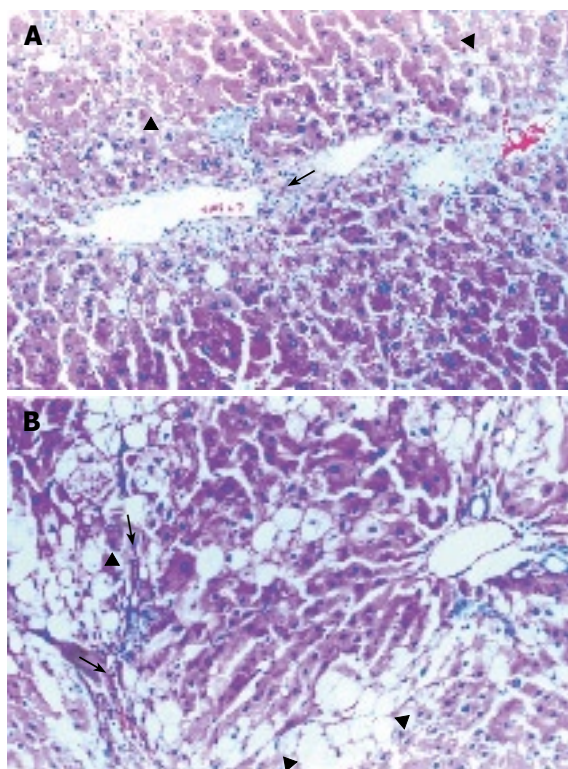


Figure 1 A: Mild bile duct proliferation (arrow) and microvesicular changes (arrowheads) in Taurine treated animals (HE, x 200); B: Severe macro and microvesicular fatty accumulation (arrowheads) and fibrosis (arrows) in Taurine untreated group (HE, x 50).

Table 2 Histopathologic scoring and organelle injury scores of Group 1 and Group 2 (mean \pm SD)

	Group 1	Group 2	P
Histopathologic scoring			
Necrosis	1.20 \pm 0.15	2.27 \pm 0.18	< 0.001 ¹
Inflammation	1.40 \pm 0.13	2.00 \pm 0.17	< 0.03 ¹
Fat accumulation	1.27 \pm 0.12	2.07 \pm 0.21	< 0.01 ¹
Fibrosis	1.40 \pm 0.16	2.27 \pm 0.18	< 0.005 ¹
Total	5.20 \pm 0.38	8.60 \pm 0.29	< 0.001 ¹
Organelle injury scores			
Mitochondrion	45.7 \pm 1.3	98.9 \pm 2.1	< 0.001 ²
Rough ER	22.1 \pm 0.8	41.6 \pm 2.1	< 0.001 ²
Smooth ER	20.4 \pm 0.8	43.3 \pm 1.6	< 0.001 ²
Nucleus	17.9 \pm 1.2	33.3 \pm 0.9	< 0.001 ²
Total	106 \pm 4	217 \pm 6	< 0.001 ²

¹Mann-Whitney *U* test; ²Student's *t*-test. ER: Endoplasmic reticulum.

microscopy evaluation of liver sections from animals treated with CCl₄ and taurine are shown in Figure 1, and Table 2. Necrosis, inflammation, fatty accumulation and fibrosis were significantly lower in Group 1 when compared with Group 2 (Figure 1B).

Transmission electron microscopy

Ultrastructural analysis of the liver sections revealed significantly lower organelle injury scores in CCl₄ plus taurine treated group when compared with CCl₄ plus saline treatment (Table 2). Mitochondrial edema was seen in both groups but it was more extensive in saline treated animals. Additionally, mitochondrial cristae were

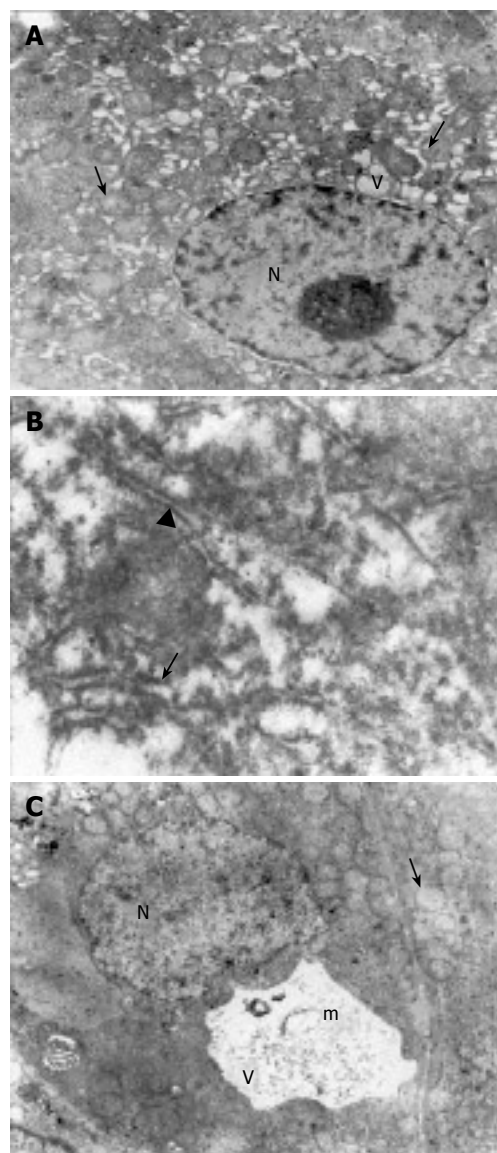
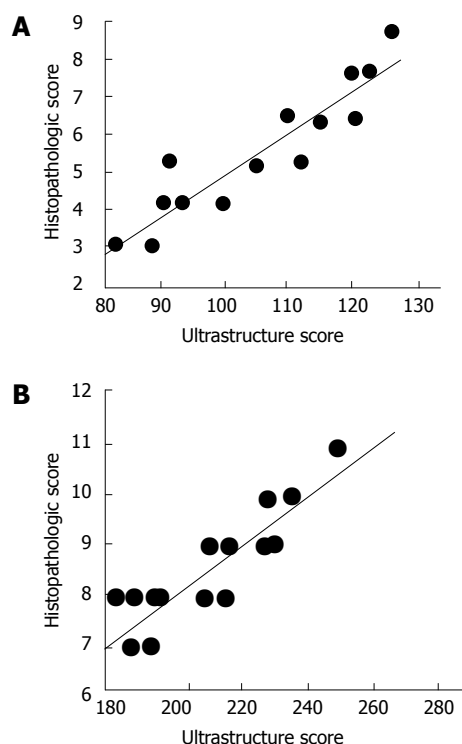


Figure 2 Electron micrographs. A: The dilations in the rER (arrow) and sER (arrowhead), large vacuoles in the hepatocytes (V), a normal nucleus (N) and mitochondria with a prominent edema (small arrow) in CCl₄ plus taurine treated group (x 4000); B: Large dilations (arrow) and focal breaks (arrowhead) in the rER in CCl₄ plus saline treated group (x 30000); C: The vacuolizations (V) and myelin figures (m) in the sER, nucleus (N) and mitochondria with prominent edema (arrow) in CCl₄ plus saline treated group (x 4000).

much more visible in taurine treated animals (Figure 2A). While there were irregular lamellar organization and large dilations with focal breaks in rERs of hepatocytes in CCl₄ plus saline treated group in many areas (Figure 2B), only some focal slight dilations were observed in taurine treated animals. Dilatations in taurine treated animals (Figure 2A) but vacuolization and myelin figures in saline treated group were sER findings (Figure 2C). Although there was irregular chromatin distribution in some areas, nuclei were almost normal in appearance with taurine treatment (Figure 2A). However, chromatin distribution was irregular and nuclei showed extensive margination and clumping in saline treated group. Other prominent findings were large vacuolization of hepatocyte cytoplasm in taurine treated animals and presence of active fibroblasts in some focal



and clumping of chromatin in saline treated animals may be morphological evidence of injury in the nucleus. However, severely degenerated nuclei were rarely detected. Nuclear content was almost normal in appearance and organization with taurine treatment, which was previously reported to prevent DNA damage^[8]. These results confirm the basic knowledge that nucleoplasmic constituents represent the structural counterpart of transcription and processing of messenger and ribosomal RNAs, and therefore constitute fine and highly sensitive indicators of cellular activity.

Electron microscope findings in hepatocytes after hepatotoxin have not been defined systematically to date. Moreover, which changes in each organelle are reversible or not is not clear. Dincer *et al.*, previously reported partly the ultrastructural changes in hepatocytes after taurine treatment^[31]. However, the present study not only defines the organelle changes in more detail; it also provides a better assessment and measurement of ultrastructural injury.

The change in ultrastructural scores between the two groups was nearly the same when compared with histopathological scores. This indicates that the current histopathological scoring system used to describe tissue injury in the present study successfully reflects organelle based ultrastructural changes in hepatocytes. On the other hand, the results obtained in this study should not be overwhelmed, because taurine's most significant action is directly counteracting the effect of CCl₄. Protective effects of this antioxidant in clinical conditions related to other injury mechanisms in the liver might not be so strongly evident.

In conclusion, this study brought us direct view evidence of changes in morphology of hepatocyte organelles after induction of a certain hepatotoxin. Taurine preserves morphology of major organelles of hepatocytes and delays the development of fibrosis. Structural changes in hepatocyte organelles we observed in this study are likely the cause of significant histological improvements. Since transmission electron microscopy is the highest magnification tool at present, modeling new ultrastructural scoring systems including more organelles and parameters can be useful in estimating the degree of injury and outcome of alternative treatment strategies in management of chronic liver diseases.

COMMENTS

Background

Liver fibrosis and cirrhosis are untreatable conditions at present. Antioxidant medications including taurine have been reported to possess antifibrotic efficacy in experimental liver fibrosis. Taurine is one of the main components of energy drinks, which are widely consumed by healthy people around the world. The present study addresses organelle based changes in hepatocytes after taurine treatment in rat liver fibrosis.

Research frontiers

Preventive effect of taurine in continuing liver injury was tested. The study design does not include treatment after establishment of liver fibrosis. Antioxidants may be taken into consideration not only for their efficacy on established liver fibrosis but also for their hepatoprotective efficacy in the long term.

Innovations and breakthroughs

Organelle based effects of taurine in hepatocytes is to be shown for the first time on animals.

Applications

Taurine administration was previously shown to be effective in delaying the development of fibrosis in experimental conditions. The present findings support the idea that conduction of large scale clinical studies on the efficacy of taurine in human liver fibrosis or cirrhosis should be encouraged.

Terminology

Liver fibrosis refers to invasion of normal liver by collagen deposits in chronic liver injury leading to destruction of normal tissue architecture and functional insufficiency. Taurine is a potent antioxidant not present in the market as a drug but is included in the majority of energy drinks.

Peer review

This paper is an experimental study in rats demonstrating that taurine protects the liver at the ultrastructural level after the administration of carbon tetrachloride. The paper is novel, well written and well organized, particularly the discussion which is comprehensive and easy to read.

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Up-regulation of α -catenin is associated with increased lymph node involvement in colorectal cancer

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SWM, but no such effect on disease free survival (DFS) or disease specific survival (DSS). As to co-expression with another member of the adhesion complex (β -catenin), high α -catenin/ β -catenin MI index was of marginal significance in predicting longer DSS ($P = 0.063$, log-rank).

CONCLUSION: The results implicate that high α -catenin expression is intimately involved in the key regulatory mechanisms leading to invasive phenotype, lymph node metastases, and progressive disease in CRC.

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Key words: Colorectal carcinoma; Alpha-catenin; Membrane staining; Cytoplasmic staining; Prognosis

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Abstract

AIM: To investigate the changing pattern of α -catenin expression and its relationship to clinical and pathological features of colorectal cancer (CRC) patients.

METHODS: Archival tumor samples were analyzed using immunohistochemistry (IHC) for α -catenin in 91 patients with advanced CRC.

RESULTS: The values of α -catenin membrane index (MI) and cytoplasmic index (CI) were significantly related to the depth of tumor invasion ($P = 0.027$, $P = 0.020$, respectively), high indices being associated with increased depth of the primary tumor invasion (T3 and T4). Similarly, patients with high α -catenin expression had a significantly increased risk of lymph node metastasis (32/39 vs 37/52 for MI and 37/45 vs 32/46 for CI) ($P = 0.001$, $P = 0.0001$, respectively, for LNN status). An altered expression (i.e., cytoplasmic pattern) was also related ($P = 0.047$) to the response to chemotherapy; patients with low CI were more responsive (CR: 7/46) than patients with high CI values (CR: 0/45). There was a marginal effect on survival in patients time with metastases (SWM) ($P = 0.087$); patients with low CI showing slightly longer

INTRODUCTION

Under normal conditions, cell-cell adhesion molecules maintain epithelial cell integrity and cellular architecture. The process of tumor invasion and metastasis is associated with alterations in the functions of several adhesion molecules. In general, tumor cells lose their capacity for normal adherence, which facilitates their detachment from their site of origin^[1,2].

Homotypic cell-cell adhesion is regulated mostly by the cadherin-catenin complex. Alterations in E-cadherin and catenins have been linked with more aggressive behavior of several human tumors^[3-6]. Catenins are divided according to their molecular weight as α -catenin (102 kDa), β -catenin (88 kDa) and γ -catenin (80 kDa)^[7-9].

α -catenin links E-cadherin to the actin cytoskeleton via its association with either β - or γ -catenin^[10,11].

Abnormal α -catenin expression has been reported in many human cancers^[12-15]. Reduced α -catenin expression was associated with tumor invasion and metastases in colorectal cancer (CRC)^[6].

In this study, we examined α -catenin expression in a series of CRC, and analyzed the relationship with clinical and pathological features of CRC patients.

MATERIAL AND METHODS

Study material

The material of the present study consists of a series of 91 patients with advanced CRC, enrolled among the consecutive CRC patients attending our clinic for therapeutic procedures. Of these 91 patients, 58 had metastases at diagnosis (Stage IV disease), while the remaining 33 patients (with stage II and III disease at baseline) subsequently developed a metastatic disease during the mean follow-up (FU) time of 25.1 ± 27.8 mo. All patients were treated for advanced and metastatic disease at the Department of Oncology and Radiotherapy, Turku University Hospital, according to the protocols used for CRC patients with stage II, III or IV disease at that time. These 91 patients included in the present study were enrolled into this prospective cohort between October 1998 and August 2003. All patients have been prospectively followed-up until death or when last seen alive at their clinical visit (March 2007), with the median FU-time of 27.6 mo (range 3-150 mo). The study was approved by the TUH Ethics Committee and was conducted in accordance with the Declaration of Helsinki. Samples were collected with the endorsement of the National Authority for Medico-legal Affairs.

The key clinical data of the patients are shown in Table 1. Of these 91 cases, 34 were women and 57 were men. The mean age was 61.5 years (range 24-78 years). The majority ($n = 38$) of the tumors were localized in the left colon, followed in order of frequency by the right colon ($n = 23$), rectum ($n = 22$), and colon transversum ($n = 7$). At the time of diagnosis, 14 patients had Stage II disease, 19 Stage III and 58 tumors were at Stage IV. The majority ($n = 59$, 64.8%) were T3 tumors, and almost half ($n = 46$) had known lymph node involvement at the time of diagnosis, including the cases with Nx status. The patients were selected into the cohort on the basis of both the diagnosis and treatment received, and were assigned to one of the two treatment arms: (1) 20 were treated with irinotecan alone; and (2) 71 received a combination of irinotecan and 5-fluorouracil (5-FU) as the first line treatment.

α -catenin immunostaining

Formalin-fixed, paraffin-embedded primary colorectal tumor tissue was obtained from 91 patients. Sections were cut serially at 5 μ m for routine haematoxylin and eosin staining and for immunohistochemical (IHC) analysis. An experienced pathologist confirmed all histological diagnoses. IHC analysis was done using the automatic system (BenchMark XT, Ventana Medical

Table 1 Key characteristics of the patients and their tumors

Variable	<i>n</i> or value	% ¹
Patients	91	
Male	57	63.0
Female	34	37.0
Age (yr)		
Median (range)	60.7 (24-80)	
Primary tumour status ²	91	
T1	1	1.0
T2	6	6.5
T3	59	64.8
T4	16	17.6
Tx	9	9.8
Primary nodal status ²	91	
N0	22	24.0
N+	49	54.0
Nx	20	22.0
Metastases at diagnosis	91	
M0	34	37.0
M1	57	63.0
Histological grade	91	
Gr I	11	12.1
Gr II	62	68.1
Gr III	18	19.8
Stage	91	
Stage II	14	15.0
Stage III	19	21.0
Stage IV	58	64.0
Survival (mo)		
From primary diagnosis		
Median (range)	27.3 (3-150)	
From metastasis		
Median (range)	21.5 (3-80)	

¹When applicable; ²TNM classification. Tx: Unknown; Nx: Unknown.

Systems, Inc. Tucson, Arizona, USA). This fully automated processing of bar code labeled slides included baking of the slides, solvent free deparaffinization, antigen retrieval in a cell conditioning buffer CC2 (Mild: 36 min conditioning, and standard: 60 min conditioning), incubation with the monoclonal mouse α -catenin antibody (clone α CAT-7A4, isotype IgG1- κ , Zymed Laboratories, San Francisco, CA), at a dilution 1:100 (32 min, 37°C). The dilution of the primary antibody was based on dilution experiments. Application of ultraView™ Universal DAB (a biotin-free, Multimer-based detection system for the specific and sensitive detection of mouse IgG, mouse IgM, and rabbit IgG primary antibodies). UltraView DAB includes: ultraView Universal HRP, ultraView Universal DAB Inhibitor, ultraView Universal DAB Chromogen, ultraView Universal DAB H₂O₂, and ultraView Universal DAB Copper. Counterstaining with haematoxylin (2021) took 4 min, and post-counterstaining with bluing reagent (2037) took 4 min as well. After staining, the sections were dehydrated in ethanol, cleared in xylene, and covered with Mountex and cover slips.

Evaluation of α -catenin staining

The α -catenin staining was evaluated by observers blinded to the clinical data using regular light microscopy. Membranous and cytoplasmic expression was evaluated separately. For the cell membrane staining,

four categories were used, (+++, ++, +, -), starting from equivalent to normal to entirely negative^[16]. The cytoplasmic staining was also graded into four categories: (0) Negative, no detectable staining; (1) Weak, but still detectable staining; (2) Moderate, clearly positive but still weak; (3) Heavy staining, intense^[17]. In calculating the staining indexes: membrane index (MI), and cytoplasmic index (CI), both the intensity of staining and the fraction of positively-stained cells were taken into account, using the following formula: $I = 0 * f_0 + 1 * f_1 + 2 * f_2 + 3 * f_3$, where I is the staining index, f_0 - f_3 are the fractions of the cells showing a defined level of staining intensity (from 0 to 3). Theoretically, the index could vary between 0 and $3n$ ^[18]. The α -catenin expression was evaluated independently by two observers (AE, AB). The agreement of the evaluation of α -catenin staining indices was tested between two observers, and the agreement was good as suggested by the correlation coefficient (Pearson's r : MI, CI, and NI were 0.77, 0.91, and 0.90, respectively, $P < 0.001$).

Statistical analysis

Statistical analyses were performed using the SPSS® (SPSS, Inc., Chicago, USA) and STATA (Stata Corp., Texas, USA) software packages (SPSS for Windows, version 14.0.1 and STATA/SE 9.2). Frequency tables were analyzed using the χ^2 test, which evaluation included likelihood ratio (LR), or Fischer's exact test to assess the significance of the correlation between the categorical variables. Odds ratios and their 95% confidence intervals (95%CI) were calculated where appropriate, using the exact method. Differences in the means of continuous variables were analyzed using non-parametric tests (Mann-Whitney or Kruskal-Wallis) for 2- and K-independent samples, respectively. ANOVA (analysis of variance) was only used for deriving the mean values (and their SD) of each individual category. Univariate survival (life-table) analysis for the outcome measure (disease specific survival, DSS; disease free survival, DFS) was based on Cox's method (indices treated as continuous variables), and/or using Kaplan-Meier analysis (indices with Median as cut-off). In all tests, $P < 0.05$ was regarded statistically significant.

RESULTS

In normal colonic mucosa, α -catenin expression was predominantly membranous but this pattern was disturbed (diffuse cytoplasmic and membranous) in the tumor tissues (Figure 1). The mean values of MI and CI were 1.3 and 1.1, respectively.

The two expression patterns of α -catenin were related to all clinical and tumor variables recorded in this series. MI was significantly ($P = 0.03$) related to the localization of the primary tumor, being more intense in carcinomas of the descending colon ($n = 38$) and rectum ($n = 22$) than in those of the ascending ($n = 23$) and transverse colon ($n = 7$). Both MI and CI were also correlated with the depth of the primary tumor (ANOVA; $P = 0.027$, $P = 0.020$, respectively), higher

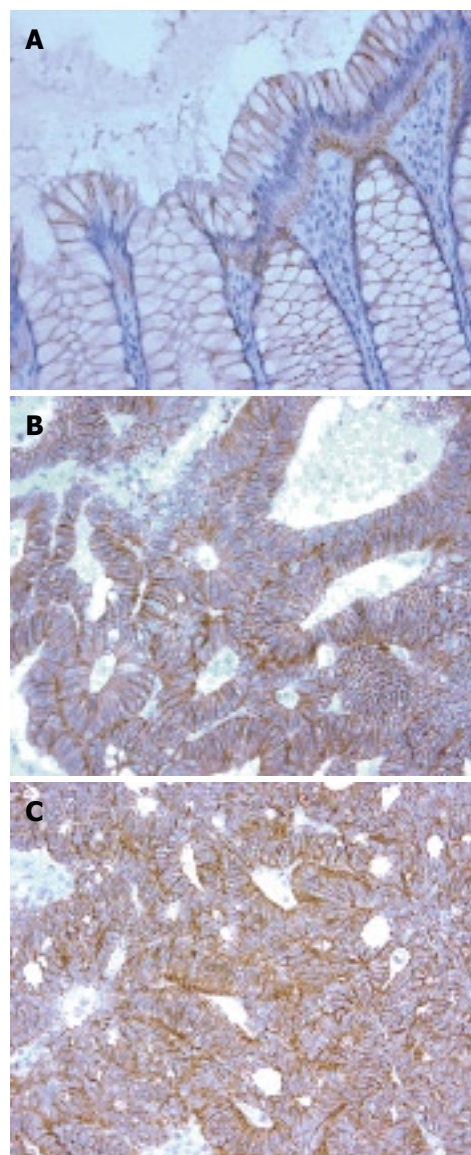


Figure 1 Different immunohistochemical staining patterns of α -catenin in colorectal carcinomas. **A:** In normal colonic epithelium, α -catenin is predominantly expressed in the cell membrane. **B:** A medium-powered view of a colonic adenocarcinoma showing membranous expression of α -catenin. **C:** This case shows both membranous and cytoplasmic expression of α -catenin.

mean index values being associated with increased depth of the primary tumor invasion (i.e., T3 and T4). Interestingly, patients with high α -catenin expression (MI, CI) had a significantly increased lymph node metastasis (32/39 *vs* 37/52 for MI and 37/45 *vs* 32/46 for CI) ($P = 0.001$, $P = 0.0001$, respectively). On the other hand, there was no correlation between α -catenin expression and most of the clinical variables (e.g. age, sex, and stage). However, there was a marginal relation between MI and CI ($P = 0.08$, $P = 0.07$, respectively) and the grade of the primary tumor.

Cytoplasmic α -catenin expression was also significantly ($P = 0.04$) related to the response to treatment in that the patients with low CI were more responsive (CR: 7/46) than patients with high CI values (CR: 0/45). No such association was established for the MI.

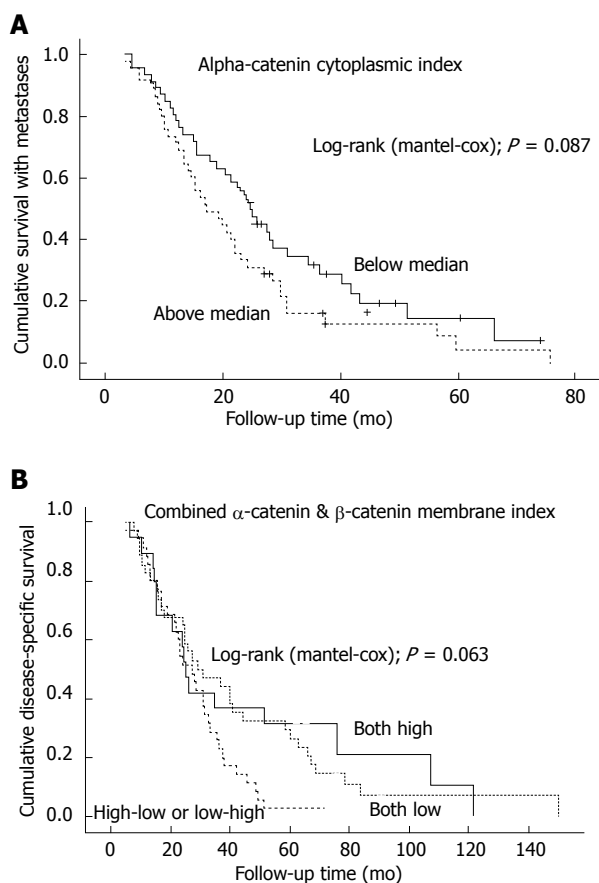


Figure 2 A: Cytoplasmic α -catenin expression and survival with metastasis in univariate (Kaplan-Meier) analysis; B: Combined α -catenin and β -catenin (MI) expression and disease-specific overall survival in Kaplan-Meier analysis.

In univariate survival analysis, neither MI nor CI was a significant predictor of disease outcome. As to the survival with metastases, there was a marginal difference in the survival curves, patients with low CI showing slightly longer survival with metastases (SWM) (Figure 2A). Finally, when α -catenin expression was analyzed jointly with β -catenin expression (with the combined MI and CI indices built up using the same criteria as described for α -catenin), only the combined MI index was of marginal prognostic value in predicting DSS, those with high MI surviving longer (Figure 2B).

DISCUSSION

As compared with the sub-cellular distribution of α -catenin in normal colonic mucosa, neoplastic cells demonstrated a distinct shift from the membranous localization to more widespread distribution (membranous, cytoplasmic) in cancer cells. This is in line with the previous reports describing this type of altered pattern of α -catenin expression in cancer cells^[19,20].

Interestingly, α -catenin expression (MI) was more intense in carcinomas of the descending colon and rectum as compared with the lesions localized in the ascending and transverse colon. Similar observation was reported for β -catenin^[21,22], but not for α -catenin. There is increasing evidence to suggest that molecular mechanisms and molecular phenotypes differ in

carcinomas arising in the proximal and distal segments of the large bowel^[23]. The involvement of different molecular pathways in colorectal carcinogenesis is exemplified by the fact that cancers of “mutator” phenotypes preferentially occur in the proximal colon, whereas the adenoma-to-carcinoma sequence phenotype is characteristic of carcinomas in the distal colon and rectum^[24,25]. Corresponding differences have also been demonstrated with other potential prognosticators^[26].

The correlation between α -catenin expression pattern and the TNM categories is a controversial issue. Some studies report that there is no correlation between α -catenin and these clinicopathological variables^[3]. On the other hand, Gofuku *et al* 1999 demonstrated that reduced expression of α -catenin was significantly correlated with the depth of invasion. Moreover, the frequency of lymph node metastases was significantly higher in those tumors with reduced α -catenin expression^[6]. Our data show that higher indices in both (MI, CI) have a correlation with increased depth of tumor invasion and to increased lymph node involvement. There are multiple reasons that could explain these inconsistent and in part discrepant results reported in these different studies^[3,6]. Such potential confounding factors might include the size of tissue sample, intrinsic tumor heterogeneity, lack of standardization of the positive and negative results, and different immunohistochemical staining and grading methods, with varying degree of sensitivity. In addition, our patients represent advanced CRC, the majority of patients with stage IV disease, as compared e.g. with Gofuku’s material, in which only 24/100 patients had stage IV CRC^[6].

To our knowledge, this is the first study to investigate the relation between α -catenin expression and response to treatment. Interestingly, a significant relation was observed between CI and the response to treatment. Accordingly, the patients with low CI were more responsive to treatment than patients with high CI values. The significance of these observations remains to be elucidated in a larger study, however.

As to the relation between α -catenin expression pattern and clinical outcome, a marginally significant association was observed to survival with metastases. When we combined both MI and CI of α -catenin and MI and CI of β -catenin (using 3 categories; high/high; high/low or low/high; low/low), only the combined MI index was of marginal prognostic value, high MI predicting longer DSS. Such information has not been reported previously. As shown by the data (Figure 2B), it seems feasible to speculate that patients with high MI of both α - and β -catenin survive longer, because this is the normal expression pattern of these two adhesion molecules, and retaining this pattern could indicate a less pronounced dedifferentiation of the cancer cells.

Taken together, the present study examined the predictive and prognostic value of α -catenin expression in advanced CRC. Our results substantiate the emerging evidence on different molecular events operating in CRC at different localizations, while demonstrating a

significant difference in α -catenin expression between tumors of the proximal and distal sites. High α -catenin expression was typical in advanced invasion of the primary tumour (i.e., T3 and T4) as well as in appearance of LNN metastases. An altered expression (i.e., cytoplasmic) pattern was also related to the response to treatment, and there was some marginal association with the SWM, but not with DFS or DSS. The latter was marginally predicted by the combined (α -catenin/ β -catenin) MI, however, patients with high MI showing longer DSS. The results implicate that α -catenin expression is associated with the regulatory mechanisms leading to invasive phenotype and progressive disease in CRC. The full prognostic value of this adhesion molecule as a single marker remains to be established, however, and there is some circumstantial evidence (Figure 2B) that probably more valuable prognostic information could be obtained when α -catenin is combined with the other constituents of the cell adhesion complex, e.g. β -catenin and the cadherins.

COMMENTS

Background

Process of tumor invasion and metastasis is associated with alterations in the functions of several adhesion molecules. In general, tumor cells lose their capacity for normal adherence, which facilitates their detachment from their site of origin. α -catenin links E-cadherin to the actin cytoskeleton via its association with either β - or γ -catenin. Reduced α -catenin expression was associated with tumor invasion and metastases in colorectal cancer (CRC). We examined α -catenin expression in a series of 91 patients with advanced colorectal carcinoma, and analyzed the relationship with clinical and pathological features of CRC patients.

Research frontiers

CRC is one of the commonest malignant tumours and has a relatively poor prognosis, where the outcome depends on the extent of local and particularly metastatic tumour spread. For example, in locally advanced disease (Dukes C), the 5-year relative survival rate is 65% but goes down to 8% in metastatic disease (Dukes D). Metastatic disease causes the majority of cancer-related deaths, either as a result of tumour involvement of critical organs or due to complications of therapy to control tumour growth and spread. This dramatic difference emphasizes the importance of delineating predictive factors capable of reliably distinguishing CRC patients at risk for developing a metastatic phenotype.

Innovations and breakthroughs

Patients with high α -catenin expression (MI, CI) had a significantly increased lymph node metastasis. There is also significant difference in α -catenin expression between tumors of the proximal and distal sites. Interestingly, α -catenin expression (MI) was more intense in carcinomas of the descending colon and rectum as compared with the lesions localized in the ascending and transverse colon. Similar observation was reported for β -catenin, but not for α -catenin. There is increasing evidence to suggest that molecular mechanisms and molecular phenotypes differ in carcinomas arising in the proximal and distal segments of the large bowel. The involvement of different molecular pathways in colorectal carcinogenesis is exemplified by the fact that cancers of "mutator" phenotypes preferentially occur in the proximal colon, whereas the adenoma-to-carcinoma sequence phenotype is characteristic of carcinomas in the distal colon and rectum. Corresponding differences have also been demonstrated with other potential prognosticators. To our knowledge, this is the first study to investigate the relation between α -catenin expression and response to treatment. Interestingly, a significant relation was observed between CI and the response to treatment. Accordingly, the patients with low CI were more responsive to treatment than patients with high CI values. The significance of these observations remains to be elucidated in a larger study, however.

Applications

Valuable prognostic information could be obtained when α -catenin is combined

with the other constituents of the cell adhesion complex, e.g. β -catenin and the cadherins.

Peer review

The manuscript is well written and the results implicate that high α -catenin expression is associated with invasive phenotype, lymph node metastases, and progressive disease in CRC. It includes interesting new data of α -catenin expression in colon carcinomas of different locations. This is also the first study to investigate the relation between α -catenin expression and the response to treatment.

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Laparoscopic *versus* open appendectomy: Which way to go?

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Abstract

AIM: To compare the outcome of laparoscopic *versus* open appendectomy.

METHODS: Prospectively collected data from 293 consecutive patients with acute appendicitis were studied. These comprised of 165 patients who underwent conventional appendectomy and 128 patients treated laparoscopically. The two groups were compared with respect to operative time, length of hospital stay, postoperative pain, complication rate and cost.

RESULTS: There were no statistical differences regarding patient characteristics between the two groups. Conversion to laparotomy was necessary in 2 patients (1.5%). Laparoscopic appendectomy was associated with a shorter hospital stay (2.2 d *vs* 3.1 d, $P = 0.04$), and lower incidence of wound infection (5.3% *vs* 12.8%, $P = 0.03$). However, in patients with complicated disease, intra-abdominal abscess formation was more common after laparoscopic appendectomy (5.3% *vs* 2.1%, $P = 0.002$). The operative time and analgesia requirements were similar in the two groups. The cost of treatment was higher by 370 € in the laparoscopic group.

CONCLUSION: Laparoscopic appendectomy is as safe and efficient as open appendectomy, provided surgical experience and equipment are available.

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Key words: Laparoscopy; Appendicitis; Appendectomy; Conventional appendectomy

INTRODUCTION

The introduction of laparoscopic surgery has dramatically changed the field of surgery. With improvements in the equipment and increasing clinical experience it is now possible to perform almost any kind of procedure under laparoscopic visualization.

Although more than a century has elapsed since McBurney first performed open appendectomy^[1], this procedure remains the treatment of choice for acute appendicitis for most surgeons.

In 1983, Semm performed the first laparoscopic appendectomy^[2]. Ever since then, the efficiency and superiority of laparoscopic approach compared to the open technique has been the subject of much debate^[3-23]. The idea of minimal surgical trauma, resulting in significantly shorter hospital stay, less postoperative pain, faster return to daily activities, and better cosmetic outcome has made laparoscopic surgery for acute appendicitis very attractive. However, several retrospective studies^[3-12], several randomized trials^[13-19] and meta-analyses^[20-24] comparing laparoscopic with open appendectomy have provided conflicting results. Some of these studies have demonstrated better clinical outcomes with the laparoscopic approach^[13-17], while other studies have shown marginal or no clinical benefit^[18-22] and higher surgical costs^[19,23].

At present, although there is no consensus regarding the superiority of the laparoscopic approach over the conventional technique, there is trend towards greater utilization of laparoscopic appendectomy^[24,25].

In the present study, we aim to compare the laparoscopic approach and the conventional technique in the treatment of acute appendicitis, using prospectively collected data from patients subjected to appendectomy between January 2006 and January 2008.

MATERIALS AND METHODS

Data was collected prospectively on patients with acute appendicitis who underwent open or laparoscopic appendectomy from January 2006 to January 2008 in the surgery department of the University Teaching Hospital at Patras. The clinical data base contained information such as patient characteristics, postoperative course, length of hospital stay, postoperative morbidity and mortality, 30-d readmission and hospital charges.

All human studies were performed according to the principles of the declaration of Helsinki. The study was approved by the research and ethics committee at the University Hospital of Patras.

The diagnosis of appendicitis was made in the emergency department and was based on the presence of right lower quadrant pain, nausea or vomiting, and abdominal guarding on physical examination. In patients where a clinical diagnosis could not be established, imaging studies such as abdominal ultrasound or CT were performed. Exclusion criteria included pregnancy, hemodynamic instability, chronic medical or psychiatric illness, cirrhosis, coagulation disorders, previous laparotomy for small bowel obstruction, and ascites. In order to increase the homogeneity of the group, a total of 37 patients (11.2%), who underwent elective interval appendectomy or had incidental appendectomy in the presence of other intra-abdominal pathology were excluded from the study. The decision about the type of the operation was made according to the preference and experience of the surgical team on duty.

Prior to the surgery, all the patients received a standard regimen of intravenous antibiotics (1.5 g of Cefuroxime and 500 mg of Metronidazole). Provided purulent appendicitis was not observed at surgery, two additional doses were given. In patients with complicated appendicitis, antibiotics were not discontinued but were modified according to the culture results.

Open appendectomy was typically performed through a 3 cm McBurney muscle splitting incision in the right lower quadrant. Following appendectomy the stump was double ligated with an absorbable suture. In the presence of complicated appendicitis the abdomen was irrigated with warm saline solution and the skin incision was closed loosely.

In the laparoscopic group, pneumoperitoneum was produced by continuous pressure of 10-12 mmHg of carbon dioxide *via* a Verres canula, positioned in the left subcostal area. Following gas insufflation, a 12 mm trocar for the 30 degree angled laparoscope was placed in the left periumbilical area and two additional trocars, a 12 mm trocar in the suprapubic area to accommodate the stapling device and to facilitate specimen extraction, and a third 5 mm trocar in the left lower abdominal quadrant were introduced under direct visualization. The patient was placed in a Trendelenburg position, with a slight rotation to the left. The abdominal cavity was thoroughly inspected in order to exclude other intra-abdominal or pelvic pathology. After the mesoappendix was divided with bipolar forceps, the base of the

Table 1 Patient demographics

	Open appendectomy	Laparoscopic appendectomy	P
Number of patients	165	128	
Male (%)	55.1	44.5	0.33
Female (%)	44.9	55.5	0.38
Mean age	33.4 ± 18	33.8 ± 17.8	0.44
WBC count	15497 ± 3000/mm ³	15728 ± 2793/mm ³	0.80
Co-morbidities (%)			
CAD	6 (3.6)	5 (3.9)	0.63
Hypertension	13 (7.8)	9 (7)	0.71
COPD	5 (3)	4 (3.1)	0.27
DM	6 (3.6)	3 (2.3)	0.14

WBC: White blood cell; CAD: Coronary artery disease; COPD: Chronic obstructive pulmonary disease; DM: Diabetes mellitus.

appendix was secured with two ligating loops, followed by dissection distal to the second loop using a curved dissector. In patients with severe inflammation, a stapling device was used for the dissection of the appendix. The specimen was placed in an endobag and was extracted through the suprapubic trocar. All specimens were sent for histopathology.

The parameters examined in this study included patient's characteristics (age, sex), operation time (from skin incision to wound closure), conversion to open procedure, and intraoperative findings (normal, gangrenous or perforated appendix). Furthermore, during the post-operative follow up, pain was assessed both by the patient's requirements for analgesia, and with a visual analog score. The length of hospital stay, complications and cost were also added to the plot. The discharge criteria were met once the patients' were afebrile, with audible bowel sounds and were able to tolerate a liquid diet.

Statistical analysis was performed using SPSS statistical software, version 12.0 (SPSS Inc., Chicago, IL). The data were expressed as mean and standard deviation. Parameters such as length of hospitalization, mortality and morbidity, and hospital cost are given as mean variable. Bivariate analyses were performed to determine the differences between laparoscopic versus open appendectomy in patient characteristics, length of hospital stay and costs using independent sample *t* tests for continuous variables and chi-square analysis for categorical variables. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

A total of 293 patients with acute appendicitis were admitted during the study period. 165 patients were subjected to open appendectomy and 128 patients to laparoscopic appendectomy. The patient characteristics are shown in Table 1. There were no significant differences with respect to gender, age, white blood cell count at presentation, and associated co-morbidities.

Out of the total 165 open procedures, 118 (71.5%) were performed for uncomplicated appendicitis and 47 (28.5%) for complicated disease including appendiceal perforation with local or widespread peritonitis.

Table 2 Intraoperative variables

	Open appendectomy	Laparoscopic appendectomy	P
Intraoperative findings (%)			
Normal appendix	16 (9.6)	8 (6.2)	0.20
Acute appendicitis	102 (61.8)	82 (64)	0.72
Gangrenous appendicitis	19 (11.5)	20 (15.6)	0.17
Appendiceal abscess	19 (11.5)	12 (9.3)	0.32
Peritonitis	9 (5.4)	6 (4.6)	0.14
Mean operative time (min)	47 ± 19.7	44.3 ± 24	0.31

Table 3 Postoperative complications *n* (%)

	Open appendectomy	Laparoscopic appendectomy	P
Uncomplicated disease	118	90	
Wound infection	1 (0.8)	0 (0)	0.01
Bowel injury	0 (0)	1 (1.1)	< 0.001
Morbidity (%)	0.8	1.1	0.5
Complicated disease	47	38	
Wound infection	6 (12.8)	2 (5.3)	0.03
Intra-abdominal abscess	1 (2.1)	2 (5.3)	0.002
Bowel obstruction	5 (10.6)	3 (7.9)	0.37
Respiratory infection	4 (8.5)	2 (5.3)	0.18
Morbidity	34	23.7	0.12
Total morbidity (%)	10.3	7.8	0.43

In the laparoscopic group, 90 (70.3%) procedures involved uncomplicated disease and 38 (29.7%) complicated appendicitis (Table 2). Additionally, in 16 (9.6%) open and 8 (6.2%) laparoscopic procedures, no pathology was observed in the appendix and other intra-abdominal structures (Table 2).

The actual operating room time was similar between the two groups (47 ± 19.7 min in the open group *vs* 44.3 ± 24 min in the laparoscopic group; *P* = 0.31, Table 2). Conversion to an open procedure was required in two patients (1.5%) with extensive cecal adhesions secondary to severe inflammation rendering appendiceal mobilization and visualization difficult and dangerous.

There was no mortality in either group and the overall morbidity was not significantly different (10.3% in the open group *vs* 7.8% in the laparoscopic group; *P* = 0.43, Table 3).

In patients with uncomplicated disease, the morbidity rates were low (0.8% in open appendectomy and 1.1% in laparoscopic appendectomy; *P* = 0.5, Table 3). One patient subjected to open appendectomy developed wound infection. The culture of pus revealed *E. coli* and the patient was successfully treated with antibiotics and wound debridement. Similarly, in one patient in the laparoscopic group, intestinal injury occurred during insertion of the visiport. The lesion was recognized intraoperatively and was successfully managed with endoscopic sutures. The end result was favorable and no further manipulations were required.

In contrast to uncomplicated disease, patients with complicated appendicitis were prone to postoperative complications (34% after open appendectomy and 23.7% after laparoscopic approach; *P* = 0.12, Table 3). Postop-

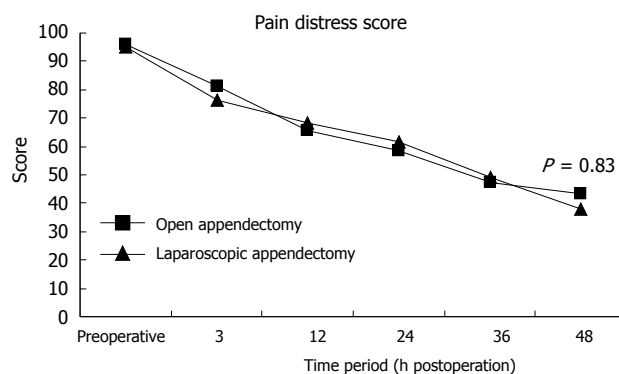


Figure 1 Visual analogue score (VAS) for pain assessment.

erative bowel obstruction was observed in patients with complicated disease in both study groups (10.6% after conventional appendectomy and 7.8% after laparoscopic appendectomy; *P* = 0.37, Table 3). In addition, complicated appendicitis was associated postoperatively with respiratory infection in 4 patients subjected to open appendectomy, and 2 patients treated laparoscopically (*P* = 0.18, Table 3).

Infectious complications were seen in both study groups in patients with complicated disease. Open appendectomy was associated with a significantly higher incidence of wound infection compared with the laparoscopic group (12.8% *vs* 5.3%; *P* = 0.03, Table 3).

On the other hand, the incidence of intra-abdominal abscess formation was higher in patients with severe peritonitis who were treated laparoscopically (5.3% *vs* 2.1%; *P* = 0.002, Table 3). All patients who developed intra-abdominal abscess were treated successfully with antibiotics and CT-guided drainage of the collection, and had an uneventful recovery.

Bowel movements in the first postoperative day were observed in 92% patients subjected to laparoscopic appendectomy and 67% in the open group (*P* < 0.001). As a result, 78% patients in the laparoscopic group and 51% in the open group were able to tolerate a liquid diet within the first 24 postoperative hours (*P* < 0.001). The mean postoperative hospital stay was 2.2 d (range, 1-17 d) after laparoscopic appendectomy and 3.1 d (range, 1-18 d) after open appendectomy (*P* = 0.04).

Visual analogue pain scores were similar in the two groups for the first two postoperative days (Figure 1). There was a significant decline after the first 3 postoperative hours to 48 h in both groups. There was no difference between open and laparoscopic groups with respect to either overall pain level (*P* = 0.93) or degree of pain remission (*P* = 0.82). Eventually, the need for analgesic medication usage for the control of postoperative pain was similar in the two groups.

Finally, the operative costs were higher by 370 € in the laparoscopic group. In the present study, the costs were calculated based on the most cost effective materials used such as laparoscopic equipment, versatile laparoscopic instruments, endoloops and collection bags. Hospital charges regarding anesthesia were not added to the plot since there was no difference in the operative times.

DISCUSSION

Acute appendicitis is the most common intra-abdominal condition requiring emergency surgery^[26]. Although more than 20 years have elapsed since the introduction of laparoscopic appendectomy, there is no consensus on its advantages and disadvantages compared to the conventional technique.

Recent studies have shown significant advantages of laparoscopic appendectomy with respect to the length of hospital stay, postoperative pain and infectious complications^[5,8,12,14,18]. These findings have been challenged by other authors who observed no significant difference in the outcome between the two procedures, and moreover noted higher costs with laparoscopic appendectomy^[3,17,19,25,27].

Bearing in mind that laparoscopic appendectomy, unlike other laparoscopic procedures, has not been found superior to open surgery for acute appendicitis, we designed the present study to determine any possible benefits of the laparoscopic approach.

Operation time remains a topic of much debate among experts. Preliminary studies^[28-30] have shown significantly longer operative times for laparoscopic appendectomy. The inexperience of the surgeons with the new technique may contribute to the longer duration of the operation in the early studies. However, recent studies^[16-18] have supported the initial findings. Because in these studies, most of the operations were performed by residents, the longer operation times can be attributed to the learning curve. By contrast, in the present study, the operation times were nearly similar in the two techniques, and the learning curve effect was minimal as the surgeons performing the procedures were highly experienced with a wide spectrum of laparoscopic procedures, including laparoscopic bariatric surgery and laparoscopic colectomy. This experience is reflected in our study by the relatively narrow range of operative times in the laparoscopic group (44.3 ± 24).

Previous studies have given conflicting results with respect to the length of hospital stay after laparoscopic appendectomy. Guller *et al*^[12] in a population-based analysis using a national administrative data base showed that laparoscopic appendectomy is associated with significantly shorter hospital stay. These findings were supported by the Cochrane Collaboration large scale meta-analysis^[24]. In agreement with these studies, we found that hospital stay was significantly shorter in patients subjected to laparoscopic appendectomy ($P = 0.004$). In the present study, bowel movements were observed significantly earlier in patients managed laparoscopically, leading to earlier feeding and discharge from hospital.

In the present study, pain was assessed both subjectively *via* a visual analogue scale and objectively by the tabulation of analgesic use. Although some studies have reported less pain in the first 48 h after laparoscopic appendectomy^[20,21,24,25,31], in our series there was no difference between the two groups with respect to either the visual analogue scores or the use of analgesics. Our study suffered from the drawback that it

was not blinded. As a result, the perception of pain may have been influenced by the patient's enthusiasm for a novel technique.

There was no mortality in our study. This is consistent with the majority of previous publications. It has been reported that the mortality rate is 0.05% and 0.3% in laparoscopic and open appendectomy respectively^[12]. The low mortality rates indicate that appendectomy, especially in the absence of complicated disease, is a safe procedure regardless of the technique used.

In the present study, the overall complication rates were 10.6% and 8.1% for open and laparoscopic appendectomy respectively. These results are in agreement with previous reports, which vary from 5.7% to 25.8% for open appendectomy and 3% to 19% for laparoscopic appendectomy^[13-15,20-23].

Complicated appendicitis was initially considered as a contraindication to laparoscopic appendectomy^[32,33]. However, recent studies have shown that laparoscopic approach in complicated disease is feasible and may even be superior to the conventional approach^[6,7,10].

In our series, 28.5% patients in the open group and 29.7% in the laparoscopic group had complicated disease. These patients are considered to be at increased risk of postoperative infections such as wound infection and intra-abdominal abscess formation^[34,35]. According to the Cochrane systemic review of the literature^[24], wound infection is about one-half after laparoscopic appendectomy, while intra-abdominal abscess formation is 3 times higher after laparoscopic appendectomy.

In the present study, the rate of wound infection in patients with complicated disease was significantly lower after laparoscopic appendectomy (5.3% *vs* 12.8%, $P = 0.03$). Placement of the detached appendix into an endobag before its removal from the abdominal cavity reduces contact with the fascial surfaces and minimizes contamination.

Intra-abdominal abscess formation was more common after laparoscopic appendectomy in complicated disease (5.3% *vs* 2.1%, $P = 0.002$). It has been suggested that carbon dioxide insufflation may promote mechanical spread of bacteria in the peritoneal cavity, especially in cases of ruptured appendix^[21,36-38]. In order to decrease the bacterial load and hence the risk of abscess formation, we advocate extensive irrigation of the abdominal cavity. However, in our practice, we observed that meticulous irrigation was unnecessary and even more dangerous as it leads to contamination of the entire abdominal cavity, which is difficult to aspirate latter. That was the case in two patients with severe peritonitis where intra-abdominal abscess formation occurred. Ever since we have changed our practice to simple suctioning of the infected area, we have not observed any postoperative abscess formation, even in patients with severe peritonitis.

The higher cost of laparoscopic appendectomy compared to the conventional technique is considered as an obstacle to its greater use. However, hospital charges for laparoscopic appendectomy have reduced

dramatically over the past several years^[39]. Surgical expertise and the abundance of laparoscopic equipment have significantly reduced the economical mismatch in favor of the conventional technique. In addition, Moore and coworkers, using a decision analysis model, have demonstrated an economic benefit of laparoscopic appendectomy from a social perspective, since shorter hospital stay and earlier return to daily activities is very important, especially for patients who are young and lead a productive life^[40].

In the present study, the operative costs for laparoscopic appendectomy were only 370 € higher. The greater cost of laparoscopic appendectomy observed in various studies^[3,14,25] can be attributed to the use of disposable laparoscopic instruments and the longer operative time. In our series, we were able to minimize the operative costs, mainly by employing reusable laparoscopic instruments.

Although there is no consensus with regard to the advantages of the laparoscopic approach compared to the conventional technique, the use of laparoscopic appendectomy has increased significantly in the last several years. In the present study, we were able to demonstrate the superiority of the laparoscopic approach in terms of hospital stay and wound infection, with only marginally higher hospital costs. Although the incidence of intra-abdominal abscess formation was higher after laparoscopic appendectomy, all complications occurred early in our practice. Greater experience and improvements in our technique has made it possible to eradicate this catastrophic complication.

Provided that surgical experience and equipment are available, laparoscopic appendectomy is safe and equally efficient compared to the conventional technique. However, as long as there is no consensus to the best approach for appendicitis, the choice of the procedure will be based on the preference of the surgeons and patients.

COMMENTS

Background

Laparoscopic surgery has been available for a long time. Today, even the most complicated procedures can be performed laparoscopically. However, laparoscopic appendectomy, a relatively easy procedure, has not gained wide acceptance among surgeons, and the conventional technique remains the procedure of choice in many centres worldwide.

Research frontiers

Intra-abdominal abscess formation is the most catastrophic complication of laparoscopic appendectomy. By simple suctioning of the infected area, rather than using widespread irrigation we were able to decrease the incidence of postoperative abscess formation.

Innovations and breakthroughs

In the present study, we were able to demonstrate that laparoscopic appendectomy is superior to the conventional technique in terms of hospital stay and wound infection. Additionally, in expert hands, even the most serious complications such as an intra-abdominal abscess formation can be minimized. Furthermore, in the present study, we were able to decrease medical costs by employing reusable laparoscopic equipment.

Applications

The present study has shown that laparoscopic surgery should be considered in every patient with appendicitis.

Peer review

The authors demonstrated a prospective study of laparoscopic versus open appendectomy and concluded "Provided that surgical experience and equipment are available, laparoscopic appendectomy is safe and equally efficient alternative to conventional technique." This present study is an interesting and novel.

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Relationship between microvessel count and post-hepatectomy survival in patients with hepatocellular carcinoma

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analysis (risk ratio, 1.64; $P = 0.024$), in addition to tumor size, vascular invasion, macroscopic finding and hepatic dysfunction. Significant differences in disease-free and overall survivals by MVC were observed in HCC patients with mJIS 2 ($P = 0.046$ and $P = 0.0014$, respectively), but not in those with other scores.

CONCLUSION: Tumor MVC appears to offer a useful prognostic marker of HCC patient survival, particularly in HCC patients with mJIS 2.

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Key words: Hepatocellular carcinoma; Hepatic resection; Microvessel count; CD34; Modified Japan integrated staging score

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Abstract

AIM: To elucidate the relationship between the microvessel count (MVC) by CD34 analyzed by immunohistochemical method and prognosis in hepatocellular carcinoma (HCC) patients who underwent hepatectomy based on our preliminary study.

METHODS: We examined relationships between MVC and clinicopathological factors in 128 HCC patients. The modified Japan Integrated Staging score (mJIS) was applied to examine subsets of HCC patients.

RESULTS: Median MVC was 178/mm², which was used as a cut-off value. MVC was not significantly associated with any clinicopathologic factors or postoperative recurrent rate. Lower MVC was associated with poor disease-free and overall survivals by univariate analysis ($P = 0.039$ and $P = 0.087$, respectively) and lower MVC represented an independent poor prognostic factor in disease-free survival by Cox's multivariate

INTRODUCTION

Hepatic resection is a useful option for radical treatment of hepatocellular carcinoma (HCC). However, recurrence rate after resection remains high^[1,2]. Patient survival thus remains unsatisfactory due to high recurrence rate even at this stage. Clinicopathological factors in HCC are related to tumor recurrence^[3] and tumor biological characteristics provide useful information regarding the activity of HCC. According to previous reports, candidates for tumor biological factors and molecular markers include abnormal expression of p53^[4], nm23 (a tumor suppressor gene)^[5], tumor angiogenesis^[6], proliferative activity^[7], growth factors^[8], DNA ploidy^[9] and other molecular markers^[10]. Some of

these markers are related to prognosis in HCC patients. Combination of conventional clinicopathological factors and prognostic factors of tumor biology may improve prediction of prognosis after hepatectomy for HCC and may contribute to a new staging classification.

Tumor angiogenesis may be important to support tumor growth^[11], and HCC is a hypervascular tumor expressing several angiogenic factors^[12]. Levels of angiogenic factors such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (b-FGF) are increased in HCC and might affect patient survival^[12,13]. Recent studies have also shown that microvessel density (MVD) in HCC or non-cancerous liver surrounding tumor correlates with tumor aggressiveness and prognosis^[6,14-16]. We have previously provided a preliminary demonstration of the comprehensive analysis of various biological factors, revealing that microvessel count (MVC) using CD34 antibody was independently associated with poor prognosis in HCC patients undergoing hepatic resection by multivariate analysis^[17]. However, contrary to other reports, poor patient prognosis was related to lower MVCs. We hypothesize that hypovascularity in HCC represents a factor associated with treatment-resistance such as chemoembolization, which causes poor prognosis.

The present study examined the relationship between MVC in HCC using immunohistochemical stains and conventional clinicopathological factors and prognosis in a larger number of HCC patients with longer follow-up period to clarify our hypothesis. Furthermore, we examined this relationship in subsets of patients after applying the modified Japan Integrated Staging score (mJIS).

MATERIALS AND METHODS

Patients

HCC specimens from 128 patients (104 men, 24 women) were obtained during surgery on patients admitted to the Division of Surgical Oncology at Nagasaki University Graduate School of Biomedical Sciences (NUGSBS) between 1990 and 2005. Mean age for patients at the time of surgery was 62.9 ± 8.2 years (range, 28-78 yr). Prior to surgery for HCC, 36 patients (28.1%) were treated using either chemoembolization ($n = 30$) or local ablation ($n = 6$), including alcohol injection in 2 patients and radiofrequency ablation (RFA) in 4 patients. After surgery, 3 patients (2.3%) received adjuvant 5-fluorouracil chemotherapy by intra-arterial injection through a subcutaneously implanted reservoir. Child-Pugh classification was B in 11 patients (8.6%) and A in 117 patients. The liver damage grade by the Liver Cancer Study Group (LCSG) of Japan in 2000 was B in 26 patients and A in 102 (Table 1)^[18]. The operative procedures included lobectomy or extended lobectomy ($n = 54$), segmentectomy or subsegmentectomy ($n = 43$) and partial resection ($n = 31$). Radical hepatectomy was performed to remove hepatic tumor without leaving any residual tumor. All hepatic tumors were completely

Table 1 Definition and criteria of Child-Pugh classification and liver damage grade

	A	B	C
Child-Pugh classification			
Encephalopathy	None	Mild	Coma
Ascites	None	Responsive	Unresponsive
Serum bilirubin (mg/dL)	< 2.0	2.0-3.0	> 3.0
Serum albumin (g/dL)	> 3.5	2.8-3.5	< 2.8
Prothrombin activity (%)	> 70	40-70	< 40
Liver damage grade ^[18]			
Ascites	None	Responsive	Unresponsive
Serum bilirubin (mg/dL)	< 2.0	2.0-3.0	> 3.0
Serum albumin (g/dL)	> 3.5	3.0-3.5	< 3.0
ICG R15 (%)	< 15	15-40	> 40
Prothrombin activity (%)	> 80	50-80	< 50

ICG R15: Indocyanine green retention rate at 15 min.

resected without macroscopic exposure of the amputated section to the remaining liver. The present series included no in-hospital deaths and the only causes of death were cancer-related. Minimum follow-up period after hepatic resection of HCC was 24 mo.

We used the classification system of the General Rules for the Clinical and Pathological Study of Primary Liver Cancer^[19]. This system provides a clinicopathological evaluation of HCC. Macroscopic classification as described by Classification of Primary Liver Cancer^[19] was also applied in the study. All study protocols were approved by the Human Ethics Review Board of our institution. Informed consent for data collection was obtained from each patient during this period. Anesthetic and patient data were retrieved from the NUGSBS database.

Immunohistochemical staining

Resected specimens were fixed in 10% formalin and embedded in paraffin. Thin sections (4 μ m) were deparaffinized twice using xylene and rehydrated in a series of ethanol solutions (100%, 90% and 80%). Sections were placed in 0.01 mol/L trisodium citrate dehydrate buffer (pH 6.0) and treated in a microwave oven for 10 min at 500 W. For CD34 staining^[17,20], tissue sections were digested with 0.2% trypsin in 0.01 mol/L phosphate-buffered saline (PBS) for 20 min at 37°C. In the next step, tissues were immersed in 3% H₂O₂ with distilled water for 10 min to inactivate endogenous peroxidases. After blocking non-specific binding by normal goat serum, sections were incubated overnight at 4°C with mouse anti-monoclonal CD34 antibody (1:25; QB-END/10, Novocastra Laboratories, Newcastle, United Kingdom) as the primary antibody. This was followed by reaction with biotinylated anti-immunoglobulin and reagent using labeled streptavidin-biotin (LSAB) kit peroxidase (Dako, Carpinteria, CA). The peroxidase reaction was visualized with 0.01% H₂O₂ and 3,3'-diaminobenzidine under light microscopy ($\times 200$). For MVCs using CD34 staining, average count was determined in the 5 most-vascular areas in the HCC examined at $200 \times$ magnification^[17,20]. Two pathologists blindly assessed each slide.

Table 2 Definition and criteria of TNM stage for HCC according to the Liver Cancer Study Group of Japan^[18]

Criteria for TNM categories	
(1) Number of tumors: 1	
(2) Tumor size: ≤ 2 cm	
(3) No vascular or bile duct invasion	
T category	T1: Fulfilling all three criteria T2: Fulfilling two criteria T3: Fulfilling one criterion T4: Fulfilling none of the criteria
N category	N0: Absence of lymph node metastasis N1: Presence of lymph node metastasis
M category	M0: Absence of distant metastasis M1: Presence of distant metastasis
Stage I	T1 N0 M0
Stage II	T2 N0 M0
Stage III	T3 N0 M0
Stage IV-A	T4 N0 M0 or T1-T4, N1M0
Stage IV-B	T1-4, N0 or N1, M1

Table 3 Definition and criteria for JIS and mJIS

	Score			
	0	1	2	3
Original JIS score ^[21]				
Japanese TNM stage	I	II	III	IV
Child-Pugh Classification	A	B	C	
Modified JIS score ^[22]				
Japanese TNM stage	I	II	III	IV
Liver damage grade	A	B	C	

TNM: tumor-node-metastasis.

Staging criteria for the mJIS

We used the pathological tumor-node-metastasis (pTNM) classification system as defined by the Liver Cancer Study Group (LCSG) of Japan in 2000^[18]. T category was determined based on 3 factors: number, size, and vascular or bile duct invasion. N category was determined as the presence of lymph node metastasis, while M category represented the presence of distant metastases. TNM staging comprises 4 stages based on the combination of T, N, and M categories (Table 2). The original Japan Integrated Staging score proposed by Kudo *et al.*^[21] comprised the sum of scores for the two variables of Japanese TNM classification and Child-Pugh classification. In the mJIS proposed by our institute^[18,22], Child-Pugh classification was replaced by the score for liver damage grade as defined by the LCSG of Japan (Table 3).

Statistical analysis

Continuous data are expressed as mean ± standard deviation. Data from different groups were compared using one-way analysis of variance (ANOVA) and examined by Student's *t*-test or Dunnett's multiple comparison test. For univariate analysis, categorical data were analyzed using Fisher's exact test. Disease-free and overall survival rates were calculated according to the Kaplan-Meier method, and differences between groups were tested for significance using the log-rank test.

Multivariate analysis was performed using proportional hazards regression modeling. A two-tailed value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SAS software (Statistical Analysis System, Cary, NC).

RESULTS

Among the 128 patients in the present study, disease-free 1-, 3- and 5-year survival rates were 63%, 39% and 29%, respectively, and median disease-free survival was 3.5 years. Overall 1-, 3- and 5-year survival rates were 89%, 65% and 48%, respectively, and median overall survival was 5.9 years. Of 94 patients (75.0%) who displayed tumor recurrence after hepatectomy, 85 (90.4%) received chemoembolization ($n = 81$) or alcohol injection ($n = 4$).

Median MVC within the tumor area was 178/mm², and this value was applied as a cut-off value. Table 4 shows the relationship between MVC and clinicopathological features in 128 HCC patients. However, MVC was not significantly associated with any clinicopathological factors, TNM stage or postoperative recurrence.

Figure 1 shows disease-free and overall survival after hepatectomy compared to MVC. Disease-free survival rate was significantly lower in patients with lower MVC than in patients with higher MVC ($P = 0.039$). Overall survival rates tended to be lower in patients with lower MVC than in patients with higher MVC, but this difference was not significant. Table 5 shows the results of multivariate analysis for disease-free and overall survival after hepatectomy for various factors identified as displaying significant associations on univariate analysis. Multiple tumors, vascular involvement of the tumor, liver damage grade B and lower MVC represented independent risk factors for poor disease-free survival after hepatectomy ($P = 0.0004$, 0.003, 0.007 and 0.024, respectively). Multiple tumors, vascular involvement of the tumor and macroscopic findings were identified as independent risk factors for poor overall survival after hepatectomy ($P = 0.025$, 0.014 and 0.017, respectively), whereas MVC was not associated with overall survival ($P = 0.266$).

Figure 2 shows disease-free survival by comparing MVC for each mJIS score. In HCC with mJIS 0 or 1, MVC was not associated with disease-free survival. Lower MVC was significantly associated with poor disease-free survival in HCC with mJIS 2. Lower MVC tended to be associated with poor disease-free survival in HCC with mJIS ≥ 3, but this result was not significant. Figure 3 shows overall survival by comparing MVC at each mJIS score. In HCC with mJIS 0, 1 or ≥ 3, MVC was not associated with overall survival. Lower MVC was significantly associated with poor disease-free survival in HCC with mJIS 2.

DISCUSSION

Previous studies have investigated prognostic factors in HCC for patients who underwent radical hepatectomy

Table 4 Relationship between microvessel density and clinicopathological factors in HCC

	Microvessel count > 178/mm ² /≤ 178/mm ²	P
Pretreatment		
No (n = 98)	53/45	1.0
Yes (n = 30)	16/14	
Liver damage		
A (n = 102)	54/48	0.969
B (n = 26)	15/11	
Background liver		
Normal liver (n = 7)	3/4	0.459
Chronic hepatitis (n = 75)	38/37	
Cirrhosis (n = 46)	28/18	
Viral status		
Hepatitis virus B (n = 50)	32/18	0.152
Hepatitis virus C (n = 61)	31/30	
Both hepatitis virus B and C (n = 4)	2/2	
Non-B, non-C (n = 13)	4/9	
Number of tumors		
Solitary (n = 92)	50/42	1.0
Multiple (n = 36)	19/17	
Tumor size		
< 3 cm (n = 31)	15/16	0.371
3-5 cm (n = 49)	24/25	
> 5 cm (n = 48)	30/18	
Macroscopic finding ¹		
SN (n = 37)	16/21	0.207
SNEG (n = 39)	25/14	
CMN (n = 52)	28/24	
Vascular involvement		
No (n = 83)	42/41	0.476
Yes (n = 45)	27/18	
Histopathological differentiation		
Well (n = 19)	12/7	0.437
Moderately (n = 97)	55/42	
Poorly (n = 6)	2/4	
Japan tumor-node-metastasis stage ²		
I (n = 14)	5/9	0.542
II (n = 51)	27/25	
III (n = 44)	25/19	
VI-A (n = 19)	12/7	
Recurrence		
No (n = 32)	16/16	0.386
Yes (n = 96)	53/43	

¹Macroscopic classification by Liver Cancer Study Group of Japan^[19]; ²The General Rules for the Clinical and Pathological Study of Primary Liver Cancer^[18]. SN: Single nodular; SNEG: Single nodular with extranodular growth; CMN: Confluent multinodular group.

and in whom tumor stage had been determined^[23,24]. The International Union against Cancer (UICC) and Japanese TNM classification systems for predicting patient prognosis in HCC did not provide good reflection of patient survival in HCC patients^[25,26]. Combined staging systems with hepatic function have recently been applied^[21,22,27,28], and among these, the JIS score system was applied in Japan^[21]. Child-Pugh classification was used in this system, but liver damage grade by the LCSG of Japan offers a better reflection of patient survival^[18,29]. The mJIS score system thus includes liver damage grade and has been applied in a few reports^[22]. The present study also applied mJIS score. In the next step, a staging system for HCC may need additional useful factors comprising tumor biological or molecular markers. The present study identified angiogenic factors of MVC

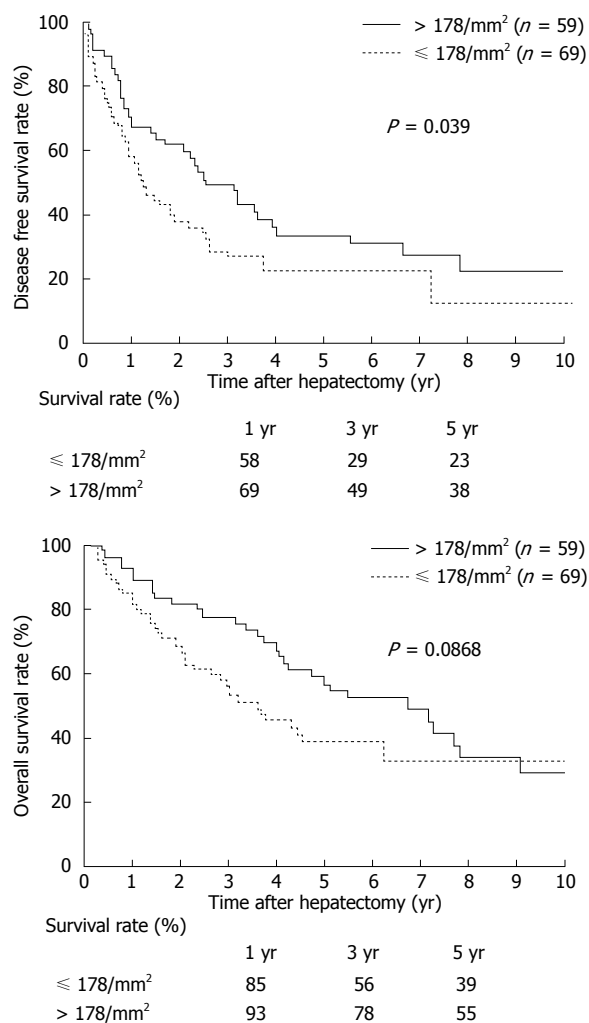


Figure 1 Relationship between microvessel count (MVC) and disease-free and overall survival in patients with hepatocellular carcinoma (HCC) who underwent hepatic resection.

as potentially useful. In the last decade, microvessel density using CD34 as a prognostic parameter in HCC patients has been reported and appears consistently very useful^[6,16,30,31]. We also performed a preliminary study of the significance of MVC for survival in HCC patients and, unlike other reports, revealed hypovascularity (i.e., lower MVC) as a poor prognostic parameter^[17].

MVC was not associated with any clinicopathological factors or recurrence rate after hepatectomy in either the present study or our pilot study^[17]. In contrast to our results, El-Assal *et al*^[30] and other investigators^[6,14,15,30-32] have reported that microvessel density in HCC is increased in larger tumors, tumors with poor differentiation and cirrhotic patients, while higher microvessel density is associated with intra-hepatic recurrence. In the present study, however, MVC did not correlate with co-existing cirrhosis or with various etiological factors related to chronic hepatitis such as viral status. Conversely, Sun *et al*^[33] found no relationship between MVC and either clinicopathological factors or patient prognosis. The relationship between tumor vascularity and clinicopathological features thus remains controversial. Increased micro-angiogenesis is definitely

Table 5 Multivariate analysis by Cox’s proportional hazard test of prognostic factors influencing disease-free survival and overall survival in HCC after hepatectomy

	Disease-free survival			Overall survival		
	Risk ratio	95% CI	P	Risk ratio	95% CI	P
Number ≥ 2 lesions	2.28	1.44-3.60	0.0004	1.87	1.08-3.24	0.025
Vessel involvement positive	2.04	1.28-3.24	0.003	2.07	1.16-3.68	0.014
Macroscopic finding SNEG or CMN	1.43	0.89-2.29	0.135	2.33	1.17-4.66	0.017
Surgical margin positive	1.75	0.93-3.33	0.086	1.59	0.65-3.91	0.310
Blood loss > 1500 mL	1.36	0.81-2.29	0.135	1.50	0.82-2.74	0.189
Liver damage grade B	1.99	1.20-3.31	0.007	1.44	0.77-2.69	0.255
PIVKA-II > 400 mAU/mL	1.30	0.81-2.13	0.274	1.15	0.65-2.04	0.636
Microvessel count ≤ 178/mm ²	1.64	1.06-2.50	0.024	1.35	0.71-2.04	0.266

Macroscopic classification by Liver Cancer Study Group of Japan^[19]. SN: Single nodular; SNEG: Single nodular with extranodular growth; CMN: Confluent multinodular group; CI: Confidence interval.

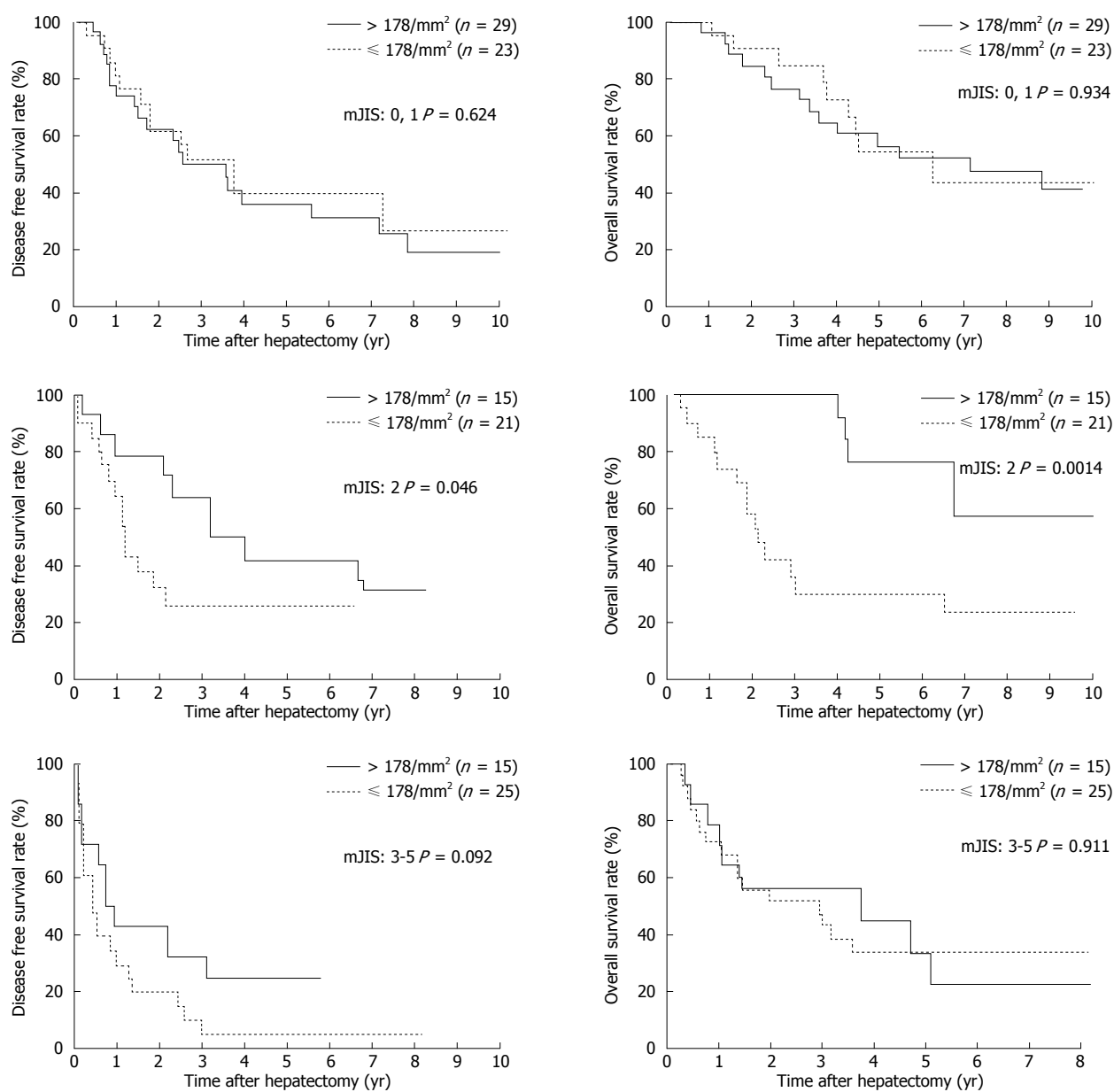


Figure 2 Relationship between MVC and disease-free survival using the modified Japan Integrated Staging score (mJIS) in HCC patients who underwent hepatic resection.

Figure 3 Relationship between MVC and overall survival using the mJIS in HCC patients who underwent hepatic resection.

associated with carcinogenesis of HCC and development to advanced tumor^[12,32]. At the stage of smaller-sized HCC, tumor vascularity is already rich on computed

tomography or enhanced ultrasonography^[34,35]. Considering the mechanisms of HCC characterized above, increased microvessel density of the tumor is a logical result. When tumor demonstrates a large tumor size or poor histologic differentiation, tumor vascularity may be decreased. Previous reports also showed that HCC tumor vascularity decreased with the progression of histopathological grade^[36,37]. Our results showed that lower MVC tended to be higher in poorly differentiated HCC, but this finding was not significant. A larger sample of poorly differentiated HCCs is needed to clarify this issue, as only 6 cases were included in the present series.

With respect to disease-free and overall survival after hepatectomy^[1,2,6,24], the results from our series were favorable. Counts $\leq 178/\text{mm}^2$ for HCC were associated with worsened prognosis in patients undergoing hepatectomy, particularly in disease-free survival by univariate and multivariate analysis in the present study. Compared to other independent risk factors, the odds ratio was lower for MVC than for other factors such as a number of tumors, vascular involvement, macroscopic findings or liver damage grade. The role of MVC for tumor relapse or progression might not be particularly strong. This result contradicts findings in other reports described above^[6,14,16,30,31]. As explained above, HCC naturally acquires rich tumor vascularity in the early stages and most clinically treated HCCs represent tumor with radiological enhancement^[34,35,38]. Hypervascularity is observed in the majority of HCC^[36]. As tumor vascularity decreases in HCC with deterioration of histological differentiation and/or aggressive invasiveness^[36,37], the malignant potential of hypovascular HCC could increase relative to that of hypervascular HCC, thus leading to poorer prognosis. In cases of hypervascular HCC, initial or recurrent tumor can be observed in earlier stages under various imaging modalities, and treatments combined with chemoembolization or chemotherapy via tumor microvessels may well prove effective^[39]. We speculate that hypovascular HCC is difficult to detect by conventional imaging and to treat by chemoembolization or other therapy, and treatment selection is therefore limited. Previous studies showed that tumor vascularity correlates with the response to chemotherapy, which in turn correlates with survival^[40-42]. Furthermore, tumor hypervascularity, which is observed in most HCC, correlated with good response to chemoembolization, whereas hypovascularity of early or sclerosing HCC did not correlate with good response to chemoembolization^[40,41]. Thus, a better response to arterial chemoembolization is associated with prolonged survival^[40,41]. In our series, most patients with postoperative recurrence of HCC received arterial chemoembolization and, therefore, the prognosis of these patients could have been influenced by tumor vascularity and response to chemoembolization. Although we could not provide such evidence in recurrent tumors in our follow-up study, it is possible that the vascularity of primary HCC could influence the biological characteristics of recurrent HCC. For these

reasons, the survival of patients with hypovascular HCC logically would be worse after recurrence. Our results concerning MVC are thus biologically quite feasible. Anti-angiogenic therapy is a promising treatment for HCC^[43]; however, the response of hypovascular HCC to such treatment remains problematic.

In addition to tumor-associated factors, hepatic functional reserve and liver function after surgery influence postoperative prognosis^[3,6,44]. In the present series, we applied mJIS score to examine subsets of HCC patients. Poon *et al*^[6] also reported that the significance of MVC differed between subsets of HCC patients. Our results showed no marked differences in disease-free and overall survival according to MVC in the early stage of mJIS 0 or the advanced stage of mJIS 3-5. A significant difference in MVC was only observed for mJIS 2. Poon *et al*^[6] found microvessel density by CD34 as the only significant factor predictive of disease-free survival in patients with HCC (5 cm, but no significant prognostic influence was seen for larger HCC. In early-stage HCC, tumor can be sufficiently cured by hepatic resection even in the presence of malignant potential. Conversely, in severely advanced HCC, other significant prognostic factors might exert greater influence on tumor aggressiveness and survival, such as number of tumors, vascular involvement, macroscopic findings or liver damage grade, as indicated in the present study. For mJIS 2, patient survival with hepatic resection or ablation therapy was not particularly satisfactory compared to mJIS 0 or 1 according to our recent report^[45]. In this stage of mJIS 2, treatment indications need to be defined according to the appropriate prognostic factors. In cases where lower vessel count was observed at initial resection, liver transplantation following recurrence may be a good option. Our results showed that MVC did not correlate with other clinicopathological parameters and is an independent risk factor for prognosis. Thus, according to the present results, MVC would represent a useful parameter to decide treatment modality.

In conclusion, we have demonstrated that lower MVC by CD34 in HCC offers an independent predictor of disease-free and overall survival in patients with HCC, particularly in HCC with mJIS 2. As a tumor biological factor, MVC representing tumor angiogenesis offer a new candidate prognostic factor in HCC to predict tumor recurrence and patient survival in combination with traditional pathological factors. Furthermore, this marker can be applied as a predictive marker to select molecular targeting treatments in future.

COMMENTS

Background

Tumor biological characteristics provide useful information on the activity of hepatocellular carcinoma (HCC). The combination of conventional clinicopathological factors and prognostic factors related to tumor biology may improve prediction of prognosis of patients with HCC. HCC is a hypervascular tumor that expresses various tumor angiogenic factors. Microvessel density (MVD) in HCC may correlate with tumor aggressiveness and prognosis.

Research frontiers

Tumor angiogenesis may be important for tumor growth. High levels of

angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF) have been described in HCC, and they correlate with patient survival. Microvessel counts (MVC) in HCC or non-tumorous liver tissue that surrounds HCC correlate with tumor aggressiveness and prognosis. On the other hand, it should be difficult to chemoembolize hypovascular HCC. Therefore, we hypothesized that low MVCs correlates with poor prognosis.

Innovations and breakthroughs

Our results presented new findings that were different than studies published previously by other investigators. However, we found that a low MVC is an independent prognostic factor for tumor relapse. This was particularly significant in HCC patients with mJIS 2.

Applications

MVC could be a potentially useful marker of prognosis in HCC by predicting tumor recurrence and patient survival, in addition to traditional pathological factors. Furthermore, MVC could help in clinical decision making with respect to the selection of treatment modality.

Peer review

Our study identified a novel prognostic factor that can be used to predict tumor recurrence and survival of patients with HCC. This marker can be potentially used to select the most appropriate surgical treatment modality, such as a liver transplantation particularly in patients with mJIS 2.

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OCTN and CARD15 gene polymorphism in Chinese patients with inflammatory bowel disease

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Abstract

AIM: To investigate the single nucleotide polymorphism (SNPs) distribution of NOD2/CARD15 (R702W, G908R), OCTN1 1672C/T and OCTN2-207G/C in Chinese patients with inflammatory bowel disease (IBD).

METHODS: A total of 61 patients with Crohn's disease (CD), 151 patients with ulcerative colitis (UC), and 200 unrelated healthy controls were genotyped. Genotyping was performed by sequence specific primer polymerase chain reaction (PCR-SSP) or by restriction fragment length polymorphism (PCR-RFLP) analysis.

RESULTS: Among the subjects in our study groups, including patients with CD, UC and healthy controls, none had OCTN and CARD15 variants and very rare IBD family history was found in our patients with the percentage of 0 (0/61 with CD) and 1.3% (2/151 with UC).

CONCLUSION: Our results indicate that although OCTN or CARD15 variation is associated with susceptibility to IBD in Western populations, these might be rare and may not be associated with susceptibility to IBD in Chinese patients.

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Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; CARD15; Carnitine/organic cation transporter gene

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC), the two common forms of idiopathic inflammatory bowel disease (IBD), are chronic, relapsing inflammatory disorders of the gastrointestinal tract. CD and UC are very common in developed countries with a prevalence of 0.7-11.6 per 100 000 and 2.0-14.3 per 100 000, while it is relatively uncommon in Asian countries with a prevalence of 0.08 per 100 000 and 0.5 per 100 000 in Japan^[1]. However, the incidence of IBD has been increasing in some Asian countries in recent years, and China is one of the notable countries^[2].

The precise etiology of the disease is unknown, but interplay of environmental risk factors and immunologic changes will trigger the onset of the disease in a genetically susceptible host. Epidemiological studies in the past suggested a genetic susceptibility that has been confirmed by total genome scans and candidate gene studies. After the IBD1 locus in the chromosome 16 was identified as a CD locus by Hugot *et al*^[3], fine mapping of the IBD1 locus and following candidate gene approach led people to identify the CARD15 (previously NOD2) as a susceptibility gene of CD. The NOD2/CARD15 gene product is expressed in monocytes. It is involved in the binding of bacteria lipopolysaccharides and peptidoglycans so that it played an important role in activation of nuclear transcription factor kappa-B (NF-κB) in inflammatory response. Two missense mutations Arg702Trp (2104C→T), Gly908Arg (2722G→C) and one frame-shift mutation (3020insC) of the NOD2/CARD15 gene affecting the function of binding microbial pathogens are independently associated with the development of CD^[4].

Numerous genome-wide scans and replication studies have identified IBD susceptibility loci since the initial

study was published in 1996 by Hugot *et al*^[3]. Recent studies suggested that *OCTN* (Carnitine/organic cation transporter gene) 1 and 2 in the IBD5 locus on chromosome 5 both encoded organic cation transporters and revealed significant associations with CD. The *OCTN* family is a family of transporter proteins for organic cations, and may also transport carnitine, an essential cofactor of the metabolism of lipids. *OCTNs* are therefore important in the maintenance of intracellular homeostasis and play an important role in the energy production of the cell. A C1672T substitution in exon 9 of the *OCTN1* gene and a G-207C in the *OCTN2* promoter region were indicated as functional and causative mutations to increase susceptibility to CD^[5].

The aim of the present study was to investigate the single nucleotide polymorphism (SNPs) distribution of *NOD2/CARD15* (R702W, G908R), *OCTN1* 1672C/T, *OCTN2*-207G/C and its association with IBD in Chinese patients.

MATERIALS AND METHODS

Study population

Blood samples from 61 patients with CD and 151 patients with UC were prospectively collected at the IBD Outpatient Clinic of the first affiliated hospital of Zhongshan University (Guangzhou, Guangdong Province, China) and Xijing Hospital of the Fourth Military Medical University (Xi'an, Shaanxi Province, China) between March 2005 and June 2006. All patients were followed up at least for one year and registered with an integrated clinical and epidemiological registry. A total of 212 healthy controls matched for age, sex and geography were healthy physical examinees in the two hospitals. All patients and healthy controls were of unrelated Chinese Han nationality. The diagnosis of either CD or UC was in accordance with previously established international criteria^[6] based upon clinical, endoscopic, radiological and histopathological findings. All patients gave informed consent to participate in the study that was approved by the Ethics Committee of Zhongshan University and the Fourth Military Medical University.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the spin column technique (TIANamp Blood DNA kit, Tiangen Biotech, China).

Gene polymorphisms (R702W, G908R, *OCTN1* 1672C/T, *OCTN2*-207G/C) were determined using the sequence specific primers by polymerase chain reaction (SSP-PCR). Primer sequences and methods are depicted in Table 1. The PCR cycling parameters were a denaturing step at 94°C (3 min); 5 cycles of 94°C (30 s), 70°C (45 s), 72°C (30 s); 18 cycles of 94°C (30 s), 65°C (50 s), 72°C (30 s); 10 cycles of 94°C (30 s), 55°C (1 min), 72°C (1 min); and a final elongation step of 72°C (10 min). The PCR products were electrophoresed on 2% agarose gels containing ethidium bromide and viewed under ultraviolet light.

Restriction fragment length polymorphism (RFLP)

Table 1 Primer sequences for SSP-PCR genotyping

SNP	Primers	PCR product (bp)
<i>OCTN1</i> 1672C/T (rs1050152)	W: TCTGACTGTCCTGATTGGA ATCC	Allel C: 518 bp
	S: TAGTCTGACTGTCCTGATT GGAATCT	Allel T: 520 bp
	C: TTTTGAGACGGAGTTT TGCTCTTGT	
<i>OCTN2</i> -207G/C (rs2631367)	W: GCACGACCAGGGAAGGTTG	Allel G: 493 bp
	S: GCACGACCAGGGAAGGTTT C: TCCCAGCCTCTCTAC TAGGGTAGTT	Allel C: 493 bp
R702W 2104C/T (rs2066844)	W: CTGAGAAGGCCCTGCTCC	Allel C: 396 bp
	S: CATCTGAGAAGGCCCTGCTCT C: CAATGCCCAGTAAC ACTCACTACAG	Allel T: 399 bp
G908R 2722G/C (rs2066845)	W: TGGCCTTTTCAGATTCTGGG	Allel G: 308 bp
	S: TGGCCTTTTCAGATTCTGGC C: TGTATCAAAACCTG AGAGGACAA	Allel C: 308 bp

and sequencing were performed as means of verifying the PCR results. Five samples were chosen from each allele (including homozygous wild-type, heterozygous SNP and homozygous SNP) to perform RFLP and sequencing. Sequencing was performed by AuGCT Corporation of Beijing. The primers of sequencing were the same with RFLP. RFLP was performed as follows: (1) PCR amplification: initially a denaturing step at 96°C (1 min); 25 cycles of 96°C (30 s), 70°C (40 s), 72°C (30 s); 10 cycles of 96°C (30 s), 65°C (30 s), 72°C (30 s); and a final elongation step of 72°C (10 min). (2) RFLP: 10 µL of the PCR products mixed with 2 µL 10 × buffer and 2 µL restriction enzyme, then adding water to 30 µL, incubated for 12 h at 37°C and electrophoresed on 20% non-denaturing polyacrylamide gels, finally viewed under ultraviolet light after stained with ethidium bromide solution for 30 min. Primer sequences and restriction enzymes are depicted in Table 2.

The results of gene sequence and RFLP are identical. The result of SSP-PCR about G908R and *OCTN2* -207G/C was consistent with sequence and RFLP, so we chose 30 samples to perform RFLP and obtained the same result. But the result of SSP-PCR about R702W and *OCTN1* 1672C/T was not consistent with sequence or RFLP, so we changed all samples to perform RFLP.

Statistics analysis

Comparison between cases and controls was made using the Chi-square test for categorical data with the SPSS software ver.13.0.

RESULTS

In this study, we first performed genotyping by SSP-PCR with less cost and time than RFLP or sequencing. But the shortage of SSP-PCR is easy to result in false positivity. So after we completed the SSP-PCR, we used

Table 2 Primer sequences and restriction enzymes used for RFLP genotyping

SNP	Primers	Restriction enzyme	Length of restriction fragments
OCTN1 1672C/T (rs1050152)	F: CGTCATGGGTAGTCTGACTGTCTGATTGGGATC R: TCCTACTTACCATTTCACTTTCIGCATCTGCTCTAAGG	<i>Bam</i> H I	Allel C: 30 + 88 bp Allel T: 118 bp
OCTN2 -207G/C (rs2631367)	F: GCGCCGCTCTGCCTGCCAG R: AGGGTAGGCTCGCGAGCTGACACC	<i>Msp</i> I	Allel G: 44 + 83 bp Allel C: 127 bp
R702W 2104C/T (rs2066844)	F: TGGGGCCTGCTGGCTGAGTG R: GTGCAGCTGGCGGGATGGAG	<i>Msp</i> I	Allel C: 76 + 45 bp Allel T: 121 bp
G908R 2722G/C (rs2066845)	F: TCTGGCTGGGACTGCAGAGG R: CCCCTCGTACCCACTCTGTCGC	<i>Bst</i> U I	Allel G: 131 bp Allel C: 109 + 22 bp

Table 3 Demographics and phenotype of IBD patients

	CD patients (n = 61)	UC patients (n = 151)
Sex (M:F)	40:21	91:60
Median age (yr, mean \pm SD)	36.9 \pm 13.7	43.8 \pm 13.4
Patients with relative(s) who have IBD	0	2 (1.3%)
Location of CD		Location of ulcerative colitis
Small bowel only (%)	25 (41.0)	Rectum sigmoid colon 86 (57.0)
Colon only (%)	7 (11.5)	Left hemicolon 18 (11.9)
Small bowel & colon (%)	29 (47.5)	Extensive 47 (31.1)
Behaviour of Crohn's disease (%)		Severe criteria of ulcerative colitis
Non-stricturing, non-penetrating (%)	26 (42.6)	Mild 74 (49.0)
Penetrating (%)	15 (24.6)	Moderate 57 (37.7)
Stricturing (%)	19 (31.1)	Severe 20 (13.2)
Stricturing & penetrating (%)	1 (1.6)	

RFLP and sequencing to verify the result. The SSP-PCR results of G908R and OCTN2-207G/C were consistent with RFLP or sequencing so that the SSP-PCR is successful in genotyping these alleles. On the contrary, we failed to genotype alleles of R702W and OCTN1 1672C/T by SSP-PCR, we therefore changed to use RFLP which had been used to detect polymorphism for a long time with reliable result.

We found very rare IBD family history in our patients with the percentage of 0 (0/61 with CD) and 1.3% (2/151 with UC). The demographics and phenotype of the IBD patients are described in Table 3.

As shown in Table 4, we found that the four SNPs, OCTN1 1672C/T, OCTN2-207G/C, R702W and G908R were completely absent in the Chinese Han nation population, in either the IBD patients or the control group. These results demonstrated that OCTN and CARD15 variations might be rare and may not be associated with susceptibility to IBD in Chinese patients of Han nation.

DISCUSSION

In the present study, we found that the polymorphism of C1672T in exon 9 of OCTN1, G-207C in the

Table 4 Genotype and allele results

SNP	Group	Cases	Genotype			Allele	
OCTN1			C/C	C/T	T/T	C	T
1672C/T (rs1050152)	CD	61	61	0	0	122	0
	UC	151	151	0	0	302	0
	Control	200	200	0	0	400	0
OCTN2			G/G	G/C	C/C	G	C
-207G/C (rs2631367)	CD	61	61	0	0	122	0
	UC	151	151	0	0	302	0
	Control	200	200	0	0	400	0
R702W			C/C	C/T	T/T	C	T
2104C/T (rs2066844)	CD	61	61	0	0	122	0
	UC	151	151	0	0	302	0
	Control	200	200	0	0	400	0
G908R			G/G	G/C	C/C	G	C
2722G/C (rs2066845)	CD	61	61	0	0	122	0
	UC	151	151	0	0	302	0
	Control	200	200	0	0	400	0

OCTN2 promoter region, and 2104C/T(R702W), 2722G/C(G908R) in CARD15 were completely absent in Chinese patients with IBD and healthy controls. The study suggested that the four SNPs might not play a role in susceptibility of IBD in Chinese patients, thereby differing from the case of Western populations, but consistent with the results in Asian population. In our study, there were 212 IBD patients (61 CD, 151 UC) and 200 healthy controls. Compared with the studies in the Chinese population before, we had the largest number of cases. In Asia, it was the first study to detect the polymorphism of OCTN in UC patients.

In the last ten years, there have been tremendous researches on genetic susceptibility of inflammatory bowel diseases (IBD) and over 10 chromosomal regions have been identified by genome-wide scanning. The regions on chromosomes 16, 12, 6, 14, 5, 19 and 1 have been renamed IBD 1-7, respectively^[7]. Further fine mapping as well as candidate gene studies have already led to the identification of a number of susceptibility genes including CARD15, DLG5, OCTN1 and 2, NOD1, HLA, TLR4, TNF- α , IL-1RA, and ICAM-1^[8]. The CARD15 gene is undoubtedly replicated most widely at present. The three NOD2/CARD15 variant alleles, Arg702Trp, Gly908Arg and 3020insC were found to increase the risk of CD in Caucasians, including those from Germany, England^[9], Australia^[10] and America^[11]. When OCTN1 and 2 were reported to be associated to IBD, the west-

ern countries such as Canada^[5], England^[12], German^[13], Greek^[14], Spain^[15] and New Zealand^[16] carried out experiments and proved that the SNPs of *OCTN1* and 2 independently or the haplotype OCTN-TC (SNPs of *OCTN1* and 2 create a two-allele risk haplotype, TC) were positively associated with IBD (with CD only in most studies). On the contrary, studies performed in Asian population differed from the case in Caucasians. The three NOD2 mutations were proved to be totally absent in the studies of Yamazaki *et al*^[17] in Japan with 483 CD patients, Lee *et al*^[18] in Korea with 128 CD and 47 UC by sequencing, and Leong *et al*^[19] in Hong Kong with 65 CD and 63 UC, Gao *et al*^[20] in Zhejiang University of China with 32 CD and 110 UC by SSP-PCR. Guo QS in Wuhan University of China found Two heterozygotes of the 3020insC mutation in 74 UC patients and one in 15 CD, and only one in healthy controls by SSP-PCR. So they concluded that the NOD2 3020insC mutation was not associated with CD or UC in Hubei Han population^[21]. Similar to the *CARD* gene, Yamazaki *et al*^[22] in Japan found the SNPs of *OCTN1* and 2 were completely absent with 484 CD patients and 345 healthy control by means of sequencing. The studies above showed that there was apparent genetic heterogeneity among Caucasians and Asians, so there should be a presence of ethnic differences in susceptibility to IBD in Chinese population.

In our study, familial clustering was rare in IBD patients. Although Chinese, Korean and Japanese races differ, the familial aggregation was similarly rare in IBD patients from the three countries. Does low prevalence of familial clustering and absence of NOD2/*CARD15* and OCTN gene variants suggest that genetic factors may play a less important role in the development of IBD in the Asian population? The study of Kim *et al*^[23] in Korea did not support this point. He found that although a positive family history [21 of 1043 (2.01%) with UC and 6 of 397 (1.51%) with CD] is much lower than that with Western patients, the population relative risk was 13.8 in first-degree relatives, indicating that a positive family history is an important risk factor for IBD in Koreans. Montgomery *et al*^[24] found that young Asians who were born in Britain are at a significantly higher risk of developing IBD than the indigenous European population with relative odds of 6.1. This may reflect a greater genetic predisposition to IBD when uncovered by exposure to environmental factors. Undoubtedly, genetic susceptibility plays a most important role in the etiology of IBD. Recently, IL23R has been regarded as a milestone in unraveling etiology of CD and SNPs of IL23R was reported to be associated with CD^[25]. We are recruiting more cases and controls to investigate whether SNPs of IL23R would also play a protective role in Chinese CD patients. Since there is great genetic heterogeneity between Chinese and the Caucasians, further studies including total genome-wide scans among Chinese patients are warranted to identify genes susceptible to IBD which would shed more light on the etiology of this disease in our own country.

COMMENTS

Background

Inflammatory bowel disease (IBD), including two clinical subtypes: Crohn's disease (CD) and ulcerative colitis (UC), has been increasing in some Asian countries in recent years, and China is one of the notable countries. Previous epidemiological studies suggested a genetic susceptibility in IBD which has been confirmed by molecular biology techniques. *CARD15* (previously NOD2) was first confirmed to be a susceptible gene of CD. Two missense mutations Arg702Trp (2104C→T), Gly908Arg (2722G→C) and one frame-shift mutation (3020insC) of the NOD2/*CARD15* gene affecting the function of binding microbial pathogens are independently associated with the development of CD. In recent years, A C1672T substitution in exon 9 of the *OCTN1* gene and a G-207C in the *OCTN2* promoter region were indicated as functional and causative mutations to enhance the susceptibility to IBD.

Research frontiers

In the last ten years, there have been tremendous researches on genetic susceptibility of IBD and over 10 chromosomal regions have been identified by genome-wide scanning. Further fine mapping as well as candidate gene studies have already led to the identification of a number of susceptible genes. When *CARD15* was first confirmed to be a susceptible gene of CD, its three variant alleles, Arg702Trp, Gly908Arg and 3020insC were found to increase the risk of CD in many western countries but completely different in Asian countries. Similar to the *CARD* gene, many western countries have proved that the SNPs of *OCTN1* and 2 were positively associated with IBD but absent in Japanese. These studies showed that there was apparent genetic heterogeneity between Caucasian and Asian as well as Chinese population.

Innovations and breakthroughs

The study suggested that the four SNPs, C1672T, G-207C, 2104C/T and 2722G/C, might not play a role in susceptibility of IBD in Chinese patients, thereby differing from the case of Western populations, but consistent with the results in Asian population. Compared with the studies in the Chinese population before, we had the largest number of cases. In Asia, it was the first study to detect the polymorphism of OCTN in UC patients.

Applications

With great genetic heterogeneity between Chinese and the Caucasian, further studies including total genome-wide scans among Chinese patients are warranted to identify genes susceptible to IBD which would shed more light on the etiology of this disease in China.

Peer review

In this study, the authors found that of the Chinese patients with IBD as well as the healthy controls none had OCTN and *CARD15* variants. These results suggest that OCTN and *CARD15* are rare in the Chinese population and may not be associated with susceptibility to IBD in Chinese patients. This was an interesting study with an aim that was well justified.

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RAPID COMMUNICATION

Permeabilities of rebamipide *via* rat intestinal membranes and its colon specific delivery using chitosan capsule as a carrier

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totally within 6 h. The area under concentration-time profile of drug in the colon mucosa using chitosan capsules (AUC_{LI}, 16011.2 ng·h/g) was 2.5 times and 4.4 times greater than using gelatin capsules and CMC suspension, respectively. Meanwhile, the area under concentration-time profile of drug in the plasma (AUC_{PL}) was 1016.0 ng·h/mL for chitosan capsule, 1887.9 ng·h/mL for CMC suspension p and 2163.5 ng·h/mL for gelatin capsule. Overall, both AUC_{LI} and AUC_{PL} were increased when C12 was co-administrated, but the increase of AUC_{LI} was much greater; the drug delivery index (DDI) was more than 1 compared with simple chitosan capsule group.

CONCLUSION: There was a regional difference in the permeability of Rebamipide across the jejunum, ileum and the colon, and passive diffusion seems to be one of the major transport mechanisms of rebamipide. Absorption enhancers can increase the permeability of rebamipide across the colon tissue significantly. In addition, chitosan capsule may be a useful carrier to deliver rebamipide to the colon specifically and the co-administration of C12 with rebamipide may also be very useful in local treatment.

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Key words: Rebamipide; Diffusion chamber; Permeability; Sodium laurate; Chitosan capsule; Colon-specific delivery

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Abstract

AIM: To investigate the permeability characteristics of rebamipide across intestinal mucosa, and examine the effects of some absorption enhancers on the permeability across the colonic tissue. Another purpose is to demonstrate the colon-specific delivery of rebamipide with or without absorption enhancers using chitosan capsule as a carrier.

METHODS: The permeability of rebamipide was evaluated using an *in vitro* diffusion chamber system, and the effects of some absorption enhancers on the permeability *via* colon were further investigated. The release of rebamipide from chitosan or gelatin capsule was studied by Japan Pharmacopoeia rotating basket method. The colonic and plasma concentrations were analyzed by high performance liquid chromatography (HPLC) to evaluate colon-targeting action after oral administration of various dosage forms, and rebamipide with absorption enhancers in chitosan dosage forms.

RESULTS: The permeability of rebamipide across the jejunal or ileal membranes was higher than the colonic membranes. Both sodium laurate (C12) and labrasol significantly increased permeability across the colon membranes. On the other hand, the release of rebamipide from chitosan capsule was less than 10%

INTRODUCTION

Rebamipide (2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinon-4-yl] propionic acid), a novel anti-ulcer drug, has been reported to prevent various acute

experimental gastric lesions and accelerate healing of chronic gastric ulcers^[1]. This drug has been marketed in Japan since 1990 as a therapeutic agent treating gastric ulcer and acute and/or chronic gastritis. Although the characteristics of rebamipide in preclinical and clinical area^[2,3], which is one of key factors to develop its reasonable oral dosage form at the initial stage, has been investigated fully, little was known about the permeability of this drug in different gastrointestinal tissues. On the other hand, recent studies also have shown the beneficial effect of this drug on experimental colitis. It has been demonstrated that the attenuation of colitis indices induced by rebamipide was associated with its inhibition of inflammatory cytokine-mediated granulocyte (neutrophil) infiltration into the colon^[4]. Other groups demonstrated that rebamipide can suppress chemically induced colitis in rodents, which appeared to be largely related with the inhibition of the production of reactive oxygen species. In clinical practice, rebamipide has been used to treat patients with proctitis^[5]. Therefore, we hope to find some absorption enhancers to augment permeability of rebamipide across the colonic tissues, and in this case, this drug should be specifically localized in the large intestine by its colon-specific delivery to improve its therapeutic effect on colitis and to decrease its side-effects, as well. There are many investigations on absorption enhancers; meanwhile, it has been demonstrated previously that chitosan capsule could act as useful carriers for colon-specific delivery of peptide and anti-inflammatory drugs including insulin, calcitonin, 5-aminosalicylic acid and ridogrel^[6-9]. However, the effects of absorption enhancers increasing the distribution on colon and chitosan capsule on the colon-specific delivery of rebamipide ought to be established.

Therefore, we evaluated the permeability of rebamipide across different gastrointestinal membranes in the present study, analyzed potential transport mode, and investigated the effect of some absorption enhancers, such as sodium laurate (C12) and labrasol, on the permeability of rebamipide across colonic tissues. In addition, the effectiveness of chitosan capsule to the colon-specific delivery of rebamipide and the influence of rebamipide chitosan capsules with absorption enhancers on colon specific delivery were evaluated.

MATERIALS AND METHODS

Materials

Rebamipide was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid) and HPMCP (Hydroxypropyl methylcellulose phthalate) were purchased from Wako Pure Chemical Industries Co. C12 was from Tokyo Kasei Kogyo Co. Ltd, while labrasol was from Saint-Priest, France. Chitosan capsules and gelatin capsules were obtained from Aicello Chemical Company Ltd. (Toyohashi, Japan), and the mean diameters and weight of these capsules were 3.5 mm × 1.6 mm and about 1.0 mg, respectively. All other chemicals were of analytical grade.

Instruments

Diffusion chamber apparatus was purchased from Harvard, US. The Shimadzu LC-10A system (Japan) as high performance liquid chromatography (HPLC) instrument was used in this study. The pH measuring instrument was from DKK.TOA Corporation in Japan. NTR-6000 Dissolution Tester made in Japan was chosen to test the dissolution rate of drug dosage forms.

Animals

Male Wistar rats (280 ± 20 g) were purchased from Laboratory Animal Center, Southern Medical University. All of the animal experiments were performed according to the guideline of Experimental Animal Ethics Committee of Southern Medical University.

Preparation of drug solutions for the *in vitro* permeability studies

For *in vitro* permeability studies, rebamipide was dissolved in oxygenated (O₂/CO₂, 95/5) HEPES buffer adjusted to pH 7.4, which was prepared daily, to yield final concentration of 80 µmol/L. In certain experiments, the dosing solutions were added to labrasol (0.4, 1, 2 g/L) or C12 (0.5, 1, 2 mmol/L).

Permeability studies

The *in vitro* transport of rebamipide across different intestinal membranes was evaluated by a diffusion chamber method using stripped rat intestine for 2 h^[10,11]. Male Wistar rats, weighing 280 ± 20 g, were fasted overnight and anesthetized with sodium pentobarbital (32 mg/kg, IP). The intestine of each rat was excised and rinsed in PBS of pH 7.4 and, avoiding Peyer's patches, experimental segments were obtained. The first 5 cm of the top of small intestine was cut away, the next 10 cm was used as the jejunum and the final 10 cm was considered to be the ileum. The first 2 cm of the large intestine was removed and the next 6 cm was used as the colon. The underlying muscularis from the serosal side of the tissue was removed and the final intestinal segments were mounted in the diffusion chamber in which a surface area of 1.78 cm² was exposed and preheated to 37°C. Immediately following tissue mounting, 7 mL of HEPES buffer at pH 7.4 was added to serosal side or mucosal side, while an equal volume of the rebamipide solution with or without an absorption enhancer was added to the opposite side. Each side of the chamber was bubbled with a mixture of 95 mL/L O₂ and 5 mL/L CO₂ to maintain the viability of the membrane. The heating unit was capable of holding six cells and the temperature was kept at 37°C during the whole procedure using a circulating water bath. At predetermined time intervals, 0.4 mL of solution at receiver side was sampled and it was immediately added to an equal volume of the HEPES buffer kept at the same temperature. The concentration of rebamipide in the samples was determined by HPLC. The apparent permeability coefficient (P_{app}) was calculated by the equation $P_{app} = dC/dt \times (1/A \cdot C_0)$, where P_{app} is expressed in cm/s, dC/dt is the slope of the linear portion of the permeation curves, A is

the diffusion area, and C_0 is the initial concentration of rebamipide in the donor side.

Preparation of various rebamipide dosage forms

One mg of rebamipide or a mixture of C12 (0.05, 0.15 and 0.25 mg) with rebamipide (1 mg) was filled in one chitosan capsule. Then, the surface of the chitosan capsule was coated with hydroxypropylmethylcellulose phthalate (HPMCP) as an enteric coating material. 150 g/L HPMCP dissolved in acetone/ethanol (1/1) solvent was used in the whole procedure. In addition, 1 mg of rebamipide was filled in one gelatin capsule as one control dosage form. The procedure of preparation of this dosage form was the same as that of chitosan capsule. For another control dosage form, 4 mg/mL of rebamipide solution in 5 g/L CMC (Carboxymethylcellulose) containing 10 mmol/L NaOH was also prepared.

In vitro dissolution test

The dissolution tests of rebamipide from chitosan capsules and gelatin capsules were carried out using the Japanese Pharmacopoeia (JP) rotating basket method with some slight modifications. Liquid 1 (a model medium of an artificial gastric juice for the Japanese Pharmacopoeia disintegration test) and liquid 2 (a model medium of an artificial intestinal juice for the Japanese Pharmacopoeia disintegration test) were used as media in these experiments. The rotation speed of the baskets was 100 r/min. Samples (0.2 mL) were taken every 60 min and the amount of rebamipide released from the capsules was determined by HPLC.

Establishment of rebamipide determination in rat plasma and colonic tissue using HPLC

HPLC conditions: Rebamipide in colonic tissue and plasma was assayed by reversed phase HPLC system containing 5- μ m Cosmosil (4.6 mm \times 150 mm) particles in an analytical column from Nacalai Tesque, a Shimadzu LC-10 pump system, a Shimadzu SIL-10A autoinjector and a Shimadzu CR-6A integrator. The mobile phase was mixture of 15 mL/L HAc solution (mobile phase A) and acetonitrile containing 100 mL/L tetrahydrofuran (mobile phase B). The gradient system was programmed by linearly increasing the proportion of mobile phase B from 18% to 40% within 55 min. The ultraviolet detector was set at 240 nm.

Preparation of colon tissue sample: Male Wistar rats weighing 280 ± 20 g were fasted for 16 h prior to experiments but allowed water *ad libitum*. They were anesthetized with sodium pentobarbital (32 mg/kg, ip). The abdomen was opened through a midline incision and the whole colon was removed from the body. After being washed with PBS, the colon tissue was cut into small pieces. The specimens were weighed. Methanol (5 mL) was added and the specimens were homogenized at ice-water bath using a POLYTRON homogenizer. The homogenate was centrifuged at 12000 r/min for 15 min. The supernatant was evaporated at 60°C under nitrogen flow. The residue was re-dissolved in 0.25 mL

of 5 mmol/L NaOH solution by ultrasound for 15 min. The suspension was centrifuged at 12000 r/min for 15 min. The resulting supernatant was taken as colon tissue sample to be analyzed by HPLC.

Preparation of plasma sample: Male Wistar rats, 280 ± 20 g, were fasted for 16 h prior to experiments but allowed water *ad libitum*. They were anesthetized with sodium pentobarbital (32 mg/kg, IP). The abdomen was opened through a midline incision and 5 mL blood was collected into heparinized syringes *via* the abdominal vein. Samples were immediately centrifuged at 10000 r/min for 5 min to obtain plasma fraction. 5 mL methanol was added in 1 mL plasma, and the mixture was vortexed for 1 min and then centrifuged at 3600 r/min for 15 min. 4.5 mL of supernatant was evaporated at 60°C under nitrogen flow. The residue was re-dissolved in 0.25 mL 5 mmol/L NaOH solution by ultrasound for 15 min. The suspension was centrifuged at 12000 r/min for 15 min. The resulting supernatant was taken as plasma sample to be analyzed by HPLC.

In vivo absorption experiments

Male Wistar rats, 280 ± 20 g, were fasted for 16 h before the experiments, and then four chitosan or gelatin capsules (4 mg rebamipide) were administered orally to the stomach *via* polyethylene tubing under light ether anesthesia. One mL of distilled water was administered after that. For the CMC group, 1 mL of rebamipide CMC solution (4 mg rebamipide) was taken orally under the same conditions. At the predetermined time interval, blood samples and colonic tissue samples were prepared, and then the rebamipide contents in the samples were determined by HPLC. Drug delivery index (DDI) was calculated from the following equation: $DDI = (AUC1LI/AUC2LI)/(AUC1PL/AUC2PL)$, where AUC1LI and AUC1PL represent the area under concentration-time profiles of rebamipide in the large intestinal mucosa and the area under the plasma concentration-time profiles of rebamipide after the oral administration of its chitosan capsules, respectively, while AUC2LI and AUC2PL represent the area under concentration-time profiles of rebamipide in the large intestinal mucosa and the area under the plasma concentration-time profiles of rebamipide, respectively, after the oral administration of its gelatin capsules or its CMC solution. The *in vivo* absorption experiments were run with C12, and then four chitosan with C12 were orally administered to the stomach, other procedures same as above, $DDI = (AUC3LI/AUC1LI)/(AUC3PL/AUC1PL)$. AUC3LI and AUC3PL represent the area under concentration-time profiles of rebamipide in the large intestinal mucosa and the area under the plasma concentration-time profiles of rebamipide, respectively, after the oral administration of chitosan capsules with different dosage of C12.

Statistical analyses

Results were expressed as the mean \pm SE and statistical significance was performed by the Student's *t*-test

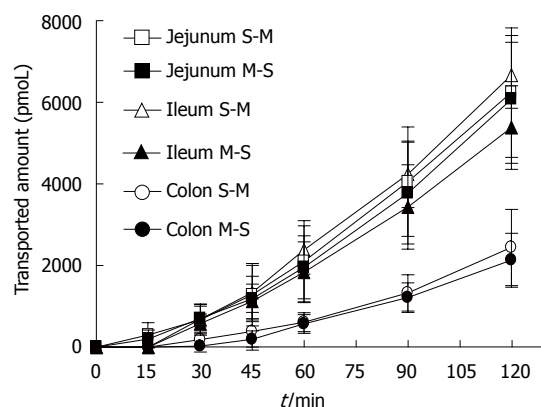


Figure 1 Time course of M-S and S-M transport of rebamipide across the different intestinal tissues. Each value represents mean \pm SE ($n = 10$).

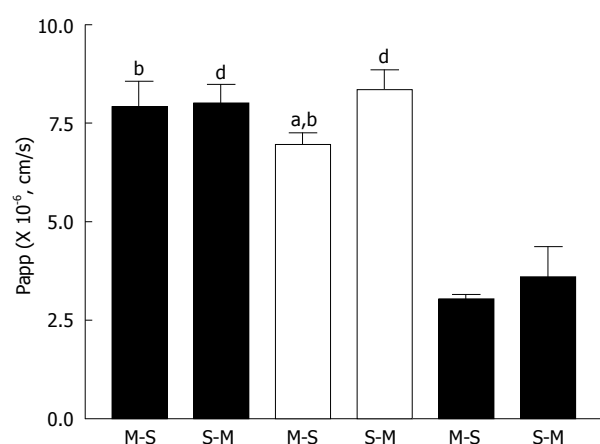


Figure 2 The Papps of rebamipide across the different intestinal regions. Each value represents mean \pm SE ($n = 10$). ^b $P < 0.01$ vs colon M-S, ^a $P < 0.01$ vs colon S-M, ^a $P < 0.05$ vs ileum S-M.

or Dunnett's test for multiple comparisons with the minimum levels of significance, $P < 0.05$.

RESULTS

Regional differences in the permeability of rebamipide across different intestinal membranes

Figure 1 shows the time course of absorptive (mucosal to serosal, M-S) and secretory (serosal to mucosal, S-M) transport of rebamipide across the rat various intestinal membranes. As shown, there were regional differences in the *in vitro* permeability of rebamipide. The permeability of rebamipide across the jejunal or ileal membranes was higher than that across the colonic membrane. The S-M transport of rebamipide in the jejunum and colon was almost as same as its M-S transport, while the S-M transport of rebamipide across ileal tissues was slightly greater than that from M-S transport. Figure 2 summarizes the Papps of rebamipide across the different intestinal regions. As shown in Figure 2, Papp in jejunum and ileum was higher than that in colon, but no significant difference of drug permeability was observed between jejunal and ileal region. Based on the regionally different absorption studies, we selected colon as a model region to estimate the permeability of rebamipide in the presence of some

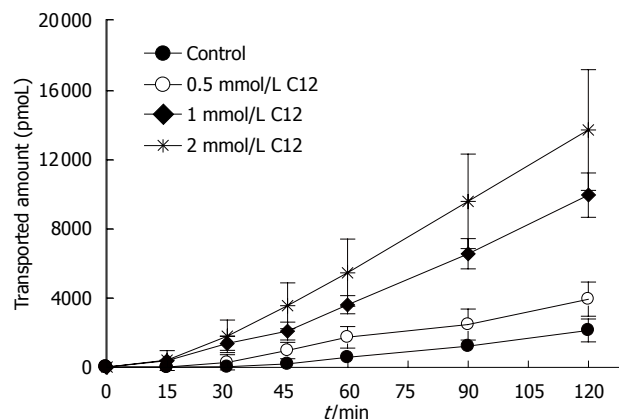


Figure 3 Time course of transport of rebamipide across the colonic tissues in the presence of various concentrations of C12. Each value represents mean \pm SE ($n = 10$).

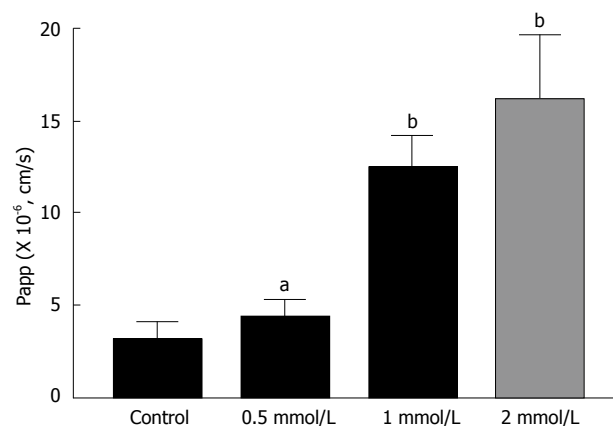


Figure 4 Effect of various concentrations of C12 on the Papps of rebamipide in the colonic tissues. Each value represents mean \pm SE ($n = 10$). ^a $P < 0.05$, ^b $P < 0.01$ vs control.

absorption enhancers in the following, because the colon is the pharmacodynamic position of rebamipide.

Effect of C12 on the permeability of rebamipide across the colonic membranes

Figures 3 and 4 show the effects of different concentrations of C12 on the cumulative amount and Papp of rebamipide across the colonic region. As showed in Figures 3 and 4, the permeability of rebamipide from the colonic region was remarkably enhanced by the addition of C12. Also, there exists a concentration-dependent effect of C12 on the absorptive transport of rebamipide over the range of 0.5 to 2 mmol/L. In general, the higher concentrations of C12 gave the greater enhancement of rebamipide transport. In this experiment, we also have used 0.4% labrasol to dissolve C12, since C12 itself was not dissolved easily in the HEPES buffer. Therefore, we also investigated the influence of labrasol on the permeability of rebamipide across the colonic membranes.

Effect of labrasol on the permeability of rebamipide across the colonic membranes

The transport of rebamipide with labrasol across

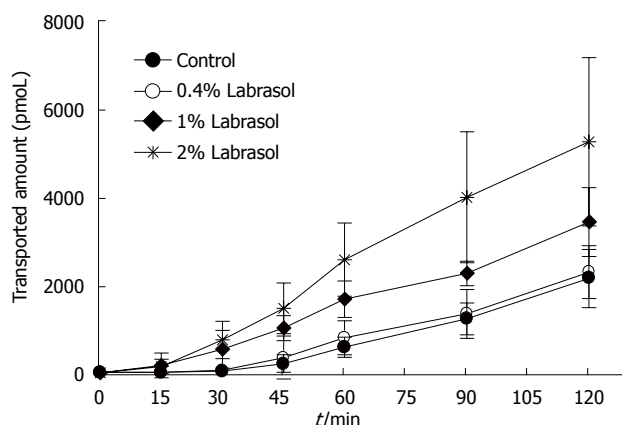


Figure 5 Time course of transport of rebamipide across the colonic tissues in the presence of various concentrations of labrasol. Each value represents mean \pm SE ($n = 10$).

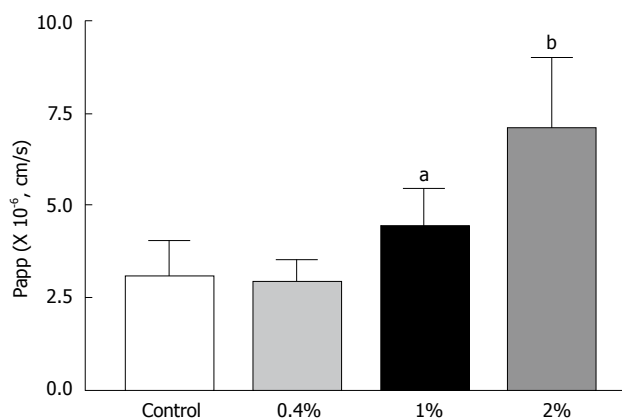


Figure 6 Effect of various concentrations of labrasol on the Papps of rebamipide in the colonic tissues. Each value represents mean \pm SE ($n = 10$). ^a $P < 0.05$, ^b $P < 0.01$ vs control.

the colonic membranes was examined. We observed a concentration-dependent effect of labrasol on the cumulative amount and Papp of rebamipide across the colonic region, as indicated in Figures 5 and 6. However, there exists no effect of labrasol at lower concentration (0.4 g/L) on the transport of rebamipide. In addition, the enhancement effect of labrasol for the permeability of rebamipide was not as strong as that with C12.

In vitro releasing pattern of rebamipide from chitosan and gelatin capsules

Figure 7 shows the release-time profiles of rebamipide from chitosan capsules and gelatin capsules. We studied drug release in liquid 1, an artificial gastric juice (pH 1), during the period 0-2 h after dosing, and in liquid 2, an artificial intestinal juice (pH 7) during the period 2-6 h after dosing. The release of rebamipide from the chitosan capsules in the protection of HPMCP was less than 10% totally within 6 h, while its release from the gelatin capsules reached about 100% in the same conditions.

Plasma concentration and colonic tissue distribution after oral administration of rebamipide in different dosage forms

Figure 8 shows the time course of rebamipide content

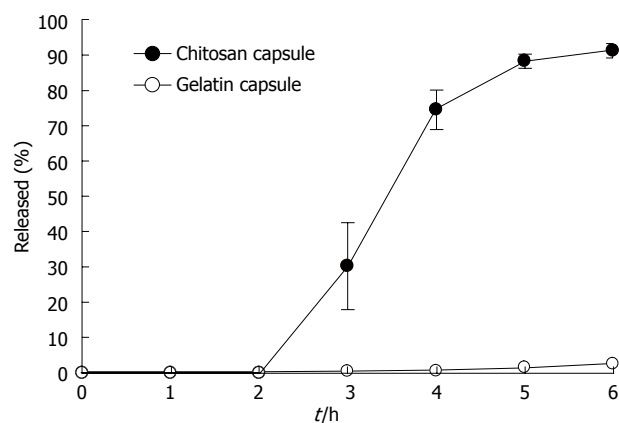


Figure 7 Release of rebamipide from chitosan capsules or gelatin capsules by Japanese pharmacopoeia rotating basket method ($n = 6$). 0-2 h: In the artificial gastric juice; 2-6 h: In the artificial small intestinal juice.

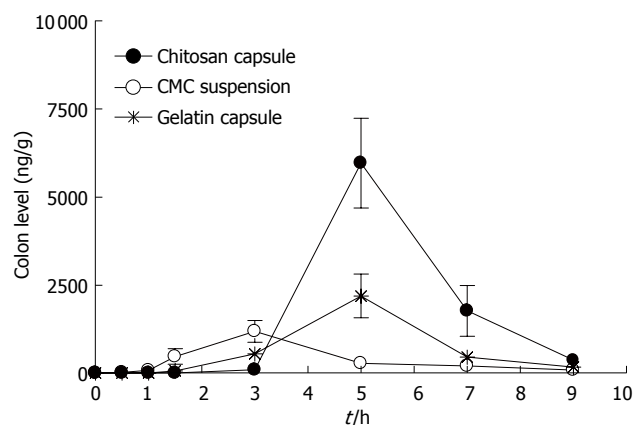


Figure 8 Concentration-time profiles of rebamipide in the colon tissue after oral administration to rat with various dosage forms. Each value represents mean \pm SE ($n = 3$).

in the large intestine after the oral administration of rebamipide in different dosage forms. The area under concentration-time profile of drug in the large intestinal mucosa (AUC_{LI}, 16011.22 ng·h/g) after the oral administration of rebamipide using chitosan capsules was 2.5 times and 4.4 times greater than that of rebamipide using gelatin capsules and CMC solution, respectively. The target site of rebamipide is the large intestine, while the transfer amount of rebamipide to the systemic circulation after the oral administration is an index of the drug level in non-targeted sites and is related to the manifestation of adverse effects. We therefore determined the plasma concentrations of rebamipide after its oral administration with chitosan capsules and gelatin capsules as well as CMC solution. Figure 9 shows the plasma concentration-time profiles of rebamipide after the oral administration of rebamipide in different dosage forms. The area under the curve in the plasma (AUC_{PL}) in CMC solution group was 1887.92 ng·h/mL, and AUC_{PL} in gelatin capsule group was 2163.52 ng·h/mL. On the other hand, we observed an at least 1 h lag time in plasma concentration after the oral administration of chitosan capsules containing rebamipide. The AUC_{PL} was 1016.02 ng·h/mL. Overall, the AUC_{PL} value of rebamipide with chitosan capsule was lower than that seen with gelatin capsule or CMC

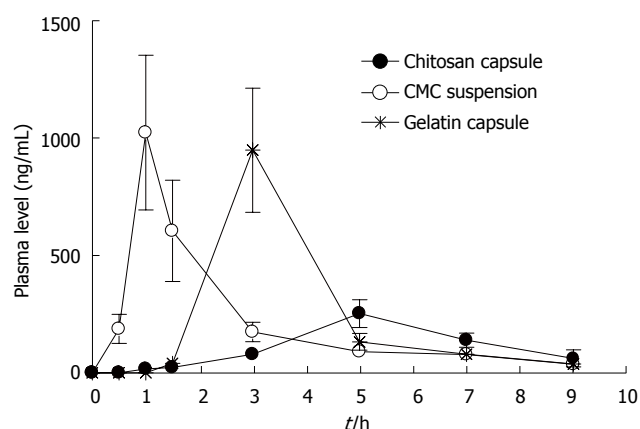


Figure 9 Plasma concentration-time profiles of rebamipide after oral administration to rat with various dosage forms. Each value represents mean \pm SE ($n = 3$).

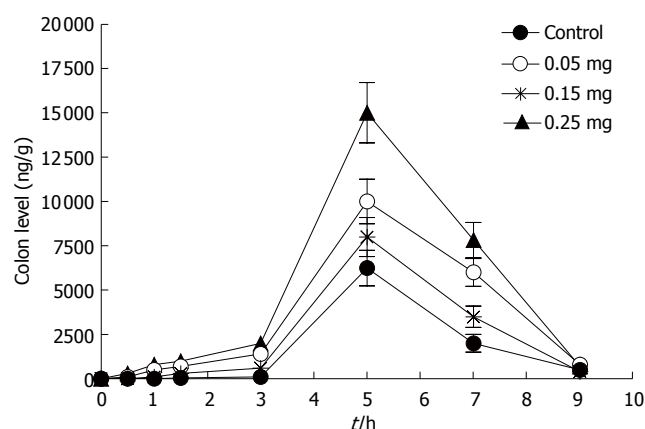


Figure 10 Concentration-time profiles of rebamipide in the colon tissue after oral administration to rat with or without C12. Each value represents mean \pm SE ($n = 3$).

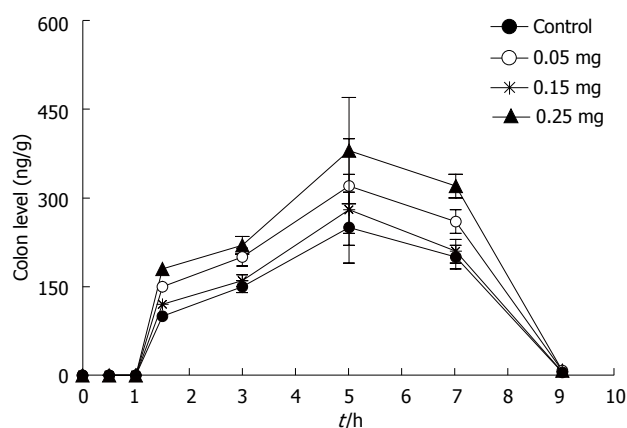


Figure 11 Plasma concentration-time profiles of rebamipide after oral administration to rat with or without C12. Each value represents mean \pm SE ($n = 3$).

solution. Thus the absorption of rebamipide from the gastrointestinal tract to the systemic circulation after the oral administration was inhibited by the use of chitosan capsules. In addition, the DDI, which shows the ratio of drug amount in targeted and non-targeted sites in different dosage forms, constitutes a good index for drug efficacy and safety. By using the equation indicated in Materials and Methods, the DDI of rebamipide in chitosan capsules was calculated to be 8.3 and 5.4, compared with CMC solution and gelatin capsules, respectively, and thus confirmed the effectiveness of chitosan capsules in ensuring the colon-specific delivery of rebamipide.

Plasma concentration and colonic tissue distribution after oral administration of rebamipide with different dosage of C12

The time course of rebamipide content in the large intestine and plasma of rebamipide after the oral administration of rebamipide with different dosage of C12 is shown in Figures 10 and 11. The area under concentration-time profile of drug in the large intestinal mucosa (AUC_{LI}) increased remarkably after the oral administration of rebamipide with C12, especially

the dosage of 0.25 mg; AUC_{LI} of chitosan capsules containing rebamipide was 38 458.2 ng \cdot h/g. Though AUC_{PL} (1536.1 ng \cdot h/mL) also enhanced, the increment of AUC_{LI} was larger than AUC_{PL} statistically. By using the three dosage of C12, the DDI of rebamipide in chitosan capsules was calculated to be 1.8, 1.4 and 1.2, respectively, compared with chitosan capsules. These indicated that absorption enhancer C12 can promote plasma concentration and colonic tissue distribution of rebamipide, but is much more profitable to colonic tissue distribution. In addition, there existed a concentration-dependent effect of C12 on the plasma concentration and colonic tissue distribution over the range of 0.05 to 0.25 mg. In general, the higher dosage of C12 gave the larger enhancement of distribution.

DISCUSSION

Most drugs (approximately 60%) treat diseases by oral administration, which is the easiest and most useful method for drug delivery, and prediction of drug absorption is therefore very important for the design of an oral preparation. Only a few experimental *in vitro* methods have so far been established for prediction of drug absorption capability *in vivo*. Caco-2 monolayers are generally accepted to be a suitable *in vitro* model for drug transport studies as these cells have been shown to express most of the enzymatic, functional and morphological characteristics of the intestinal and morphological characteristics of the intestinal mucosa^[12,13]. However, Caco-2 monolayers cannot be used to predict the regional differences in the permeability of drugs in the gastrointestinal tract. Also, the expression levels of transporters in Caco-2 cells were usually variable and were dependent on the culture condition, which is one of the major disadvantages to estimate the actual permeability of drugs, especially drugs mediated by these transporters. On the other hand, diffusion chamber technique^[14-16] is one of the other effective means to predict the absorbability of drugs in humans based on rat intestinal permeability.

Recently, Watanabe *et al* investigated the correlation between the apparent permeability coefficients (Papp) of 15 water-soluble and poorly water-soluble drugs based on the diffusion chamber experiment and the fractions absorbed (Fa) in humans. A good correlation was found between Papp and Fa of the test drugs^[17]. Therefore, in this study, we also investigated the permeability of rebamipide across different gastrointestinal membranes using diffusion chamber experiment.

The low solubility and low permeability of rebamipide indicate that it should be classified into Class IV in Biopharmaceutics Classification System (BCS)^[18]. It has been known that many drugs categorized into BCS Class IV are P-gp substrates^[19]. Also, it has also been demonstrated that the intestinal P-gp, an ATP-dependent multidrug efflux pump, can be an active secretion system or an absorption barrier by transporting some drugs from cells into intestinal lumen. Meanwhile, our results revealed that the permeability of rebamipide across the different gastrointestinal regions in M-S or S-M direction is almost equal, although S-M transport of rebamipide across the ileal tissue was slightly greater than that from M-S transport. Based on these results, it may be inferred that carrier-mediated intestinal transport was not involved in the transport of rebamipide and passive diffusion seems to be one of the major mechanisms for the intestinal transport of rebamipide. However, we have used 80 $\mu\text{mol/L}$ of rebamipide in this study to avoid the limitation of detectable amount by the present HPLC method. For most of carrier-mediated intestinal transports, it has been said that there exists a saturated effect of drug concentration on the transport of drug^[20]. Therefore, it is necessary to investigate the characteristics of the permeability of rebamipide across M-S or S-M direction at much lower concentration in order to elucidate the exact mechanism of its transport across the intestinal membrane in the future, as more sensitive determination of rebamipide will have to be set up. In addition, we found in this study that the Papp of rebamipide across the colonic membranes in the absorptive direction was about $(3.06 \pm 0.07) \times 10^{-6} \text{ cm/s}$ ($n = 15$), while Miyake *et al*^[21] reported the Papp across the same membrane was about $(2.93 \pm 0.35) \times 10^{-6} \text{ cm/s}$. The slight difference may be result from the different experimental conditions.

As far as the discordance of the absorbability or bioavailability of rebamipide in human and its *in vitro* permeability is concerned, it can be inferred that some rebamipide metabolism or any others could be involved in human intestinal tract, as the bioavailability of rebamipide was only about 10%, while the permeabilities of rebamipide across the absorptive jejunal and ileal membranes in our study reached $8.0 \times 10^{-6} \text{ cm/s}$ and $6.9 \times 10^{-6} \text{ cm/s}$, respectively. Consistent with our results, Walle *et al* also showed that the Papp of taxol was $4.4 \times 10^{-6} \text{ cm/s}$ in Caco-2 cells, and in general a Papp value in Caco-2 cells of $> 1 \times 10^{-6} \text{ cm/s}$ is associated with efficient intestinal absorption in humans. Therefore, it was hypothesized that the low oral bioavailability of taxol may be more dependent on presystemic metabolism in

the liver than on lack of absorption. Also, the authors pointed out that a novel development involving increased expression of CYP3A4 should be helpful to examine the potential contribution of CYP3A4 to the transport of drugs such as taxol using the Caco-2 cell system^[22]. Hence, these factors also should be involved to evaluate the absorption characteristics of rebamipide in human, based on the obtained permeability parameters of rebamipide using diffusion chamber in our present study.

As the improvement of drug permeability by using an absorption enhancer has been very attractive from the aspects of biopharmaceutics, pharmacology, and economics, many researchers investigated the absorption enhancement using various adjuvants^[23-25]. However, it has been very difficult to use those adjuvants for practical formulation, because they possibly cause local toxicity. Although many compounds have been reported to have absorption enhancing ability, medium-chain fatty acids and medium-chain glycerides are thought to be relatively safe because they are used as nutritional dietary supplements. Currently, only sodium caprate (C10) has been used and marketed as an absorption enhancer in ampicillin suppository marketed in Japan, Denmark, and Sweden, and in ceftizoxime suppository in Japan^[26,27]. Furthermore, Miyake *et al* demonstrated that C12 was a more effective and safer adjuvant than C10 at the same concentration. Additionally, rebamipide is used to treat the proctitis in colon; therefore, we examined the effect of C12 on the permeability of rebamipide across colonic membranes in the absorptive direction, and we found that the permeability of rebamipide from the colonic region was remarkably enhanced by the addition of C12. Generally, the effects of the absorption enhancing agents are also often intestinal site-dependent. Hence, we also determined the effect of C12 on the permeability of rebamipide across the absorptive ileum membranes. We found the increased Papp ratio of rebamipide with 1 mmol/L of C12, compared with no C12 in this region was 1.92 (data was not shown in the results section), while it was 4.04 in the colon region, where Papp ratio = Papp (with C12)/Papp (without C12). It seems that using C12 as absorption enhancer to increase the absorption of rebamipide is feasible. On the other hand, C12 is not easily dissolved in the experimental HEPES buffer solution; therefore, labrasol was used to increase the solubility of C12 in this test medium. Labrasol is a surfactant that contains saturated polyglycolized C6-C14 glycerides, and its NMR characterization indicated that it is a mixture consisting of 30% mono-, di- and triglycerides of C8 and C10 fatty acids, 50% of mono- and diesters of poly (ethylene glycol) (PEG) and 20% of free PEG 400^[28]. It shows high tolerance and low toxicity, and has a LD50 of 22 g/kg in rats. Labrasol has been included as a pharmaceutical excipient in European Pharmacopoeia in 1998. As a result, 0.4 g/L labrasol can make 2 mmol/L C12 dissolve in the HEPES buffer. Also, it was found that there was no influence on the permeability of rebamipide at this low concentration, though increased transport of rebamipide with 1 g/L or 2 g/L of labrasol across the colon membrane was found

and also showed concentration-dependent effect of labrasol on the permeability of rebamipide.

As the action and some mechanisms of rebamipide on treating colitis in animals and in human beings have been demonstrated, we investigated the colon specific delivery of rebamipide using chitosan capsule. Chitosan is a high molecular weight cationic polysaccharide, which can be prepared by alkaline N-deacetylation of chitin, the second most abundant natural polymer^[29]. It has been known that chitosan possesses many advantages including low toxicity, with an oral LD50 in mice of > 16 g/kg, moderate immunostimulating effect, and inert and biodegradable characteristics. In addition, chitosan has been widely applied as a potential formulation excipient in conventional pharmaceutical devices. This polymer also has been investigated as a potential adjuvant for orally controlled release systems^[30] and colon targeting^[4-7]. In addition, it was reported that chitosan had mucoadhesive properties, which probably was mediated through ionic interactions with negative charges in mucus or on cell surfaces. In this study, the chitosan material with average molecular weight of 43000 and average deacetyl degree of 83% was chosen to prepare the capsules. As results, we also have demonstrated that this kind of chitosan capsule coated with HPMCP could be a useful carrier to the colon specific delivery of rebamipide, as has been shown in that the DDI of rebamipide chitosan capsule was calculated to be 8.3 or 5.4, compared with CMC solution group or gelatin capsule group, respectively. Based on our findings, we proposed mechanisms for the colon-specific delivery of rebamipide using chitosan capsule. In the case of rebamipide CMC solution, rebamipide has been absorbed from the small intestine region. Additionally, rebamipide in the gelatin capsule also is absorbed from the small intestinal region, though HPMCP coating the surface of gelatin capsule can protect this kind of capsule from the attack of strongly acidic surroundings in the stomach. However, both HPMCP and gelatin material are easily dissolved in the small medium. Therefore, rebamipide is released and absorbed from the small region when HPMCP-coated gelatin capsule reaches this region. On the other hand, when rebamipide was orally administered using HPMCP-coated chitosan capsules, HPMCP first protected the dissolution of chitosan capsule in stomach. Then, the HPMCP is rapidly dissolved and there is little influence of the intestinal medium on the chitosan capsule when HPMCP-coated chitosan capsule enters into the small region. After that, this dosage form reaches the cecum and then the colon. The capsule was disintegrated by microorganisms richly distributed in these regions, and hence rebamipide was released and exerted its local action on colitis. Alternatively, it was reported recently that the pH may actually fall in the colon, when compared with the pH of the small intestine. This low pH, which is caused by acidification of the colonic contents by the products of bacterial fermentation, may be also related to the degradation of chitosan capsule in rat cecal contents and in colon, since chitosan is

unstable and easily degraded under acidic conditions. For one thing, rebamipide has weak acidity and can be dissolved in basic solution. Therefore, it can be dissolved in the colonic medium due to the basic property of this medium, which makes rebamipide much easier to distribute into the colon tissue and exert its local action.

Based on these mechanisms, it is also necessary to make the model drug released from the chitosan capsule easily dissolve in the colon medium by some means to insure that a large amount of drug is distributed in the tissue or absorbed into the circulation system, if we carry out the colon-specific delivery of some other model drug using chitosan capsule in the future. Since there were many experiments done to demonstrate the possibility of chitosan capsule as a specific colon delivery carrier in animals, we should pay close attention to the large scale production of drug-filled chitosan capsules and its prospects in clinical practice from now on. Further investigation should involve: (1) the difference of colon-specific delivery from other chitosan capsules with different molecular weights and degrees of deacetylation; (2) besides HPMCP, the possibility of some other enteric compounds as the coating material and the effect of the coating thickness on colon specific delivery of drugs; (3) the toxicity of cyanoacrylate adhesive used to seal the body and cap in the preparation of drug-filled chitosan capsules; (4) the evaluation of the advantages and disadvantages of chitosan capsules, compared with other colon specific delivery carriers.

Moreover, it is an important method to co-fill with some absorption enhancer in chitosan capsule to increase the drug absorption when systemic action is expected, while it is doubtful that some absorption enhancer is co-administered if the goal is to exert drug's local treatment and reduce the systemic side-effects. In our experiment, rebamipide content in the large intestine increasing notably after the oral administration of rebamipide with C12, which may indicate that absorption enhancer can enhance effect of therapeutic on colitis. On the other hand, DDI got raised, but absolutely speaking, the increasing of plasma concentration may produce adverse effects. Therefore, considering such factors, we should choose a reasonable dosage of C12, which can improve the distribution in colon of rebamipide and control the level of blood concentration; thus, rebamipide with chitosan capsule and C12 produces the best treatment outcome. In future, we will study the effects on colon absorption of rebamipide with various kinds of absorption enhancers, and the safety and toxicity of combinations of absorption enhancer and chitosan capsule.

In addition, in the case of ulcerative colitis, it is reported^[31,32] that rebamipide enema treatment is useful; however, the treatment is mainly effective in local inflammation of rectum and descending colon due to the limitation of enema technique. Using chitosan capsule, rebamipide could be first distributed in the ascending colon, and then in the transverse colon and other colon. Therefore, rebamipide chitosan capsule may be more helpful in treating the extensive colitis or pancolitis.

In conclusion, we demonstrated a regional difference in the *in vitro* permeability of rebamipide across the small intestine and the colon in rats. Also, absorption enhancers such as C12 and labrasol can increase the permeability of rebamipide across the colon tissue significantly. We demonstrated that rebamipide can be specifically delivered to the colon using HPMCP-coated chitosan capsule. Meanwhile, we also confirm that rebamipide chitosan capsule, when added with C12, is more useful in treating colitis.

COMMENTS

Background

Rebamipide has been used to treat patients with proctitis using enema, but this treatment is inconvenient and circumscribed. It has been demonstrated previously that chitosan capsules could act as useful carriers for colon-specific delivery of peptide and anti-inflammatory drugs. However, the effects of absorption enhancement of the distribution on colon and of chitosan capsule on the colon-specific delivery of rebamipide ought to be established.

Research frontiers

It is very important to improve therapeutic effects of rebamipide on colitis and decrease its side-effects, so it is suggested to find some absorption enhancers to augment permeability of rebamipide across the colonic tissues, and to localize specifically the drug in the large intestine by colon-specific delivery.

Innovations and breakthroughs

Rebamipide chitosan capsule is more convenient and helpful than enema in treating colitis, especially extensive colitis or pancolitis.

Applications

Since there were many experiments done that have demonstrated the possibility of chitosan capsule as a specific colon delivery carrier in animal, the large scale production of drug-filled chitosan capsules and its prospects in clinical practice from now on should be paid much attention. Rebamipide chitosan capsule, added with absorption enhancer, can be using in treating colitis in future.

Terminology

The apparent permeability coefficient (Papp) is the apparent parameter of permeability (cm/s), was calculated by the equation $Papp = dC/dT \times (1/A \cdot C_0)$, where dC/dT is the slope of the linear portion of the permeation curves, A is the diffusion area, and C_0 is the initial concentration of rebamipide in the donor side.

Peer review

The authors explained rebamipide delivery using chitosan capsule. The study seems to be interesting and promising.

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RAPID COMMUNICATION

Compression anastomosis clip for gastrointestinal anastomosis

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INTRODUCTION

Compression anastomosis was first reported in 1826 by Denan, who conceived a sutureless bowel anastomosis that encompassed the inverting technique proposed by Lembert^[1]. The idea is to compress two bowel walls together and induce a simultaneous necrosis and healing process leading to the joining of the two lumens. In 1892, Murphy introduced a mechanical device known as "Murphy's Button", which has been used for years^[2-4].

Additional compression devices, such as magnetic ring, AKA-2 device, and biofragmentable anastomosis ring (BAR) were developed after almost 100 years^[5-7]. Although animal experiments and clinical trials have reported the high efficacy and safety of these anastomotic devices, they are not widely accepted because of their narrow inner caliber and high expenses. Finally, these compression anastomotic devices were substituted by staplers which are used routinely at present^[3,8].

However, compression anastomosis with no foreign bodies left at the anastomotic site permanently, is considered an ideal anastomotic method. Many surgeons still make effort to improve this compression device. Recently, a new device, compression anastomosis clip (CAC) emerged in 2000, was approved for its use by the FDA^[9]. Clinical trials performed in Israeli Medical Center have confirmed its safety and efficacy for colonic anastomosis in laparoscopic procedures^[10-12]. However, its application in gastrointestinal anastomosis proximal to the ileocecal junction is limited at an experimental level^[9]. This prompts us to study its clinical effects on gastrointestinal anastomosis proximal to the ileocecal junction.

MATERIALS AND METHODS

Sample size and randomization

This study was approved by the institutional committee on ethics in clinical trials. Sixty-six patients at the age of 35-83 years, who underwent gastroenterostomy or enteroenterostomy in July 2005 - December 2006,

Abstract

AIM: To investigate the feasibility of compression anastomosis clip (CAC) for gastrointestinal anastomosis proximal to the ileocecal junction.

METHODS: Sixty-six patients undergoing gastrointestinal anastomosis proximal to the ileocecal junction were randomized into two groups according to the anastomotic method, CAC or stapler.

RESULTS: The postoperative recovery of patients in CAC and stapled anastomosis groups was similar. No postoperative complication related to the anastomotic method was found in either group. Both upper gastrointestinal contrast radiography at the early postoperative course and endoscopic examination after a 6-mo follow-up showed a better healing at the compression anastomosis.

CONCLUSION: CAC can be used not only in colonic surgery but also in gastrointestinal anastomosis. Our result strongly suggests that CAC anastomosis is safe in various complication circumstances. However, it should be further confirmed with a larger patient sample.

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Key words: Gastrointestinal anastomosis; Compression anastomosis clip; Stapler

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Table 1 Clinical data obtained from the patients (mean \pm SD)

	Study group (<i>n</i> = 33)	Control group (<i>n</i> = 33)
Sex (M:F)	24:9	21:12
Age (yr)	57.0 \pm 11.85	58.6 \pm 13.23
Height (cm)	167.7 \pm 7.54	166.4 \pm 8.59
Body weight (kg)	61.7 \pm 10.92	62.7 \pm 7.49
BMI (kg/m ²)	21.9 \pm 3.13	22.7 \pm 3.54
Albumin (g/L)	41.6 \pm 4.44	39.9 \pm 4.77
Hemoglobin (g/L)	125.8 \pm 17.48	118.6 \pm 16.34
Prealbumin (mg/L)	276.1 \pm 105.74	301.9 \pm 100.18

Table 2 Primary disease and distribution of patients

Dignosis	Study group	Control group	Total
Malignant			
Gastric cancer	21	24	45
Gastric stump cancer	1	0	1
Reflux esophagitis	1	0	1
Anastomotic stenosis	1	1	2
Intestinal tumor	2	0	2
Total	26	25	51
Benign			
Intestinal adhesion	3	2	5
Intestinal tumor	1	1	2
Peptic ulcer disease	1	3	4
Annular pancreas	1	0	1
Inflammatory bowel disease	1	2	3
Total	7	8	15

were recruited into the study. Patients with an emergency surgical history or a nickel allergy history were excluded. All participants signed an informed consent form and were randomly divided into a study group or a control group according to the anastomotic method. Randomization was based on computer-generated codes that were maintained in sequentially numbered opaque envelopes. Compression anastomosis clip (CAC) (NiTi Medical Technologies, Netanya, Israel), 30 mm in diameter, was used for anastomosis in the study group (*n* = 33). In the control group, an anastomosis was performed with a stapler. Bowel preparation, consisting of oral magnesium sulphate, gentamycin, and metronidazole, was performed on the day prior to surgery in all patients except for seven patients with complete intestinal obstruction. There was no statistical difference in preoperative conditions between the two groups (Table 1). The main indication for underlying disease was gastric cancer in both groups (Table 2). One patient in the study group was complicated by postgastrectomy reflux esophagitis due to a previous radical gastrectomy and Brown's reconstruction. She complained of serious nausea and vomiting after surgery and was readmitted to our hospital for reconstruction. The afferent loop was resected and a side-to-side anastomosis to distal jejunum was performed with CAC. The distribution of anastomotic sites and operative procedures are listed in Table 3. Two anastomoses were performed for the patient who underwent total gastrectomy and Roux-en-Y reconstruction during esophagojejunostomy and jejunojejunostomy. Only the difference in the latter was compared in both

Table 3 Anastomotic site and operative procedure

Site	Study group	Control group	Total
Stomach-small bowel			
Subtotal gastrectomy with a Billroth II reconstruction	12	11	23
Gastrojejunostomy	6	3	9
Total	18	14	32
Small bowel-small bowel			
Total gastrectomy with a Roux-en-Y reconstruction	9	14	23
Enterointerostomy	6	5	11
Total	15	19	34

groups based on the different anastomotic methods.

The compression anastomosis was completed with CAC which is a shape memory nitinol double ring clip. The hollow viscera to be anastomosed were parallel to each other and two 5-mm incisions were made at both sides. The CAC mounted on a deployment device was cooled in ice-cold water for 2 min before use. After the clip was opened at an angle of approximately 30°, it was inserted into the hollow viscera through the two 5-mm incisions. The device returned to its original closed shape when it was warmed by body temperature and compressed the two visceral walls firmly. Then, the scalpel incorporated in the applicator created a slit through the entrapped walls to allow free passage of air and feces through the anastomosis until the healing process ended. Finally, the applicator was withdrawn and the two small incisions through which the clip was inserted were sutured. The compression anastomotic procedure was completed. The postoperative parameters were followed in three aspects.

Clinical evaluation

Vital sign, recovery of bowel function, antibiotics application, duration of nasogastric drainage, time to start oral feeding and duration of postoperative hospitalization were recorded. We also recorded the time to complete the anastomosis, intraoperative and postoperative complications in all patients, and the time to expel the ring in the study group.

Radiological examination

Four days after surgery, patients in the CAC group received abdomen plain X-ray examination to locate the ring in body. In addition, the integrity of anastomosis was examined by upper gastrointestinal contrast radiography with dilute gastrografen 7 d after operation.

Endoscopy

After a six-month follow-up period, endoscopy was performed to evaluate the healing of anastomosis.

Statistical analysis

Results were expressed as mean \pm SD. Differences in means between the two groups were calculated for significance with independent-samples *t* test using SPSS 10.0. *P* < 0.05 was considered statistically significant.

Table 4 Recovery-related variables after operation (mean \pm SD)

Variable	Study group (<i>n</i> = 33)	Control group (<i>n</i> = 33)	<i>P</i>
Gases started (d)	4.3 \pm 1.24	4.7 \pm 1.28	0.230
Bowel movement (d)	5.7 \pm 2.36	6.2 \pm 2.53	0.407
Antibiotics stopped (d)	3.8 \pm 0.74	4.0 \pm 0.83	0.258
Duration of nasogastric drainage (d)	2.6 \pm 0.98	2.8 \pm 0.64	0.314
Start of oral intake (d)	4.8 \pm 1.82	5.4 \pm 1.69	0.174
Postoperative hospital duration (d)	14.9 \pm 6.01	15.1 \pm 8.35	0.946

RESULTS

All patients recovered from operation uneventfully. The parameters collected during their early postoperative course were similar in both groups (Table 4). No difference was observed in the duration of operation between the two groups (139.4 ± 41.39 min *vs* 143.2 ± 40.53 min). No intraoperative and postoperative complications were found, including anastomotic leakage, bleeding, and stenosis, which were related to the anastomotic methods. However, postoperative complications unrelated to the anastomosis occurred in two patients with compression anastomosis. One patient who underwent subtotal gastrectomy and Billroth II reconstruction showed delayed emptying of the stomach. The intestinal adherence distant from the compression anastomosis was observed at the second operation. The patient recovered finally. Another gastric cancer patient with pyloric obstruction underwent a palliative gastrojejunostomy and was refed with normal diet gradually. However, the patient's condition deteriorated suddenly and the patient died on day 35 after operation because of cardiopulmonary decompensation.

In the CAC group, all of the anastomosis clips were expelled with stool except two, and none complained of discomfort or tenesmus. The mean time of ring expulsion was 15.1 ± 6.04 d (5-29 d). One patient who died 35 d after surgery did not expel the ring. The anastomotic ring in another patient with adhesive ileus was taken out by gastroscopy on the 16th postoperative day.

Using the radiological examination, we were able to locate the anastomosis ring in the patient's body dynamically (Figure 1). The upper gastrointestinal contrast radiography showed that the anastomoses in both groups were intact. No stenosis was observed in all patients, even with the CAC left at the anastomotic site (Figure 2).

After a 6-mo follow-up period, the anastomotic healing was evaluated in all patients by gastroscopy or enteroscopy. No relevant stenosis was detected in either group. All anastomoses showed good healing without scar. The mucosa of compression anastomosis was smooth (Figure 3). However, slight edema and congestion occurred in three of the stapled mucosa.

DISCUSSION

Compression anastomosis is very close to the sutureless

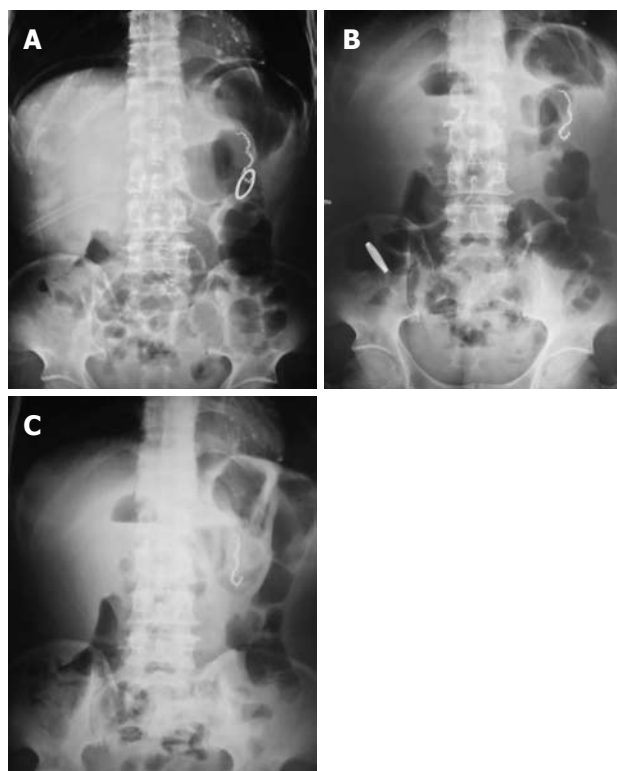


Figure 1 Plain X-ray film showing the CAC *in situ* in abdomen of patients who underwent radical resection of gastric cancer and Billroth II reconstruction 5 d after operation (A), the ring moving to the right lower abdomen 11 d after operation (B), and expelling of the ring with stools 15 d after operation (C).



Figure 2 Upper gastrointestinal contrast radiography showing no stenosis with CAC *in situ* in the patient who underwent alleviative gastrojejunostomy 7 d after operation.

anastomosis in the absence of permanent foreign bodies at the anastomotic site^[2]. Although the technique of compression anastomosis was introduced more than 180 years ago and the theoretical advantages were appreciated, the technique has not been accepted as a standard for gastrointestinal anastomosis, which could be explained by its narrow inner caliber, difficult application and assemblage, and high cost^[3,8]. It seems that these barriers can be tackled well with the new device, compression anastomosis clip.

The mechanism of CAC is similar to that of Murphy's button. The walls of hollow organs are placed between coils of the ring, pressed and clamped together. Its contin-

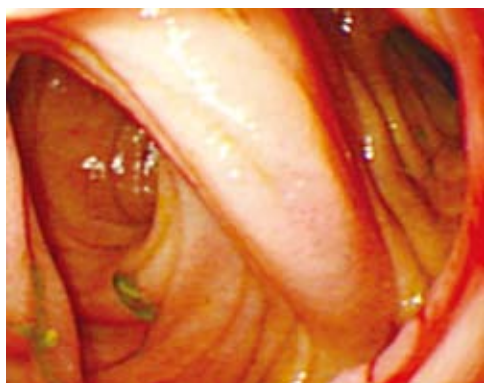


Figure 3 Gastroscope examination revealing a good healing at the compression anastomosis without scar, edema or congestion six months after subtotal gastrectomy with a Billroth II reconstruction.

uous pressure creates gradual and controlled necrosis of the tissue in the area limited by the CAC coil's perimeter while the external edges of this area become sealed, forming a smooth tight homogeneous anastomosis. The device is biofragmented or detached from the anastomotic site and is excreted from the body. The innovation of CAC is the shape memory nitinol double ring that becomes flexible in cool water and resumes its programmed shape when warmed by body temperature.

Technical failure rates of a new technique must be reduced by eliminating the learning curve on a suitable animal model^[13]. In our study, no intraoperative problems were observed and no additional supportive measures were needed, suggesting that the increasing experience with our previous animal experiments may significantly shorten the duration of technique learning. The learning process of this new technique is short, indicating that this technique is easy to learn and handle. We also confirmed that CAC could be used not only for colonic anastomosis but also for gastrointestinal anastomosis and enteroenterostomy. In addition, we also performed gastrojejunostomy and enteroenterostomy with CAC for patients with intestinal adhesion, peptic ulcer, or inflammatory bowel disease. Almost all the patients in the CAC group passed the ring without discomfort only with two exceptions. No obstruction due to anastomotic device was observed, indicating that the air and feces are able to pass through the temporary passage of anastomosis during the healing process of anastomosis. The CAC, 30 mm in diameter and 8 mm in inner caliber, could pass through the ileocecal junction successfully.

Anastomotic leakage is a serious postoperative complication of gastrointestinal anastomosis. However, surgical procedure is one of the important factors that affect the healing of anastomosis^[14]. Our data are consistent with a parallel study in Israel^[10-12]. No anastomotic leakage was observed in our study, which may be partially explained by a constant stress plateau, which is exerted by coils at about 400 Mpa and up to 6% perfect recoverable strain^[8]. The constant pressure makes the ring detached from the anastomotic site at an appropriate time. The unique physical properties of nitinol, mostly the constancy of stress, enable the device to exert

a relatively constant pressure irrespective of the bowel wall thickness.

Ischemia, inflammation, and fibrosis due to anastomosis leakage are the factors for anastomotic stenosis^[15,16]. No sign of anastomotic stenosis at compression anastomotic site was observed either in upper gastrointestinal contrast radiography early postoperatively or in the endoscopy after a long-term follow-up period. The compression anastomosis showed a smooth and intact healing. In contrast, edema and congestion occurred in three of the stapled anastomoses. These discrepancies may be explained by the following. First, the absence of foreign bodies at CAC anastomotic site may greatly decrease inflammatory stimuli and formation of fibrous tissue. Second, the compression anastomotic diameter is determined by the outer diameter of the CAC device, as opposed to staplers, which is determined by its inner diameter. Thus, the CAC is capable of creating a larger anastomosis using a small device. Finally, the raw surface at the edge of the stapler line after firing may also increase the possibility of stricture.

The mean time of expulsion of anastomosis clip was 15.1 ± 6.04 d after surgery. However, significant variations were found in different individuals. Half of the patients in the CAC group expelled the anastomotic clip in about 2 wk. The patients who passed the ring within about 1 wk were always younger and more likely to have benign diseases. They recovered from surgical stress faster and started activities earlier. In contrast, the elder patients with malignant disease often had complex conditions and malnutrition before surgery. They were bedridden for a longer time after surgery and took a longer time to expel the anastomotic ring, suggesting that many factors including anastomotic healing, enterokinesia, and distance of pathway, *etc*, can affect the duration of the ring expelling. Therefore, we hold that the duration for the ring to detach from the anastomotic site is more important. Further study is needed to confirm our findings.

The characteristics of postoperative recovery were similar in patients with CAC or stapled anastomosis. The anastomotic device did not influence the normal diet intake. None of the patients complained of abdominal pain due to dietary. However, Thiede and colleagues conducted a multicenter prospective trial of biofragmentable anastomosis ring and found that a low fiber diet until the evacuation of ring fragments from the bowel could prevent obstruction with no discomfort^[16].

The recovery course of 7 patients with a compression anastomosis who had complete intestinal obstruction before surgery, was uneventful and similar to those without complete intestinal obstruction before operation, which might be related to the characteristics of the CAC. When a CAC anastomosis was performed, the coils were inserted into the bowel lumens that were to be anastomosed through the small incisions and the compression anastomosis was completed after closure of the incisions. However, performing a stapled anastomosis needed a relatively larger surgical field and had more chances to contaminate the abdominal cavity when the applier was withdrawn. In addition, application of

the CAC may simplify the gastrointestinal bypass operation and shorten the operative time. Therefore, surgical injury to the patient could be minimized and the bowel function could recover earlier. Moreover, previous studies demonstrated that persistent foreign bodies may be responsible for anastomotic recurrence of cancer and inflammatory bowel disease and affect radiological examination^[17,18]. Although no recurrence at anastomotic site was found in our study during the 6-mo follow-up period, further study is needed.

In conclusion, CAC is an alternative method for gastrointestinal anastomosis under different disease circumstances. Further study with a larger patient sample and a longer follow-up period is needed to confirm our findings before CAC application in emergency and laparoscopic surgery.

COMMENTS

Background

Gastrointestinal anastomosis is a common surgical procedure in abdominal surgery. Compression anastomosis, which was first reported in 1826 by Denan, is one of the methods. Because there is no foreign body left at the anastomotic site permanently, it is considered an ideal anastomotic method. However, the compression anastomotic devices have been substituted by staplers due to their narrow inner caliber and high cost.

Research frontiers

Recently, a new device, compression anastomosis clip (CAC), which was emerged in 2000, was approved by the FDA. The mechanism of CAC is similar to that of Murphy's button. The innovation of CAC is the shape memory nitinol double ring that becomes flexible in cool water and resumes its programmed shape when warmed by body temperature. Clinical trials performed in Israeli Medical Center confirmed the safety and efficacy of CAC for colonic anastomosis in laparoscopic procedures.

Innovations and breakthroughs

The application of CAC in gastrointestinal anastomosis proximal to the ileocecal junction was evaluated in this study. Besides, it can also be applied in selected patients who suffered from inflammatory bowel disease, peptic ulcer disease and intestinal obstruction, etc.

Applications

CAC can be used for gastrointestinal anastomosis proximal to the ileocecal junction.

Peer review

This study elucidated the safety of CAC for gastrointestinal anastomosis under different disease circumstances, which may be an alternative method for abdominal surgery and decrease the anastomotic recurrence of cancer and inflammatory bowel disease. The study was well designed and the findings were reliable and significant.

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Expression transformation of claudin-1 in the process of gastric adenocarcinoma invasion

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Abstract

AIM: To investigate the relation of expression transformation of claudin-1 with invasiveness and metastasis of gastric carcinoma.

METHODS: By using immunohistochemistry, expression of claudin-1 in mucosa and invasive front of 136 gastric adenocarcinoma cases and proliferative index (Ki-67) were detected and analyzed.

RESULTS: In mucosa, the claudin-1 over-expression rate of mucinous adenocarcinomas (including signet-ring cell carcinomas) was the highest. It was negatively related with the differentiation but positively related with the invasiveness and metastasis of gastric cancer. In invasive front, the claudin-1 over-expression rate was positively related with the differentiation, invasiveness and metastasis of gastric carcinoma. The expression transformation of claudin-1 was found in gastric carcinoma. The expression of claudin-1 in invasive front was transformed in 28/136 gastric carcinoma cases. The transformation rate in highly differentiated tubular adenocarcinomas was the highest (51.5%, 17/33). The deeper was the invasiveness, the higher was the transformation rate. The claudin-1 expression transformation rate in serosa and omenta was significantly higher (92.9%) than in tunica muscularis of invasive gastric cancer cases, as well as in patients with

lymph node metastasis than in those without lymph node metastasis.

CONCLUSION: Up-regulation of claudin-1 expression and its transformation in invasive and metastatic gastric carcinoma suggest that claudin-1 participates in the transformation of biological behaviors in neoplasms. Further study is needed to elucidate the precise mechanism and the relation of claudin-1 expression with the neoplasm progress.

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Key words: Gastric carcinoma; Claudin-1; Expression transformation; Invasiveness; Metastasis; Immunohistochemistry

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INTRODUCTION

Gastric carcinoma is one of the most frequent malignant tumors in the world. Gastric carcinoma cells with a fibroblastic pattern in which intracellular adhesion is decreased show active mobility and invasiveness, indicating that epithelial mesenchymal transition (EMT) has occurred^[1]. Tight junction (TJ) proteins participate in EMT of tumors^[2]. The four-time transmembrane proteins of claudin family are essential components of TJ^[3], but the role of TJ proteins in the development of malignant tumor is not clear. By using immunohistochemistry, claudin-1 expression in gastric carcinoma was investigated and its relation with biological behaviors of gastric carcinoma was discussed in this study.

MATERIALS AND METHODS

Patients

A total of 136 patients (106 males and 30 females) with primary gastric carcinoma, who underwent surgery between January and December 2007 in the First Affiliated Hospital of Fujian Medical University, were enrolled in this study. Their median age was 64 years, ranging 28-80 years. All patients did not receive radiation therapy or chemotherapy prior to operation. The histological findings, lymph node metastasis and TNM stage were evaluated based on World Health Organization Classification of Tumors^[4].

Immunohistochemistry

Specimens were fixed in formalin, embedded in paraffin wax, cut into 4 μ m thick sections and stained with hematoxylin and eosin.

The sections were immunostained with anti-rabbit polyclonal antibodies for claudin-1 (1:100, ZYMED) and Ki-67 (MB67, Ready, NeoMarkers) with the EnVision method. The sections were deparaffinized and heated in a microwave oven for 10 min to retrieve the antigens. After immersed in 3% hydrogen peroxide of 100% methanol for 10 min to block the endogenous peroxidase activity, the sections were incubated with primary antibodies for 60 min at room temperature, with EnVisionTM for 20 min, and then immersed into a DAB solution. The sections were counterstained with haematoxylin, dehydrated and mounted. Between steps, the sections were washed three times with phosphate-buffered saline (PBS). As a negative control, PBS was used instead of primary antibody. Two independent observers without knowledge of the clinical outcomes evaluated the immunohistochemical staining of sections till a complete agreement on the classification.

Immunohistochemical analysis of claudin-1 and Ki-67 labeling index

Claudin-1 was expressed in the cell membrane and/or cytoplasm (Figure 1). The intensity of staining in cell membrane and cytoplasm and the percentage of immunoreactive cells over the total tumor cells were evaluated as previously described^[5]. The intensity of staining was graded as 0 when staining was not greater than negative control, 1 as light staining, and 2 as heavy staining. Immunoreactivity was scored according to the percentage of immunoreactive cells over the total tumor cells counted as 0 if < 5% cells were stained, 1 if 5%-25% cells were immunoreactive, 2 if 26%-50% cells were immunoreactive, and 3 if > 50% cells were immunoreactive. The expression of claudin-1 was finally defined according to the score obtained from the grade of intensity multiplied by the score of cell immunoreactivity, i.e. negative (-, scored 0-1), positive (+, scored 2-3), and strongly positive (++, scored 4 or above). Positive expression of Ki-67 staining was found in nuclei of carcinoma cells. Ki-67 labeling index was defined as the ratio of immunoreactive cells over 1000 tumor cells.

Statistical analysis

Chi square test was used for univariable categorical analysis. All statistical analyses were performed with SPSS 10.0. $P < 0.05$ was considered statistically significant.

RESULTS

Relation between claudin-1 expression and clinicopathological parameters of gastric carcinoma

Claudin-1 was mainly expressed in the cell membrane and/or cytoplasm of gastric carcinoma cells. The expression of claudin-1 was related with the histological type, degree of invasiveness and lymph node metastasis of gastric cancer ($P < 0.05$). However, the expression of claudin-1 was not significantly related with the sex and age of gastric cancer patients (Table 1).

The claudin-1 over-expression rate was the highest in mucinous adenocarcinomas, and lower in poorly differentiated carcinomas than in well-moderately differentiated carcinomas. It was significantly higher in mucosa of patients with their tumors invaded muscularis propria and visceral peritoneum, or with lymph node metastasis than in mucosa of patients with their tumors only invaded lamina propria or submucosa, or without lymph node metastasis.

The claudin-1 expression in invasive front was different from that in the mucosa. The claudin-1 over-expression was the highest in well-moderately differentiated carcinomas and the lowest in poorly differentiated carcinomas. The deeper was the invasive depth, the higher was the claudin-1 over-expression rate. The incidence of claudin-1 over-expression rate was 50% in invasive front with tumors invaded visceral peritoneum and significantly higher in patients with lymph node metastasis. The expression of claudin-1 was not related with the proliferation index of gastric carcinoma cells.

Relation between expression transformation of claudin-1 and biological behaviors of gastric carcinoma

The expression of claudin-1 was transformed in mucosa and invasive front of gastric carcinoma patients, which was 26.2% (28/107) in mucosa and 49.1% (28/57) in invasive front (Table 2). The expression transformation rate of claudin-1 was 51.5% (17/33) in well-moderately differentiated carcinoma patients, 16.0% (9/57) in poorly differentiated carcinoma patients, and 11.8% (2/17) in mucinous carcinoma patients, respectively (Table 3).

The deeper was the invasiveness, the higher was the transformation rate of claudin-1 expression. The claudin-1 expression transformation was significantly higher in patients (26/28) with their tumors invaded visceral peritoneum than in those with their tumors only invaded muscularis propria ($P < 0.05$), and in patients (20/61) with lymph node metastasis than in those (8/46) with no lymph node metastasis ($P < 0.05$).

DISCUSSION

TJs, adherent junctions and desmosomes form the apical

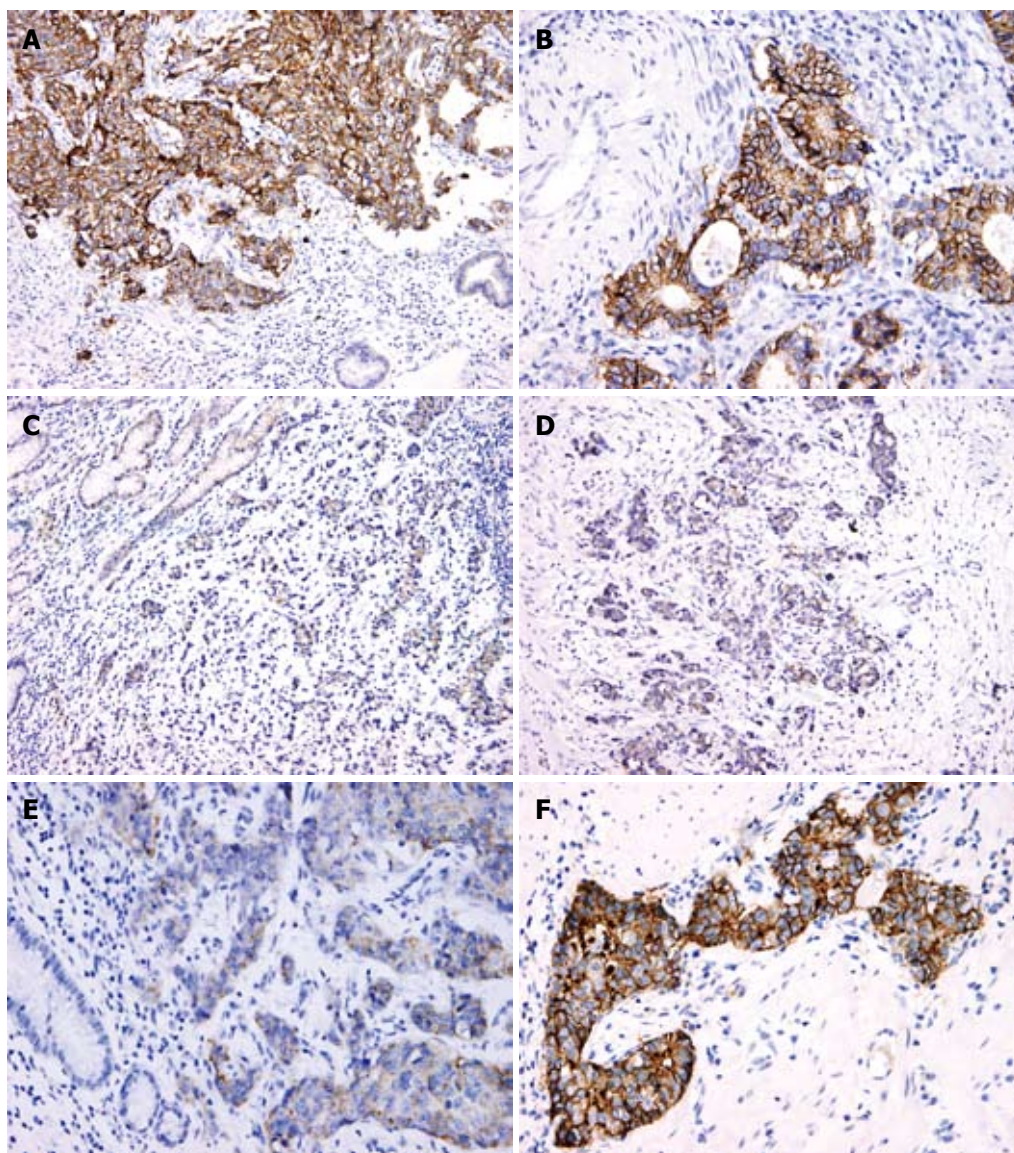


Figure 1 Immunohistochemical staining ($\times 200$) showing over-expression of claudin-1 in mucosa (A) and invasive front (B) of gastric carcinoma, low-expression in mucosa (C) and invasive front (D), and transformation of claudin-1 low-expression in mucosa (E) and over-expression in invasive front of gastric carcinoma (F).

junctional complex in epithelial cellular sheets. Adherent junctions and desmosomes are responsible for the mechanical adhesion between adjacent cells, while TJs play a mainly role in the tight sealing of cellular sheets, thus controlling the paracellular ion flux and maintaining tissue homeostasis^[6]. By forming a fence that prevents lateral diffusion of membrane proteins and lipids, TJs also play a crucial role in the maintenance of cell polarity. TJs also participate in the regulation of cell proliferation and differentiation, or other cellular functions^[7].

TJs are consisted of three major integral membrane proteins: claudins, occludin and junctional adhesion molecules. The role of these proteins has not been completely elucidated. However, it is presumed that claudins form the principal chain of TJ strands. The claudin protein family is consisted of 24 members of closely correlated transmembrane proteins. A number of tissues express multiple claudin proteins and form TJ strands by interacting through homotypic and/or heterotypic fashion, though the expression pattern of claudins is tissue specific.

The histological grade of carcinomas is a significant

prognostic parameter and depends on the differentiated degree of glandular epithelium and cellular polarity. The invasiveness in high-grade and poorly-differentiated carcinoma is stronger than that in low-grade and well-differentiated carcinoma. One of the key determiners controlling cellular adhesion and polarity is the TJs^[6]. Carcinoma cells frequently show deficiencies in structure and function of the TJs^[8]. It was supposed that the TJs play a critical role in the progress of neoplasm through acting as a connector of extracellular environment affecting the intercellular signal pathway and cellular skeleton^[9]. The changes of permeability in TJs may also permit the diffusion increase in nutrient substances and other factors for growth and survival of tumors. Otherwise, the loss of integration of TJs in the development of metastatic phenotype is also an important step.

The expression of claudin proteins may change in carcinoma cells. The expression of claudin-1 and claudin-4 in ovarian and prostatic carcinomas is increased^[10-12], claudin-4 is over-expressed in pancreatic carcinomas^[13,14], while claudin-1 expression is down-

Table 1 Claudin-1 expression and clinicopathologic characteristics of gastric carcinoma *n* (%)

	<i>n</i>	Expression in mucosa			Expression in invasive front		
		Low	Over	<i>P</i>	Low	Over	<i>P</i>
Sex							
Male	106	80 (75.5)	26 (24.5)	0.144	58 (54.7)	48 (45.3)	0.198
Female	30	27 (90)	3 (10)		21 (70)	9 (30)	
Age							
≤ 50	20	17 (85)	3 (15)	0.491	13 (65)	7 (35)	0.665
> 51	116	90 (77.6)	26 (22.4)		66 (56.9)	50 (43.1)	
Histological type							
Well-moderately differentiated	46	33 (71.7)	13 (28.3)	0.046	16 (34.8)	30 (65.2)	0.000
Poorly differentiated	65	57 (87.7)	8 (12.3)		48 (73.8)	17 (26.2)	
Mucinous	25	17 (68)	8 (32)		15 (60)	10 (40)	
Depth of invasion							
Lamina propria or submucosa	18	17 (94.4)	1 (5.6)	0.099	16 (88.9)	2 (11.1)	0.007
Muscularis propria	28	19 (67.9)	9 (32.1)		18 (64.3)	10 (35.7)	
Visceral peritoneum	90	71 (78.9)	19 (21.1)		45 (50)	45 (50)	
Lymph node metastasis							
Negative	52	46 (88.5)	6 (11.5)	0.048	38 (82.6)	14 (17.4)	0.009
Positive	84	61 (72.6)	23 (27.4)		41 (48.8)	43 (51.2)	
Ki-67 index							
I	20	17 (85)	3 (15)	0.529	14 (70)	6 (30)	0.389
II	57	43 (75.4)	14 (24.6)		33 (57.9)	24 (42.1)	
III	54	42 (77.8)	12 (22.2)		28 (51.9)	26 (48.1)	
IV	5	5 (100)	0		4 (80)	1 (20)	

Table 2 Expression transformation of claudin-1 in gastric carcinomas

Expression in mucosa	Expression in invasive front	<i>n</i>
Low-expression	Low-expression (expression invariably)	79
Low-expression	Over-expression (expression variably)	28
Over-expression	Low-expression (expression variably)	0
Over-expression	Over-expression (expression invariably)	29

regulated in breast and colon carcinomas^[15-17].

The three dimensional cultures of breast cancer cells showed that the reexpression of claudin-1 may increase apoptosis of cancer cells^[18]. It was reported that the expression of claudin-1 in stage II colon carcinomas is related with a poor prognosis^[17]. Another study showed that the expression of claudin-1 is up-regulated in colon carcinomas^[19], and the expression level of claudin-1 is negative related with the histological grade of tumors^[17].

However, investigations on the role of claudin-1 expression in the progression of gastric carcinomas are relatively few and only two studies on the expression of claudin-1 in gastric carcinomas can be found in PubMed so far. Through tissue microarray, Resnick *et al*^[20] found that claudin-1, -3, -4 and ZO-1 are expressed in non-tumor mucosa, tumor mucosa and invasive front of gastric carcinomas, the expression of calduin-1 is higher in intestinal subtype than in diffuse subtype of adenocarcinomas. Soini *et al*^[21] found that the expression of claudin-1 is significantly higher in intestinal-type gastric than in diffuse-type gastric carcinomas, indicating that claudin-1 expression is the determiner of diffuse phenotype of gastric carcinoma. Our results show that claudin-1 over-expression occurred in mucinous gastric adenocarcinomas, and was negatively related

Table 3 Relation between the expression transformation of claudin-1 and biological behaviors of gastric carcinomas

	<i>n</i>	Low-expression in mucosa	Over-expression in invasive front
Histological type			
Well-moderately differentiated	17	33	30
Poorly differentiated	9	57	17
Mucinous	2	17	10
Depth of invasion			
Lamina propria or submucosa	1	17	2
Muscularis propria	1	19	10
Visceral peritoneum	26	71	45
Lymph node metastasis			
Negative	8	46	14
Positive	20	61	43

with the differentiation degree of adenocarcinomas, but positively related with the invasiveness and metastasis of adenocarcinomas in mucosa. However, the expression of claudin-1 in invasive front was different from that in mucosa of gastric carcinoma. The claudin-1 over-expression in invasive front was positively related with the differentiated degree and the invasiveness and metastasis of gastric adenocarcinomas.

The transformation of claudin-1 expression companied the progression of gastric carcinomas. The expression of claudin-1 in invasive front of gastric carcinomas was transformed. The claudin-1 expression transformation percentage of well-differentiated adenocarcinomas was the highest (51.5%, 17/33). The deeper the invasiveness of gastric carcinomas was, the higher the transformation rate was. The transformation was significantly higher in patients with tumors invaded visceral peritoneum than in those with tumors only

invaded muscularis propria and in patients with lymph node metastasis than in those with no lymph node metastasis. These results suggest that transformation of claudin-1 expression participates in the progression of gastric carcinomas.

The role of TJ proteins in the development of cancer is not clear. Carcinoma cells, especially those exhibiting a higher potentiality of metastasis, frequently show loss of functional TJs, such as ZO-1, -2 and occludin is decreased in tumor and its metastasis^[22,23]. The exact action of claudin on cancers is not clear. It was reported that the expression of claudin-1 is decreased in invasive duct carcinoma of breast^[24] while the expression of claudin-3 and -4 is increased in some other carcinomas^[25,26]. The expression of claudin-1 may promote the activation of pro-MMP-9^[26,28], suggesting that the expression of claudin-1 may involve the invasiveness and metastasis of adenocarcinoma. Claudin-1 is regarded as a target site of β -catenin/Tcf signals, which supports that claudin-1 down-regulates the formation of colorectal carcinomas^[29]. The expression of claudin-1 mRNA is decreased in breast carcinomas^[15] while the expression of claudin-23 is down-regulated in intestinal subtype of gastric carcinomas^[30]. It was reported that the claudin-1 expression is frequently up-regulated in tumor tissues and its expression level is equal to or higher than in consecutive normal colon mucous membrane, suggesting that the expression of claudin-1 is related with poorly-differentiated adenocarcinoma. The loss of claudin-1 expression is a strongly predictive parameter for tumor recurrence and survival of patients. It was reported that expression of claudin-1 and -4 is increased in ovarian carcinomas and prostatic carcinomas^[26,31] and over-expression of claudin-4 in pancreatic carcinomas and precancerous lesion^[32] are the causative action of claudin on cellular transformation and progression of invasiveness^[33].

Usually, a low expression level of claudin may result in functional damage to TJs. However, how the over-expression of claudin promotes tumor progression remains unclear^[26,31,32]. One possible mechanism is that up-regulation or abnormal expression of some claudins may facilitate tumor formation by directly altering the function of TJ. Furuse *et al.*^[34] reported that up-regulation of claudin-2 expression in renal cells of Madin-Darby dogs decreases the function of TJ, and Tan *et al.*^[35] showed that the expression and distribution of claudin-1 are related with cell dissociation in pancreatic carcinoma by activating the mitogen-activated protein kinase-2. Up-regulation of claudins may also affect cell signaling pathways by binding domains to ZO-1^[36]. ZO-1 interacts with several signaling proteins related with the neoplastic process, such as *ras* substrate AF-6^[37], G-protein and connexin 43^[38].

In conclusion, up-regulation and transformation of claudin expression in the invasive process of gastric carcinomas are involved in the biological behavior transformation of tumors. The exact role of claudin protein in the development of malignant tumors and their prognosis remains unclear and should be further studied.

COMMENTS

Background

Tight junction (TJ) protein participates in the processes of epithelial mesenchymal transformation (EMT) of carcinomas. Claudin proteins are the major members of the TJ family. In this study, the relation between the expression transformation of claudin-1 and the invasiveness and metastasis of gastric carcinoma was evaluated.

Research frontiers

The expression of claudin-1 was found to be significantly related with the biological behavior of gastric cancers, indicating that TJ plays a role in the development of neoplasms. The mechanism of TJ underlying the progress of cancer remains to be further studied.

Innovations and breakthroughs

This study evaluated the relation between the expression of claudin-1 and the invasiveness and metastasis of gastric carcinoma.

Applications

The importance of TJ in tumor development has not been extensively studied. The role of the claudin family in the invasiveness and metastasis of cancers is controversial. The relation between the expression transformation of TJ proteins and MET in cancer was clarified in the present study, which contributes to the exploitation of its mechanism underlying the development and progress of gastric carcinoma.

Terminology

TJs, adherent junctions and desmosomes form the apical junctional complex in epithelial cellular sheets. The claudin protein family is consisted of 24 members of the transmembrane proteins, such claudins 1-4.

Peer review

This study investigated the relation between the expression transformation of claudin-1 and the invasiveness and metastasis of gastric carcinomas. The results suggest that claudin-1 participates in the transformation of the biological behaviors of gastric carcinomas. The study was well designed and the findings may be valuable for the further study on mechanism of claudin-1 underlying the development and progress of gastric carcinoma.

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Role of transforming growth factor-beta signaling pathway in pathogenesis of benign biliary stricture

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expression ratio of Smad7 in cases with benign biliary stricture was 70.0%, higher than that in normal bile duct, but this difference is not statistically significant (70.0% vs 50%, $P > 0.05$). There was a positive correlation between positive expression of TGF- β_1 , Smad4 and CTGF in cases with benign biliary stricture. **CONCLUSION:** The high expression of TGF- β /Smad/CTGF signaling pathway plays an important role in the pathogenesis of benign biliary stricture.

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Key words: Biliary stricture; Transforming growth factor-beta 1; Smad; Connective tissue growth factor; T β R

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Abstract

AIM: To characterize the expression of members of the transforming growth factor-beta (TGF- β)/Smad/connective tissue growth factor (CTGF) signaling pathway in the tissue of benign biliary stricture, and to investigate the effect of TGF- β signaling pathway in the pathogenesis of benign biliary stricture.

METHODS: Paraffin embedded materials from 23 cases of benign biliary stricture were analyzed for members of the TGF- β /Smad/CTGF signaling pathway. TGF- β_1 , T β R I, T β R II, Smad4, Smad7 and CTGF protein were detected by immunohistochemical strepto-avidinbiotin complex method, and CTGF mRNA was evaluated by hybridization *in situ*, while 6 cases of normal bile duct served as controls. The percentages of positive cells were counted. The correlation between TGF- β_1 , Smad4 and CTGF was analyzed.

RESULTS: The positive expression ratios of TGF- β_1 , T β R I, T β R II, Smad4, CTGF and CTGF mRNA in 23 cases with benign biliary stricture were 91.3%, 82.6%, 87.0%, 78.3%, 82.6% and 65.2%, respectively, significantly higher than that in 6 cases of normal bile duct respectively (vs 33.3%, 16.7%, 50.0%, 33.3%, 50.0%, 16.7%, respectively, $P < 0.05$). The positive

INTRODUCTION

Benign biliary strictures are caused by a heterogeneous group of benign conditions. They are usually iatrogenic, most frequently as a result of cholecystectomy^[1]. The incidence of this complication has increased with the widespread use of laparoscopic cholecystectomy (LC)^[2-4]. Other causes of benign biliary strictures include hepatolithiasis, recurrent cholangitis, infection with the fluke, Mirizzi syndrome, *etc*^[5]. Anastomotic strictures are seen following bile duct reconstruction or orthotopic liver transplant (OLT)^[6,7].

The main manifestations of benign biliary stricture are scar contracture and stenosis of bile duct, especially at the hepatic hilum or above^[8]. The diagnosis and treatment of benign biliary strictures remains a clinical challenge, requiring a multidisciplinary approach. The pathogenesis of benign biliary stricture is still unclear.

It is well known that the cytokine transforming growth factor beta 1 (TGF- β_1) has a key role either in

the wound healing process or induction of fibrosis. The biological effect of TGF- β_1 is regulated by a special signal transduction pathway^[9,10]. Following activation of the TGF- β receptor, intracellular signal transduction is mediated by a variety of Smad proteins^[11-13]. Connective tissue growth factor (CTGF), which promotes cell proliferation and deposition of extracellular matrix (ECM), is a downstream medium in the process in which TGF- β_1 produces a marked effect on connective tissue cell. The aim of the present study was to determine the expressions of various cytokines in TGF- β /Smad/CTGF signaling pathway in tissue of benign biliary stricture, and to further investigate the role of TGF- β signaling pathway in the pathogenesis of benign biliary stricture.

MATERIALS AND METHODS

Patients and pathologic specimens

The study population included 23 patients (10 males and 13 females; mean age 50 years) who underwent benign biliary strictures (between June 2003 and November 2005, in Department of Hepatobiliary Surgery, First Affiliated Hospital, Medical College, Xi'an Jiaotong University, Xi'an). The average time interval between the first surgery and the second surgery was 16 mo, and the average admission time was 22 d. The causes of benign biliary stricture were bile duct injury or anastomotic stricture after biliary reconstruction ($n = 17$), hepatolithiasis ($n = 3$) and recurrent cholangitis ($n = 3$). The specimens were obtained from the cicatrices of bile duct. Fibrosis was seen from HE staining. Six normal specimens of bile duct were obtained from donors in liver transplantation. The study protocol was approved by the Ethics Committee of the First Affiliated Hospital, Medical College, Xi'an Jiaotong University.

Antibodies

Antibodies to TGF- β_1 (Rabbit anti-human monoclonal antibody, Santa-dilution 1:400), T β R I (Rabbit anti-human monoclonal antibody, Santa-dilution 1:400), T β R II (Rabbit anti-human monoclonal antibody, Santa-dilution 1:300), Smad4 (Rabbit anti-human monoclonal antibody, Santa-dilution 1:400), Smad7 (Rabbit anti-human monoclonal antibody, Santa-dilution 1:400) and CTGF (Rabbit anti-human monoclonal antibody, Santa-dilution 1:300) were purchased from Wuhan Boster Biological Technology Co. Ltd.

Immunohistochemical analysis

The expression of TGF- β_1 , T β R I, T β R II, Smad4, Smad7 and CTGF was detected by SABC immunohistochemical method. The test kit of SABC was the product of Wuhan Boster Biological Technology Co. Ltd. In the control group, the primary antibody was replaced with PBS or normal rabbit serum. All paraffin embedded sections were deparaffinized and rehydrated, and pretreated for 20 min at 75°C in a microwave oven. After being treated with 1 mL/L H₂O₂ for 30 min to block the

endogenous peroxidase, the sections were incubated with 20 mL/L fetal calf serum for 30 min to reduce nonspecific binding. Then the primary TGF- β_1 , T β R I, T β R II, Smad4, Smad7 and CTGF antibodies were applied to the sections and incubated at 4°C overnight. The sections were subsequently incubated with goat anti rabbit IgG at 37°C for 30 min, followed by incubation with SABC at 37°C for 30 min, and stained with DAB-H₂O₂ for 5-10 min and counterstained with hematoxylin.

In situ hybridization for CTGF mRNA

CTGF mRNA ISH detection kit and antisense oligonucleotide probe (digoxin-labeled) were purchased from Wuhan Boster Biological Technology Co. Ltd. A 30-mer sequence that is complementary to the region of CTGF mRNA was synthesized as follows: (1) 5'-CTG CTGCCGCGTCTGCG CCAAGCAGCTGGG-3'; (2) 5'-CAACTGCCT GGTCCAGACCACAGAGTGGAG-3'; (3) 5'-TGTACTACAGGAAGATGTACGGAGACATGG-3'. In brief, deparaffinized sections were incubated with 3% hydrogen peroxide for 30 min and then with 1 g/mL pepsin for 15 min. The prehybridization was performed at 37°C for 2 h, and the hybridization was conducted in a 42°C water bath for 18 h with each section covered with a soil coverslip. After thorough washing, tissue sections were preblocked for 20 min with blocking solution. Then, mouse anti-digoxin antibody was added for 60 min at 37°C. After washed in PBS, the sections were visualized according to the manufacturer's instructions. A negative control was prepared by using a hybridization solution without the probe.

Assessment of staining reactions

A positive reaction was detected as plasmatic stain presenting in yellow or brown-yellow color. A modified Shimizu's method^[14] was used to assess staining reactions. We selected 10 visual fields under HP microscope and counted 100 cells randomly. The following scoring system was used: (1) score 0, positively stained cytoplasm in less than 5% of cells; score 1, 5% - 35% positive; score 2, 36%-65% positive; and score 3, > 66% positive; (2) The staining intensity was estimated using a 4-grade scoring system (0, 1, 2, 3): very weak (1+ staining in some cells) (Score 0); weak (1+ staining in cells) (Score 1); moderate (2+ staining in cells) (Score 2); strong (3+ staining in cells) (Score 3). The examiners were blinded to patients' clinical and histological (HE staining) profile. Two investigators evaluated the staining levels independently, after which any discordant evaluations were adjusted by connected microscopes and scored jointly. The 1st and 2nd score were added together and divided 2, then the mean was made the final score; 0, 1, 1.5-2, 2.5-3 were recorded as (-), (+), (++) respectively.

Statistical analysis

The percentages of positive cells according to the final score of TGF- β_1 , T β R I, T β R II, Smad4,

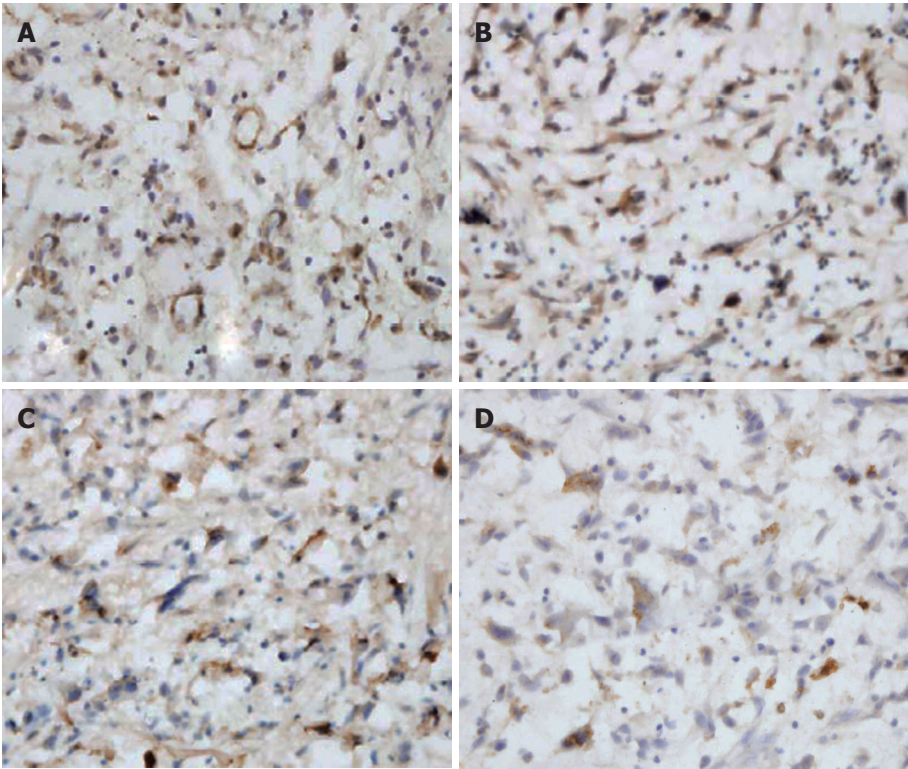


Figure 1 A: TGF-β₁ staining at stenotic bile duct (SABC, x 400); B: Smad4 staining at stenotic bile duct (SABC, x 400); C: CTGF staining at stenotic bile duct (SABC, x 400); D: CTGF mRNA staining at stenotic bile duct (INS, x 400).

Table 1 Positive expression of various cytokines in TGF-β₁/Smad/CTGF signaling pathway in benign biliary stricture group and normal control group

Group	TGF-β ₁	TβR I	TβR II	Smad4	Smad7	CTGF	CTGF mRNA
Benign biliary stricture (%)	91.3 ^a	82.6 ^a	87.0 ^a	78.3 ^a	70.0	82.6 ^a	65.2 ^a
Normal control (%)	33.3	16.7	50.0	33.3	50.0	50.0	16.7

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

Smad7, CTGF and CTGF mRNA were calculated. Fisher's exact probability test was used to analyze the relationship between stricture group and normal control group. Spearman linear correlation analysis was used to analyze correlativity between TGF-β₁, Smad4 and CTGF, respectively. *P* < 0.05 was considered statistically significant in difference. Statistical analyses were performed with SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Expression of TGF-β₁, TβR I, TβR II, Smad4, Smad7, CTGF and CTGF mRNA in benign biliary stricture group and normal control group

TGF-β₁/TβR was expressed focally or diffusely in granulation tissue and cytoplasm of fibroblasts, macrophages, vascular endothelial cells, and inflammatory cells (Figure 1A). The expression of TGF-β₁ was weak in fibrous tissue of normal bile duct wall. The positive percentages of TGF-β₁, TβR I and TβR II at benign biliary stricture group were 91.3% (21/23), 82.6% (19/23) and 87.0% (20/23), respectively. Smad4/Smad7 was mainly expressed in cytoplasm and nucleus of fibroblasts, and some was expressed

in fibrocytes (Figure 1B). The positive percentages of Smad4 and Smad7 at benign biliary stricture group were 78.3% (18/23) and 70.0% (16/23), respectively. Positive cells of CTGF were stained as yellow or brownish yellow granules in cytoplasm at immunohistochemical staining and *in situ* hybridization staining (Figure 1C and D). CTGF was expressed mainly in cytoplasm of fibroblasts in benign biliary stricture group, while it was scarcely expressed in fibrocytes in the wall of common bile duct of normal control group. The positive percentages of CTGF and CTGF mRNA of benign biliary stricture group were 82.6% (19/23) and 65.2% (15/23), respectively. The percentages of positive expression of various cytokines in TGF-β₁/Smad/CTGF signaling pathway in benign biliary stricture group and normal control group are shown in Table 1.

Linear correlation of TGF-β/ Smad/ CTGF expression

By Spearman linear correlation analysis, expression of TGF-β₁ has positive correlation with expression of Smad4 (*r* = 0.848, *P* = 0.000) and CTGF (*r* = 0.848, *P* = 0.000); furthermore, Smad4 also has positive correlation with CTGF (*r* = 0.764, *P* = 0.000). Linear correlations of TGF-β/Smad/CTGF expression are shown in Figure 2.

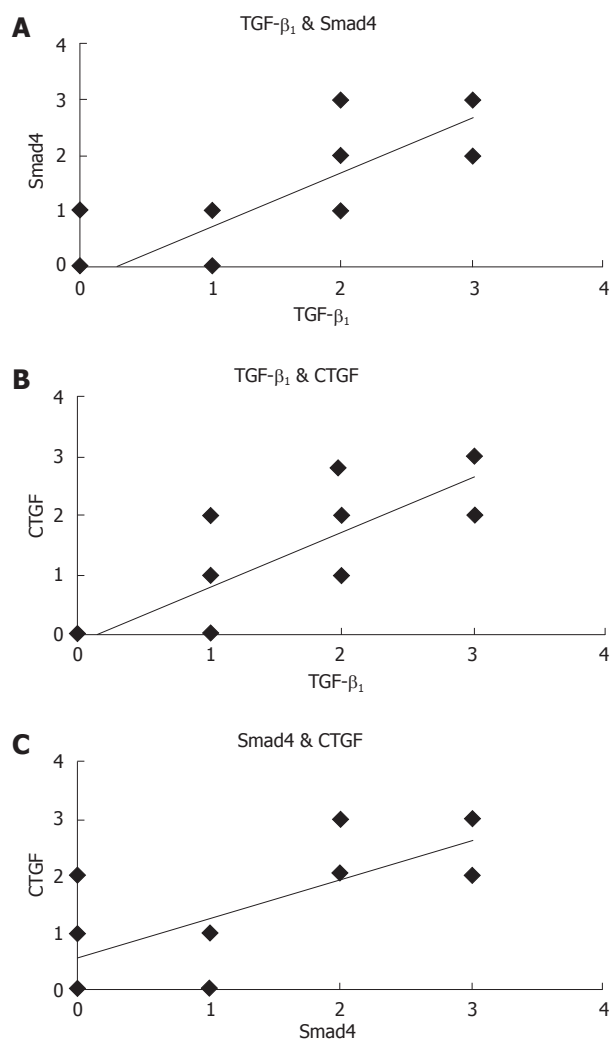


Figure 2 A: Correlation between TGF- β_1 and Smad4; B: Correlation between TGF- β_1 and CTGF; C: Correlation between Smad4 and CTGF.

DISCUSSION

In our previous study, we found that epithelial cells of the bile duct recovered poorly, chronic inflammation existed continuously, fibroblasts proliferated actively, collagens were over-deposited in submucosa, and reconstruction was poor after injury of bile duct. All these result in proliferation of cicatrix and high morbidity of stenosis of anastomosis^[8]. Many studies have shown that TGF- β_1 is the most important cytokine in the pathogenesis of over proliferation of cicatrix^[15-17]. High expression of TGF- β_1 and its receptors was found in keloid, and the expression of TGF- β_1 was significantly high in fibroblasts of hypertrophic scar tissues that were cultivated *in vitro*^[18]. In the present study, we observed that the expression of TGF- β_1 in stenotic bile duct is significantly higher than that in normal bile duct, further confirming the outcome of our previous animal experiment^[19].

Biological effects of TGF- β are regulated by specific signal transduction pathway. Smad family proteins have been identified as signal transducers for the TGF- β superfamily^[20-22]. Following activation of the TGF- β receptor, intracellular signal transduction is mediated

by a variety of Smad proteins. TGF- β_1 /Smad signal transduction pathway can regulate itself by positive and negative feedback regulation loops^[23,24]. Smad4/Smad7 are the important factors in the two circuit loops, respectively. In the T β R I, T β R II and Smad signaling pathway, TGF- β_1 can transfer and amplify signal, initiating its diverse cellular responses by binding to and activating specific cell surface receptors that have intrinsic serine/threonine kinase activity. These activated TGF- β receptors stimulate the phosphorylation of receptor-regulated Smad proteins (PSmad), which in turn form complexes with Smad4 that accumulate in the nucleus, activate the promoter of TGF- β_1 and induce the expression of endogenous TGF- β_1 by itself; this forms the positive feedback regulation loop. Meanwhile, PSmad proteins also can activate the promoter of Smad7 that inhibits TGF- β -induced transcriptional responses^[25]. Psmad2 and Psmad3 may also affect its own promoter region, inhibiting transcription itself, and down-regulate the expression of R-Smad proteins, inhibiting signal transduction^[26]. Accordingly, TGF- β_1 inhibits its signal transduction by negative feedback regulation loop made by activating Smad7 expression and down-regulating R-Smad expression.

This study indicates that T β R I, T β R II, Smad4 in tissue of stenotic bile duct also have high expression besides TGF- β_1 ; furthermore high, expression of TGF- β_1 has positive correlation with Smad4 ($r = 0.848$, $P = 0.000$). We believe that this may be because high-expression of TGF- β can promote the expression of itself and its receptor by a positive feedback regulation loop. This over-expression then could amplify bioactivity and constantly activate smads complexes, which accumulate in the nucleus and regulate the transcription of target genes, resulting in active proliferation of fibroblasts and excessive collagen deposition. So, we can presume that high-expression of R-Smad and Co-smad maybe an important mechanism in pathogenesis of benign biliary stricture. Lasting stimulus of TGF- β_1 that was constantly secreted in tissue of stenotic bile duct and high-sensitivity of the receptor to TGF- β_1 possibly result in the above consequences.

This study also has observed that the expression of Smad7 is higher in tissue of stenotic bile duct than that in normal bile duct, but there was no statistical significance between the two groups. We think that because of the tissue difference, Smad7, which is up-regulated in benign biliary cicatrix does not predominate in the competition with R-smad that was also activated and up-regulated, so Smad7 can't play a role in inhibitive function. R-smad, which predominates in the competition, is phosphorylated by T β R I and further binds to Smad4; the complex transfers into nucleus and regulates the expression of target genes.

CTGF is considered a modulator and assisted mediated factor that mediates biologic function of other molecules, and promotes fission of fibroblasts and accumulation of collagen^[27]. CTGF can promote the synthesis of ECM such as collagen I, collagen III and FN. Lasky *et al* observed that CTGF is over-expressed in

pathogenesis of fibrosis in skin, kidney, liver and heart^[28]. Igarashi *et al*^[29] thought that the expression of CTGF gene is directly regulated by TGF- β_1 . Grotendorst *et al*^[30] also believed that CTGF, which promoted cell proliferation and deposition of ECM, was a downstream medium in the process in which TGF- β_1 produced a marked effect on connective tissue cell.

The results have shown that expression of CTGF in stenotic bile duct tissue is significantly higher than in normal bile duct specimens. This difference indicated that CTGF plays an important role in the pathogenesis of over proliferation of cicatrix and benign biliary stricture. The expression of TGF- β_1 has a positive correlation with that of CTGF ($r = 0.848$, $P = 0.000$); this confirms that both of them play roles of superior and inferior grade in signal transduction pathway in the pathogenesis of benign biliary stricture, similar to other fibrotic disease, whereas the positive correlation between expression of Smad4 and CTGF ($r = 0.764$, $P = 0.000$) indicates that the relationship between TGF- β_1 and CTGF is connected by Smads signaling pathway.

Taken together, the present study indicates that the mechanism of effect in TGF- β_1 /Smad/CTGF signaling pathway is as follows. The continuity of bile duct wall is destroyed after bile duct injury, which results in changes of adjacent intra-cellular metabolism. Then, TGF- β is released and binded to the type II receptor at first; thus a binary complex is formed. Type I receptor then is recruited and phosphorylated in its GS domain by T β R II, leading to activation of its kinase activity and subsequent formation of a signaling complex. R-Smads are phosphorylated by this signaling complex, and in turn can form heteromeric complexes with Smad4. These activated Smad complexes accumulate in the nucleus, where they directly or indirectly bind to specific promoter regions on target genes together with transcription factor (TF) and/or co-activators/repressors, and downstream mediator CTGF is activated. The activation of TGF- β /Smad/CTGF signal transduction pathway can result in fibrosis in the interaction between cell and ECM, cause disorder of metabolism and regulation between inflammatory cell, repairing cell and collagen. Fibrocytes in submucosa are transformed into activated fibroblasts, which proliferate abundantly, and synthesize and secrete collagen fibers. Under the stimulus of bile and secondary infection, inflammation extends and the wound healing process disorders, chronic inflammation exists continuously, fibroblasts proliferate constantly, collagens are over-deposited in submucosa. All these result in a prolonged healing process of bile duct, proliferation of cicatrix and morbidity of benign biliary stricture.

COMMENTS

Background

Transforming growth factor beta 1 (TGF- β_1) has a key role either in the wound healing process or induction of fibrosis. The biological effects of TGF- β_1 are regulated by TGF- β /Smad/connective tissue growth factor (CTGF) signaling pathway. The main manifestations of benign biliary stricture are scar contracture

and stenosis of bile duct. The exact role of TGF- β signaling pathway in the pathogenesis of benign biliary stricture is still unknown.

Research frontiers

In the last few years, many studies have shown that TGF- β_1 is the most important cytokine in the pathogenesis of over proliferation of cicatrix. Biological effects of TGF- β are regulated by specific signal transduction pathway. Smad family proteins have been identified as signal transducers for the TGF- β superfamily. The expression of CTGF gene is regulated directly by TGF- β_1 . CTGF, which promotes cell proliferation and deposition of extracellular matrix (ECM), is a downstream medium in the process in which TGF- β_1 produces a marked effect on connective tissue cell.

Innovations and breakthroughs

The high expression of TGF- β /Smad/CTGF signaling pathway plays an important role in the pathogenesis of benign biliary stricture. These results demonstrate a new view of TGF- β signaling pathway involved in the benign biliary stricture.

Applications

This study indicates that the mechanism of TGF- β_1 /Smad/CTGF signaling pathway in pathogenesis of benign biliary stricture. It may provide new targets for further understanding of the pathogenesis of benign biliary stricture and new therapeutic targets.

Terminology

Smads are the only substrates for type I receptor kinases known to have a signalling function; they were first identified as the products of the *Drosophila* Mad and *C. elegans* Sma genes, which lie downstream of the BMP-analogous ligand-receptor systems in these organisms. Smad proteins transduce signals from TGF- β superfamily ligands that regulate cell proliferation, differentiation and death through activation of receptor serine/threonine kinases.

Peer review

The study is well designed and contains important information and novelties concerning TGF- β_1 signaling pathway in benign biliary stricture. The authors performed a great amount of experiments and a comprehensive statistical analysis of data.

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Inhibitory effects of genistein and resveratrol on guinea pig gallbladder contractility *in vitro*

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Abstract

AIM: To observe and compare the effects of phytoestrogen genistein, resveratrol and 17 β -estradiol on the tonic contraction and the phasic contraction of isolated gallbladder muscle strips and to study the underlying mechanisms.

METHODS: Isolated strips of gallbladder muscle from guinea pigs were suspended in organ baths containing Krebs's solution, and the contractilities of strips were measured before and after incubation with genistein, resveratrol and 17 β -estradiol respectively.

RESULTS: Similar to 17 β -estradiol, genistein and resveratrol could dose-dependently inhibit the phasic contractile activities, they decreased the mean contractile amplitude and the contractile frequencies of gallbladder muscle strips, and also produced a marked reduction in resting tone. The blocker of estrogen receptor ICI 182780 failed to alter the inhibitory effects induced by genistein and resveratrol, but potassium bisperoxo (1, 10 phenanthroline) oxovanadate bpV (phen), a potent protein tyrosine phosphatase inhibitor, markedly attenuated the inhibitory effects induced by genistein and resveratrol. In calcium-free Krebs's

solution containing 0.01 mmol/L egtazic acid (EGTA), genistein and resveratrol inhibited the first phasic contraction induced by acetylcholine (ACh), but did not affect the second contraction induced by CaCl₂. In addition, genistein, resveratrol and 17 β -estradiol also could reduce the contractile responses of ACh and KCl, and shift their cumulative concentration-response curves rightward.

CONCLUSION: Phytoestrogen genistein and resveratrol can directly inhibit the contractile activity of isolated gallbladder muscle both at rest and in response to stimulation. The mechanisms responsible for the inhibitory effects probably due mainly to inhibition of tyrosine kinase, Ca²⁺ influx through potential-dependent calcium channels (PDCs) and Ca²⁺ release from sarcoplasmic reticulum (SR), but were not related to the estrogen receptors.

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Key words: Phytoestrogen; Estradiol; Gallbladder; Smooth muscle; Ca²⁺ channel

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INTRODUCTION

Female sex hormones are known to affect cholesterol metabolism, gallbladder and sphincter of Oddi motility^[1-4]. Phytoestrogens represent a wide group of compounds which are naturally found in many plants, and they are defined as plant substances that are structurally or functionally similar to estradiol^[5,6]. These natural products possess a wide spectrum of physiological and pharmacological effects such as estrogenic effects^[7,8], anti-atherosclerosis^[9], anti-osteoporosis^[10], relieving menopausal symptoms^[11] and the inhibitory effect of tyrosine kinases^[12,13]. Recently, many papers indicate that phytoestrogen genistein and

resveratrol can inhibit vasocontractile responses and relax vascular smooth muscles by a Ca^{2+} antagonistic property which is very similar to estradiol^[14,15], and data from electrophysiological studies suggest genistein reversibly inhibits L-type calcium current in isolated guinea-pig ventricular myocytes in a concentration-dependent manner^[16]. Therefore, the biological actions of genistein and resveratrol are very useful for medicine and nutrition, and they are proposed to have potential as natural substitutes of estrogen therapy. Despite the increasing interest in the effects of phytoestrogen in the cardiovascular system, little is known about the effect of genistein and resveratrol on gallbladder smooth muscle. The present study was designed to observe and compare the effects of phytoestrogen genistein, resveratrol and 17 β -estradiol on the tonic contraction and the phasic contraction of isolated gallbladder muscle strips both at rest and in response to acetylcholine (ACh) and KCl and to study its underlying mechanisms.

MATERIALS AND METHODS

Animal and tissue preparation

The present work was conducted in conformity with the procedures described in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, and the procedures performed were in accordance with institutional guidelines. Adult male or non-pregnant female guinea pigs (weighing 387.8 ± 7.5 g, provided by the experimental animal center of Lanzhou University) were utilized in this study. Preliminary studies indicated that no differences existed between the sexes with respect to either the contractile responsiveness to the agonists or to the sensitivities to genistein, resveratrol and 17 β -estradiol. All animals were fasted overnight prior to being sacrificed by overdose injection of pentobarbitone and the whole gallbladders were quickly removed and placed in Krebs's solution containing the following compositions (mmol/L): NaCl 120, KCl 5.9, NaH_2PO_4 1.2, MgCl_2 1.2, NaHCO_3 15.4, CaCl_2 2.5, and glucose 11.5, buffered at pH 7.4. After removal of the mucosa by blunt dissection, muscle strips (5 mm \times 10 mm) were prepared from the body of the gallbladder by cutting parallel to the long axis of the tissue, and then were mounted horizontally in separate 5-mL tissue chambers containing $37 \pm 0.5^\circ\text{C}$ Krebs's solution, bubbled with 95% O_2 and 5% CO_2 . The muscle preparations were allowed to equilibrate for 2-3 h with a resting tension of 1.0 g and the solution was changed every 20 min. The isometric contractions were measured with force transducers and recorded with the BL-420E⁺ experimental system of biological function (TME, China) by microcomputer.

Experimental protocols

In order to observe the effects of genistein, resveratrol and 17 β -estradiol on the basal contractile activities of gallbladder, the different concentration (1, 10, 20 or 40 $\mu\text{mol/L}$) of genistein, resveratrol and 17 β -estradiol or the same dose of solvent (control) was added

respectively to the tissue chamber for 10 min. A specific antagonist of estrogen receptor ICI 182780 (10 $\mu\text{mol/L}$) or a potent protein tyrosine phosphatase inhibitor bpV (phen) (1 $\mu\text{mol/L}$) was added 10 min before administration of 10 $\mu\text{mol/L}$ genistein or resveratrol to investigate whether their actions were relevant with the estrogen receptors and tyrosine kinase inhibition in gallbladder smooth muscle.

To evaluate the possible effect of genistein, resveratrol and 17 β -estradiol on ACh-induced calcium release and calcium influx through receptor-operated calcium channels (ROCs), gallbladder muscle strips were incubated in calcium-free Krebs's solution containing 0.01 mmol/L egtazic acid (EGTA) for 30 min, and then treated with ACh (10 $\mu\text{mol/L}$). When the contractile response had reached a plateau, CaCl_2 (10 mmol/L) was added into the organ chamber and a further contraction was obtained. Tissues were washed with Ca^{2+} -free Krebs's solution and left to return to baseline tone. The strips were then treated by ACh and CaCl_2 again after being incubated with genistein (20 $\mu\text{mol/L}$) and resveratrol (20 $\mu\text{mol/L}$) or the same dose of solvent for 6-8 min.

In some experiments, to determine the effect of genistein, resveratrol and 17 β -estradiol on contractile response to ACh and potassium, the control contractile response curves to ACh (10^{-8} - 10^{-3} mol/L) and KCl (10-100 mmol/L) were obtained respectively, then the strips were washed repeatedly with Krebs's solution until the strips returned to the basal tension. The strips were then incubated with genistein (40 $\mu\text{mol/L}$), resveratrol (40 $\mu\text{mol/L}$) or 17 β -estradiol (40 $\mu\text{mol/L}$) for 10 min respectively, and ACh or KCl concentration-dependent contraction curve was obtained again.

Drugs

Genistein, resveratrol, 17 β -estradiol (Sigma, Chemical Co, USA); ACh (the Second Pharmaceutical Factory of Beijing, China); ICI 182780 (Tocris Cookson Inc., Bristol, UK); potassium bisperoxo (1,10 phenanthroline)oxovanadate bpV (phen) (Alexis Biochemicals, San Diego, CA). ICI 182780, genistein, resveratrol and 17 β -estradiol were dissolved with dimethyl sulphoxide (DMSO). The final concentration of DMSO in the bath in each case was always no more than 0.1% and had no effect on basal contraction.

Statistical analysis

All results are expressed as mean \pm SE. "*n*" refers to the number of guinea pigs used in the study. Data were expressed as % decrease in the basal tension, mean amplitude and mean frequencies of phasic contraction. In experiments involving concentration-response curves, the results were expressed as percentage of control maximal contractile responses induced by 10^{-3} mol/L ACh or 100 mmol/L KCl respectively. The EC_{50} value of each strip was determined by the Scott Method, and was expressed as negative log molar (pD_{50}) value. Statistical analysis was performed using the Student *t*-test and analysis of variance (ANOVA). Each group was compared with the solvent control. $P < 0.05$ was considered significant.

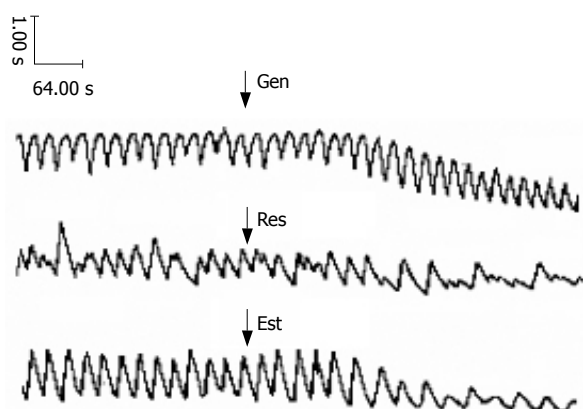


Figure 1 Sample traces showing the basal contractile activity of the gallbladder before and after the administration of 20 µmol/L genistein (Gen), resveratrol (Res) and 17β-estradiol (Est).

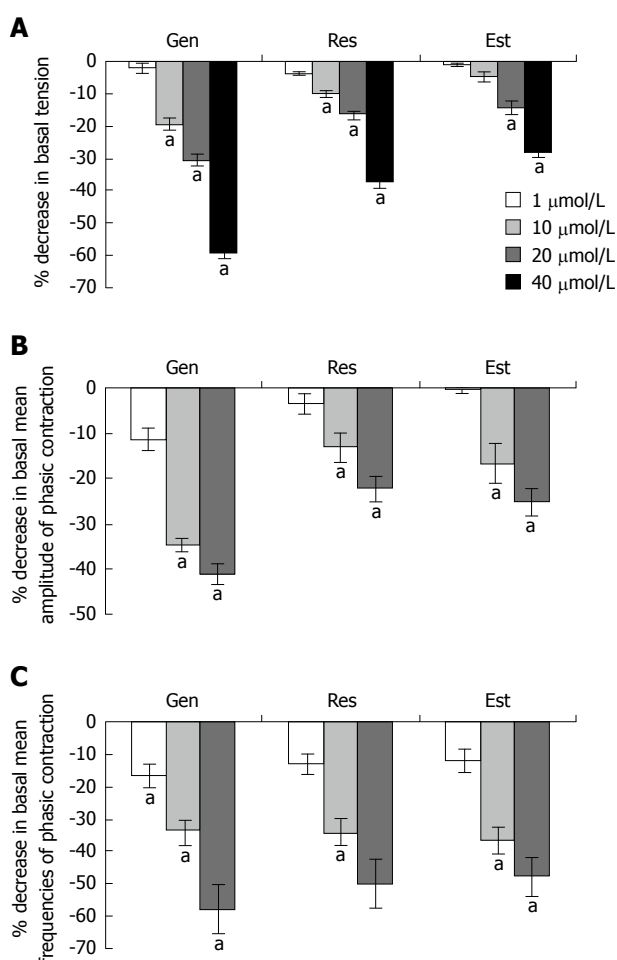


Figure 2 Effects of genistein (Gen), resveratrol (Res) and 17β-estradiol (Est) on resting tension (A), mean contractile amplitude (B) and (C) mean frequencies of phasic contraction in isolated guinea pig gallbladder muscle strips ($n = 10$). $^aP < 0.05$ vs solvent control.

RESULTS

Effects of genistein, resveratrol and 17β-estradiol on basal activities of gallbladder muscle strips

The spontaneous contractile activities of isolated gallbladder smooth muscle were not very regular, and some strips had obvious spontaneous phasic

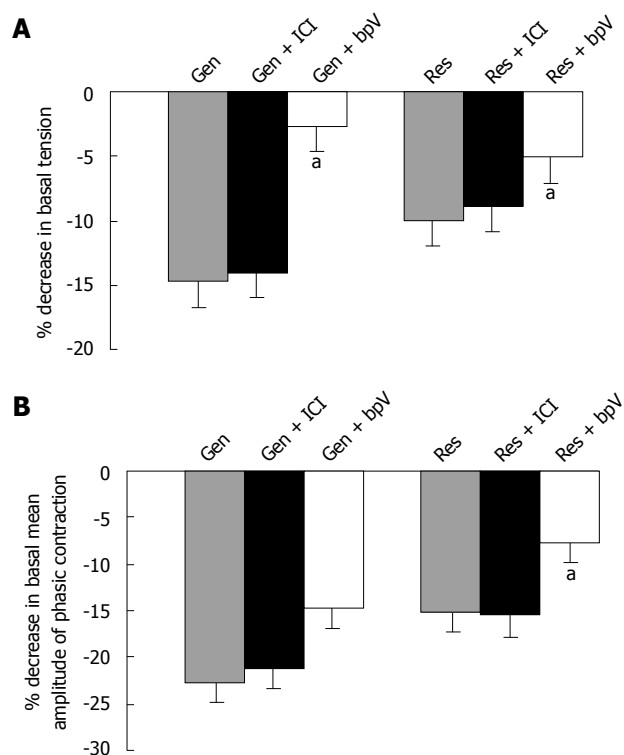


Figure 3 Effects of genistein (Gen, 10 µmol/L) and resveratrol (Res, 10 µmol/L) on the basal tension (A) and mean amplitude (B) of phasic contraction in isolated guinea pig gallbladder muscle strips after preincubation with ICI 182780 (ICI) or bpV (phen) (bpV) ($n = 5$). $^aP < 0.05$ vs corresponding Gen or Res group.

contractions with mean amplitude of 0.49 ± 0.06 g and mean frequencies of 2.80 ± 0.25 waves/min (Figure 1) while the others only possessed tonic contraction. In the strips with spontaneous phasic contractions, genistein, resveratrol and 17β-estradiol (1, 10, 20 or 40 µmol/L) could dose-dependently inhibit the phasic contractile activities, they decreased the mean contractile amplitude and the contractile frequencies and also produced a marked reduction in resting tone (Figures 1 and 2). Increasing the concentrations of the above three estrogens to 40 µmol/L, the phasic contractile activities disappeared completely, the decreased percentages of the mean contractile amplitude and the contractile frequencies all reached 100%.

Effects of genistein and resveratrol on basal activities of gallbladder in the presence of ICI 182780 and bpV (phen)

The inhibitory effects induced by genistein and resveratrol in gallbladder muscle strips had no obvious change in the presence of the specific estrogen receptor inhibitor ICI 182780 (10 µmol/L) (Figure 3), but after incubating the strips with the potent protein tyrosine phosphatase inhibitor bpV (phen) (1 µmol/L), the inhibitory effects induced by genistein and resveratrol markedly attenuated (Figure 3). ICI 182780 (10 µmol/L) and bpV (phen) (1 µmol/L) alone had no obvious effect on basal activity.

Effects of genistein and resveratrol on biphasic contraction induced by ACh and CaCl₂

In calcium-free (0.01 mmol/L EGTA) Krebs's solution,

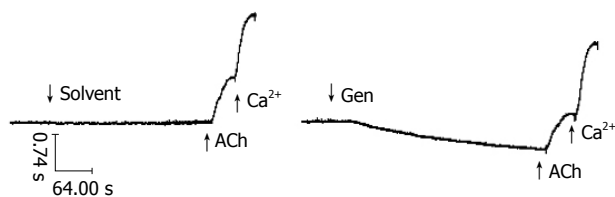


Figure 4 Traces of ACh and CaCl_2 -induced contraction of gallbladder muscle strip in Ca^{2+} -free Kreb's solution in the absence and presence of genistein (Gen, 20 $\mu\text{mol/L}$).

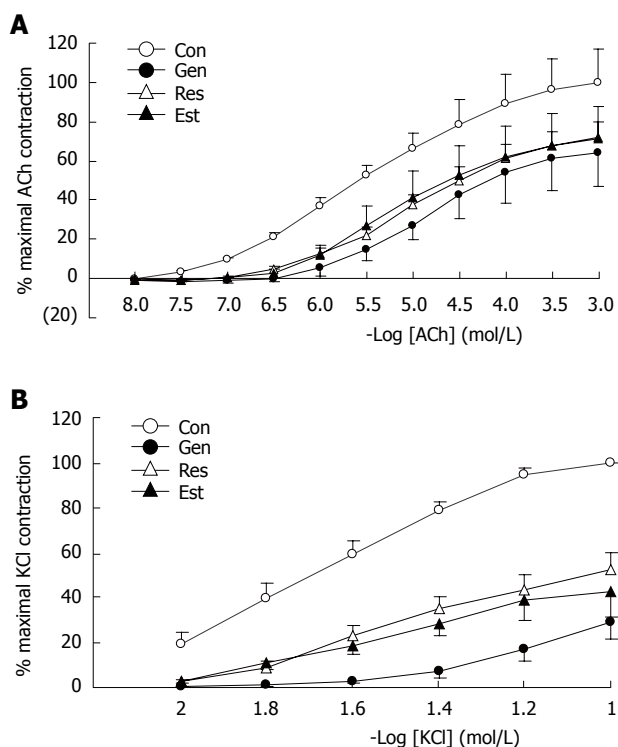


Figure 5 A: Line plots showing effects of genistein (Gen, 40 $\mu\text{mol/L}$), resveratrol (Res, 40 $\mu\text{mol/L}$) and 17 β -estradiol (Est, 40 $\mu\text{mol/L}$) on ACh concentration-dependent contraction curves in isolated guinea pig gallbladder muscle strips ($n = 6-7$); B: Effects of genistein (Gen, 40 $\mu\text{mol/L}$), resveratrol (Res, 40 $\mu\text{mol/L}$) and 17 β -estradiol (Est, 40 $\mu\text{mol/L}$) on KCl concentration-dependent contraction curves in isolated guinea pig gallbladder muscle strips ($n = 12$).

no spontaneous phasic contractions were observed, but ACh (10 $\mu\text{mol/L}$) could cause a transient contraction with the tensile increase of 0.89 ± 0.10 g. As soon as such contraction reached a plateau, CaCl_2 10 mmol/L was rapidly added into the bath and another higher contractile response occurred with the tensile increase of 1.10 ± 0.18 g ($n = 4$). Genistein (20 $\mu\text{mol/L}$; Figure 4) and resveratrol (20 $\mu\text{mol/L}$) reduced the first contraction induced by ACh from 0.89 ± 0.10 g to 0.50 ± 0.18 g and 0.64 ± 0.15 g respectively (all $P < 0.05$, $n = 4$), but did not change the second contraction caused by CaCl_2 (1.23 ± 0.25 in genistein groups and 1.18 ± 0.15 in resveratrol groups *vs* 1.10 ± 0.18 g in control groups respectively, all $P > 0.05$, $n = 4$) in Ca^{2+} -free Kreb's solution.

Effects of genistein, resveratrol and 17 β -estradiol on agonist-induced contractions

ACh (10^{-8} - 10^{-3} mol/L) and KCl (10-100 mmol/L)

elicited concentration-dependent contractile responses in isolated gallbladder muscle strips. However, genistein, resveratrol and 17 β -estradiol significantly reduced the responses to ACh and KCl, and made their concentration-dependent contraction curves shift to the right (Figure 5). The pD_2 values of ACh in control and after incubation with 40 $\mu\text{mol/L}$ genistein, 40 $\mu\text{mol/L}$ resveratrol and 40 $\mu\text{mol/L}$ 17 β -estradiol were 3.97 ± 0.16 , 3.38 ± 0.17 ($P < 0.05$ *vs* control, $n = 6$), 3.54 ± 0.08 ($P < 0.05$ *vs* control, $n = 10$) and 3.45 ± 0.14 ($P < 0.05$ *vs* control, $n = 7$), respectively. The pD_2 values of KCl in control and after incubation with 40 $\mu\text{mol/L}$ genistein, 40 $\mu\text{mol/L}$ resveratrol and 40 $\mu\text{mol/L}$ 17 β -estradiol were 1.61 ± 0.30 , 0.70 ± 0.07 ($P < 0.05$ *vs* control, $n = 11$), 1.12 ± 0.03 ($P < 0.05$ *vs* control, $n = 14$) and 1.10 ± 0.05 ($P < 0.05$ *vs* control, $n = 7$).

DISCUSSION

The gallbladder and gut should be viewed as hormonally responsive organs. The normal physiology of which may be altered by the sex hormones^[1]. Also, it is well established that cholelithiasis is more frequent in women than in men. This difference is usually explained by the effects of estrogens and progesterone on the metabolism of bile acids, biliary cholesterol secretion and saturation, and gallbladder motility^[3,4]. As we know, gallbladder motility has an important role in the regulation of bile flow, its function disturbances may prevent normal bile flow and thus enhance the probability of common bile duct stone formation. Sex steroid hormone have inhibitory effects on the contractility which may be mediated by the inhibition of the mobilization of intracellular calcium and calcium influx in gallbladder smooth muscles^[4].

Papers have shown that there are structural similarities between the steroidal nucleus of 17 β -estradiol and the rigid ring structure of phytoestrogen genistein, and because both of them are lipid-soluble compounds and their molecular weight are not large, they can easily enter cytoplasm through the cellular membrane to affect expression of some genes. The affinity of genistein to the classic estrogen α receptor (ER_α) presented on reproductive organs is less than that of estrogen^[17], but it has a similar affinity as estrogen for the novel estrogen β receptor (ER_β) in the vasculature^[18]. As well as evidence that resveratrol exhibits variable degrees of estrogen receptor agonism in different test systems, and the similarity in structure between resveratrol and the synthetic estrogen diethylstilbestrol (DES; 4,4'-dihydroxy-trans- α , β -diethylstilbene) prompted us to investigate whether resveratrol might exhibit estrogenic activity in gallbladder motility^[19]. The present study has shown that the phytoestrogen genistein and resveratrol can induce significant inhibitory effects on isolated gallbladder contractility in a similar way as 17 β -estradiol does, and the effects were dose-dependent. Gallbladder smooth muscle cells have been shown to express functional ER_α ^[2], so the effects of phytoestrogen genistein and resveratrol may be attributed to their combination with ER_α , but our study demonstrates

that the inhibitory effects induced by genistein and resveratrol are unlikely to be mediated through the ER, as the actions of genistein and resveratrol had no obvious change in the presence of the pure and specific ER antagonist ICI 182780, although it can block not only the classical ER α but also the novel ER β ^[20]. These results suggest that the acute inhibitory effects caused by genistein, resveratrol and 17- β estradiol are not mediated by the classical estrogen receptor and are independent of gene-mediated events.

It is well known that genistein and resveratrol are tyrosine kinase inhibitors^[12,13] and tyrosine kinase activity has been demonstrated to play a role in smooth muscle contractility^[4,21]. In the present experiment, a potent protein tyrosine phosphatase inhibitor bpV (phen), which can prevent the decrease of protein tyrosine phosphorylation, markedly attenuated the inhibitory effects of 10 μ mol/L genistein and 10 μ mol/L resveratrol on gallbladder smooth muscle contractile activities. Our results suggest that tyrosine kinase inhibition is probably responsible for the inhibitory effects induced by genistein and resveratrol in gallbladder smooth muscle contractility. These results are supported by the evidences that tyrosine kinase inhibition contributes to the decrease of Ca²⁺ influx and Ca²⁺ mobilization^[21,22].

The presence of cholinergic M receptors in guinea pig gallbladder smooth muscle cells has been reported^[23]. ACh can activate receptor-operated calcium channels (ROCs) in the cellular membrane of gallbladder smooth muscle and increase calcium influx, while also activating G proteins and phospholipase C to produce inositol trisphosphate (IP₃) which causes calcium release from endoplasmic reticulum^[4,23]. As we know, the contractile response to ACh comprises two distinct components in Ca²⁺-free medium: an initial phasic component that results from IP₃-mediated release of Ca²⁺ from intracellular Ca²⁺ stores followed by a tonic component that requires addition of Ca²⁺ in the continuous presence of ACh, due to Ca²⁺ influx. This is so-called biphasic contraction induced by ACh and Ca²⁺. In calcium-free Krebs's solution, genistein and resveratrol could significantly decrease ACh-induced contraction but they did not affect the latter CaCl₂-induced contraction. In normal Krebs's solution, genistein, resveratrol and 17 β -estradiol could also reduce the contractile responses of ACh and shift their cumulative concentration-response curves rightward in a parallel manner. Considering these observations, it seems reasonable to suggest that the inhibition of Ca²⁺ release may involve in the inhibitory effects of genistein and resveratrol on gallbladder smooth muscle contractility.

Potential dependent calcium channels (PDCs) are activated by depolarization of the plasma membrane when the extracellular K⁺ concentration is increased, and it has been reported that potassium-stimulated gallbladder contraction depends exclusively upon the influx of extracellular calcium^[4]. In the present experiment, genistein, resveratrol or 17 β -estradiol could shift the KCl concentration-dependent contraction

curves to the right in normal Krebs's solution and inhibited KCl concentration-dependent contractile responses in a noncompetitive manner. The results suggest that genistein and resveratrol may have Ca²⁺ antagonistic properties which are consistent with the effect of 17 β -estradiol, and inhibit extracellular Ca²⁺ influx through PDC.

In summary, similar to 17 β -estradiol, genistein and resveratrol have been shown to have a direct inhibitory effect on both the basal and agonist-stimulated contractile activity of guinea pig gallbladder *in vitro*.

COMMENTS

Background

Phytoestrogen genistein and resveratrol are structurally and functionally similar to estrogen and possess many physiological and pharmacological effects. Data indicate that genistein, resveratrol can inhibit vasocontractile responses and relax vascular smooth muscles by a Ca²⁺ antagonistic property which is similar to estradiol, but little is known about the effect of genistein and resveratrol on gallbladder smooth muscle motility.

Research frontiers

Gallbladder disease is more prevalent in women than men, and estrogen therapy has been associated with an increased incidence of gallbladder disease in both sexes, suggesting that hormones may play an important role in these conditions. Phytoestrogens such as genistein and resveratrol have estrogen agonistic/antagonistic effects, and are proposed to have potential as natural substitutes of estrogen therapy.

Innovations and breakthroughs

This study aim is to compare the direct effects of genistein and resveratrol on isolated gallbladder smooth muscle motility with that of 17 β -estradiol both at rest and in response to stimulation, and to elucidate the underlying mechanisms.

Applications

The present results reflect the pharmacological actions of genistein and resveratrol and can provide the pharmacological guidance for the application of these compounds which are very valuable for medicine and nutrition.

Peer review

This is an interesting report of effects of genistein and resveratrol on gallbladder contractility.

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Laparoscopic resection for incidentally detected Meckel diverticulum

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Abstract

The management of Meckel diverticulum found unexpectedly during an abdominal operation remains controversial. Most published reports have included only patients undergoing diverticulectomy or bowel resection through laparotomy. We report a case of a carcinoid tumor in a Meckel's diverticulum which was incidentally detected and removed during laparoscopic inguinal hernia repair. Although there is no compelling evidence in the literature to recommend prophylactic diverticulectomy, laparoscopic stapled resection represents a viable and safe approach in healthy individuals undergoing elective surgery for other purposes.

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Key words: Laparoscopy; Incidental findings; Meckel's diverticulum; Carcinoid tumor

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Bona D, Schipani LS, Nencioni M, Rubino B, Bonavina L. Laparoscopic resection for incidentally detected Meckel diverticulum. *World J Gastroenterol* 2008; 14(31): 4961-4963 Available from: URL: <http://www.wjgnet.com/1007-9327/14/4961.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.4961>

INTRODUCTION

Meckel's diverticulum (MD) is one of the most common congenital abnormalities of the gastrointestinal tract, as it occurs in approximately 2% of the population. This true diverticulum results from an incomplete obliteration of the vitelline duct during the fifth week of gestation and arises from the antimesenteric border of the distal ileum, within 100 cm of the ileocecal valve in the adult. In the majority of cases, the MD is asymptomatic and the diagnosis is made during elective surgery for other intra-abdominal disorders. In some circumstances, the MD can be associated with heterogeneous clinical manifestations ranging from recurrent abdominal pain to life-threatening problems, such as gastrointestinal bleeding or acute bowel obstruction. The diverticulum may contain areas of ectopic mucosa, more commonly of gastric type, or malignant tumors such as carcinoids^[1].

The decision to perform diverticulectomy for MD incidentally detected during an abdominal operation is still controversial. Over the past two decades, laparoscopy has been extensively used in the diagnosis and treatment of various abdominal disorders. The opportunity provided by the laparoscopic approach to perform a complete abdominal exploration may increase the number of incidental findings, and this may again pose a dilemma to the surgeon who is more and more committed to the principles of evidence-based medicine for a better and more cost-effective patient care.

CASE REPORT

A 66 year-old man was seen in the outpatient clinic because of the chronic complaint of pain on exertion and a bulge localized at his left groin. His medical history was unremarkable, except for a mild hypertension. The body mass index was 30. Physical examination showed a bilateral inguinal hernia. An abdominal ultrasonography was negative. Elective laparoscopic repair of the bilateral hernia was scheduled after the patient gave his informed consent.

Preoperative antibiotic prophylaxis (Cefazolin, 2 g i.v.) was administered. Under general anesthesia, pneumoperitoneum was established using a Veress needle. Three bladeless trocars were inserted along the transverse umbilical line, and a 30° angled scope was introduced through the umbilical port. Laparoscopic explora-

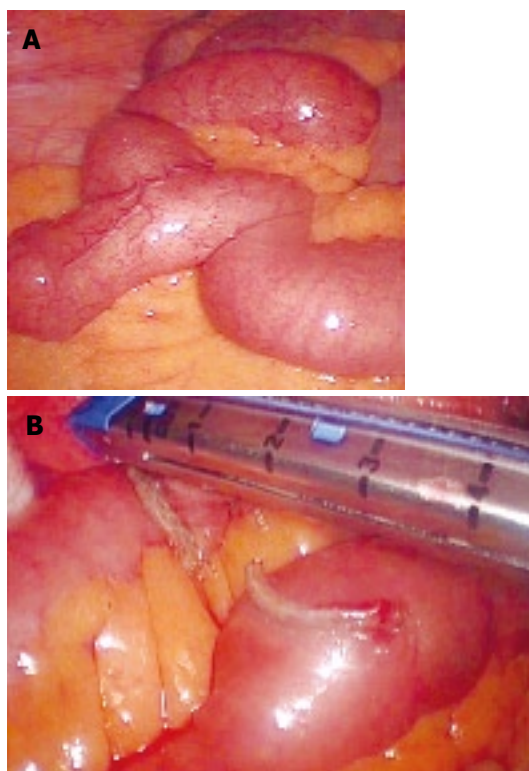


Figure 1 Meckel diverticulum found unexpectedly during laparoscopic inguinal hernia repair (A) and resection of the diverticulum with an endoscopic linear stapler (B).

tion immediately revealed a 4 cm long MD arising from the distal ileum, about 70 cm proximal to the ileocecal valve (Figure 1). Priority was given to the inguinal hernia repair. Starting from the left groin, a curvilinear incision was made in the peritoneum beginning laterally and extending to the medial umbilical ligament. A peritoneal flap was created medially by blunt dissection to expose the Cooper's ligament. A preformed polypropylene mesh was placed within the pre-peritoneal pocket and secured with staples and fibrin glue. The peritoneal layer was closed with a running suture (PDS, Ethicon). The same procedure was repeated in the right groin. Upon completion of the hernia repair, the MD was held with an atraumatic grasper through the left port and an endoscopic linear stapler (EndoGIA II, 60 mm, Covidien) was tangentially applied across the base of the diverticulum and fired. The specimen was put into a plastic bag and removed through one of the ports. The patient had an uncomplicated recovery and was discharged home on postoperative day 2. Pathologic examination of the surgical specimen showed a normal ileal mucosa lining and a nodule, 1.5 cm in diameter, with a pattern indicative for a carcinoid tumor located in the proximity of the tip of the diverticulum (Figure 2).

DISCUSSION

It has long been stated that the risk of developing complications following the incidental removal of MD can offset the potential benefits of this procedure^[2]. Opponents to incidental diverticulectomy often cite Soltero

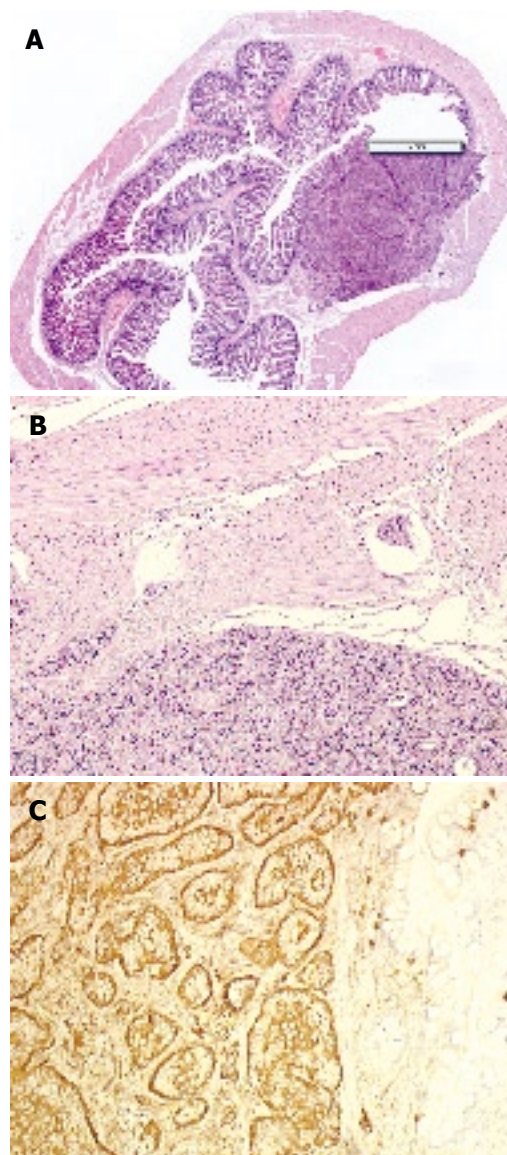


Figure 2 Meckel diverticulum with a nodule 1.5 cm in size (HE, x 1.5) (A), superficial infiltration of the diverticulum wall (HE, x 10) (B), and immunohistochemical stain with chromogranin A showing a strongly positive reaction and normal intestinal mucosa with control stain of endocrine single cells on the right (x 10) (C).

and Bill^[3] who, in 1976, estimated that the life-time risk of complications from an untreated MD was 4.2%, and that this risk decreased to zero with age. These authors recommended refraining from incidental diverticulectomy because there is only a small chance of MD-related complications in later life. In contrast, almost twenty years later, the results of a large population-based study in Olmsted County, Minnesota, provided data in support of prophylactic diverticulectomy^[4]. This study reported a 6.4% cumulative rate of developing complications of MD that required surgery over a life-time, especially in male patients up to 80 years of age. Diverticulectomy for complicated MD carried an operative mortality and morbidity of 2% and 12%, and a cumulative risk of long-term complications of 7%. The corresponding rate for incidental diverticulectomy is 1%, 2% and 2%, respectively. Interestingly, in this latter subgroup of patients,

the mortality was related to the primary operation or the patient fitness but not to the diverticulectomy itself^[4]. A subsequent report from the Mayo Clinic recommended MD resection only in male patients younger than 50 years of age, or when the diverticulum length is greater than 2 cm, or when abnormal features are detected within the diverticulum^[1]. Of interest, a carcinoid tumor was found in 2.2% of the symptomatic patients and in 2.1% of the asymptomatic ones in this series^[1]. More recently, however, a systematic review of the English literature has shown that there is no compelling evidence to support prophylactic resection^[5]. In fact, resection of incidentally detected MD has a significantly higher early complication rate than leaving the diverticulum *in situ* (5.3% *vs* 1.3%, $P < 0.0001$)^[4].

It should be noted that most of the data upon which recommendations have been based so far originate from retrospective studies in patients who underwent incidental diverticulectomy or bowel resection through laparotomy^[5]. The advent of laparoscopy may have changed this scenario. Laparoscopy allows a complete abdominal exploration in patients undergoing minimally invasive procedures and has the potential to reveal additional pathological findings. Moreover, laparoscopy has been used to diagnose and treat patients with MD complicated by small bowel obstruction or bleeding caused by occult heterotopic gastric mucosa^[6,7].

To our knowledge, this is the first reported case of carcinoid in MD which was incidentally found and removed during laparoscopy for inguinal hernia repair. Carcinoids are slow growing tumors with a malignant potential arising from the diffuse neuroendocrine system. The gastrointestinal tract is the largest neuroendocrine organ in the body and the site of origin for 90% of all carcinoids. The most common location of carcinoid is the appendix, where tumors are usually small and benign, followed by the ileum, where they are often multiple and characterized by a more aggressive biological behavior. More than 100 cases of carcinoids in MD have been reported in the literature, 72% of the tumors were located at the tip of the diverticulum, and distant metastases were

present in 24% of the patients at the time of diagnosis^[8]. Immunohistochemical studies have shown that Meckel's carcinoids are closer to the ileal rather than to the appendiceal carcinoids^[9]. Histopathological analysis in our patient demonstrated a R0 resection and the presence of limited superficial invasion of the diverticulum wall, confirming that diverticulectomy is adequate. Although there is no compelling evidence in the current literature to support prophylactic diverticulectomy for patients with MD, we believe that simple laparoscopic tangential resection with an endostapler is a viable and safe procedure during elective operations for healthy patients.

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CASE REPORT

A case of long survival in poorly differentiated small cell carcinoma of the pancreas

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Abstract

Small cell carcinoma (SCC) of the pancreas is rare. It has similar histological features to pulmonary small cell carcinoma and is equally aggressive. Most patients with SCC in the pancreas reported in case studies died within 1 year after diagnosis. We present a case of unusually long-term survival after surgery and combined chemotherapy for SCC of the pancreas. A 62-year-old woman presented with epigastric pain and jaundice. Computed tomography revealed dilated common bile duct caused by external compression of the mass in the pancreatic head. Exploratory laparotomy and pancreaticoduodenectomy (PPPD) was performed with histopathological analysis confirming a primary small cell carcinoma of the pancreas. After an uneventful postoperative recovery, the patient was treated with 6 cycles of combined chemotherapy consisting of cisplatin and ectoposide. During the follow-up, there was no evidence of recurrence and the patient has remained in a good health condition for 36 mo since the diagnosis.

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Key words: Small cell carcinoma; Pancreas; Pancreatic carcinoma; Extrapulmonary

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INTRODUCTION

Small-cell carcinoma (SCC) is common malignancy of the lung and represents 20%-25% of all bronchogenic carcinomas^[1]. It seldom originated from extrapulmonary sites (2.5%-4% of all SCC). The primary site of extrapulmonary SCC (EPSCC) can be in a variety of organs and the clinical course of these tumors has been found to be aggressive, with early dissemination and frequent recurrence. Primary SCC of the pancreas is rare, comprising about 1% of all pancreatic malignant tumors^[2]. Because of its aggressiveness, most of patients with SSC of the pancreas are diagnosed at an advanced stage of disease. According to previous reports, survival of patients with SCC in the pancreas varies between 2 and 5 mo^[3]. Here, we report a case of unusually long-term survival after curative surgery and combined chemotherapy for poorly differentiated SCC of the pancreas.

CASE REPORT

A 62-year-old woman presented to our institute with a 3-wk history of anorexia, dyspepsia, epigastric pain. She had drunken extracts of *Hovenia dulcis* for 5 mo. There was no history of smoking and regular alcohol consumption. Her family history revealed no abnormalities. She had no relevant previous medical or surgical history. Physical examination was unremarkable except for epigastric tenderness.

Laboratory studies revealed increased total bilirubin (4.9 mg/dL) and direct bilirubin (3.5 mg/dL). Liver enzymes were raised, with serum alkaline phosphatase (1072 U/L), SGOT (233 U/L) and SGPT (101 U/L). The calcium was 9.9 mg/dL and phosphate was 4.0 mg/dL. Serum viral markers, including HIV, HBsAg, and HCV were nonreactive. Tumor markers included carbohydrate antigen 19-9 (CA 19.9, 291.6 U/mL), alpha-fetoprotein (AFP, 3.6 ng/mL), and carcino-embryonic antigen (CEA, 3.2 ng/mL). Serum neuron-specific enolase (NSE) was

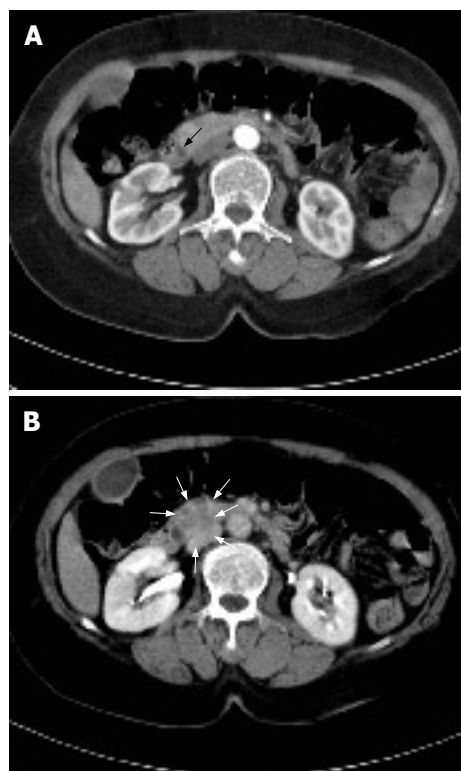


Figure 1 **A:** Abdominal computed tomography in the arterial phase shows poorly demarcated mass compressing CBD (black arrow); **B:** In delayed phase, mass (white arrows) at the pancreatic head revealed with relatively well delineated margin.

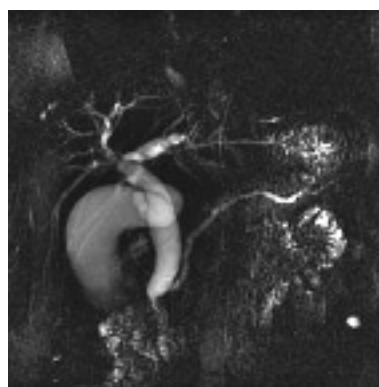


Figure 2 Magnetic resonance cholangiopancreatography (MRCP) shows abrupt narrowing of the distal common bile duct and mild dilatation of pancreatic duct.



Figure 3 Gross appearance of the specimen shows a tumor nodule 2 cm in diameter at the head of the pancreas.

12.5 ng/mL (normal, 0.1-16.3 ng/mL).

A contrast-enhanced computed tomography (CT) of the abdomen showed dilatation of intrahepatic duct, common bile duct (CBD), and pancreatic duct. There was 1.3 cm sized mass at the head of pancreas compressing the distal CBD (Figure 1). There was no evidence of pancreatitis. Magnetic resonance cholangiopancreatography and endoscopic-retrograde cholangiopancreatography (ERCP) was performed and confirmed abrupt CBD narrowing due to extrinsic compression (Figure 2). During the ERCP, it was possible to obtain tissue from the CBD.

The histopathologic study revealed small uniform nuclei with inconspicuous nucleoli and scanty cytoplasm. The morphology of the cells was similar to that of small cell carcinoma of the lung. The immunohistochemical (IHC) stain results were positive for CD56 and thyroid transcription factor-1 (TTF-1) stain and negative for NSE. Typical microscopic features confirmed small cell carcinoma. Chest X-ray and bone scan were normal.

The tumor was localized in the head of the pancreas and no extension beyond the locoregional boundaries; we performed PPPD (Longmire III operation). During the operation, there was no peritoneal seeding or invasion to adjacent organs. Common hepatic artery (No 8) and para-aortic (No 16) lymph node were enlarged but they were found out to have no metastasis in frozen section biopsy. The patient recovered without any postoperative complications.

Examination of the surgical specimen showed an

ill demarcated grayish white and firm mass measuring 2.0 cm × 1.2 cm in size in the head of the pancreas. 1 out of 16 lymph nodes showed tumor metastasis (Figure 3). There was lymphatic and perineural invasion. The tumor was composed of small monotonous and hyperchromatic poorly differentiated cells with higher nuclear to cytoplasmic ratio, and were positive for CD56, cytokeratin, chromagranin, TTF-1 and CEA, but negative for NSE, CD99 and equivocal for synaptophysin (Figure 4).

Four weeks after the operation, the patient received chemotherapy consisting of cisplatin (100 mg/m²) and ectoposide (60 mg/m², day 1-3) for 4 wk intervals. After first cycle of chemotherapy CA 19-9 decreased to 19.0 U/mL. The chemotherapy was tolerated well and was continued for 6 cycles. During the follow-up there was no evidence of recurrence and the patient has remained in a good health condition for 36 mo since the diagnosis.

DISCUSSION

Extrapulmonary small cell carcinomas (EPSCC) are rare with an incidence between 0.1%-0.4% of all cancers^[1]. Approximately 2.5% of all SCC's arise in extrapulmonary sites such as head and neck region, esophagus, stomach, colon, rectum, gallbladder, uterine cervix, breast, urinary bladder, liver, and prostate^[4-8].

SCC of the pancreas is a rare entity. Since the earliest case report of pancreatic SCC with clinical

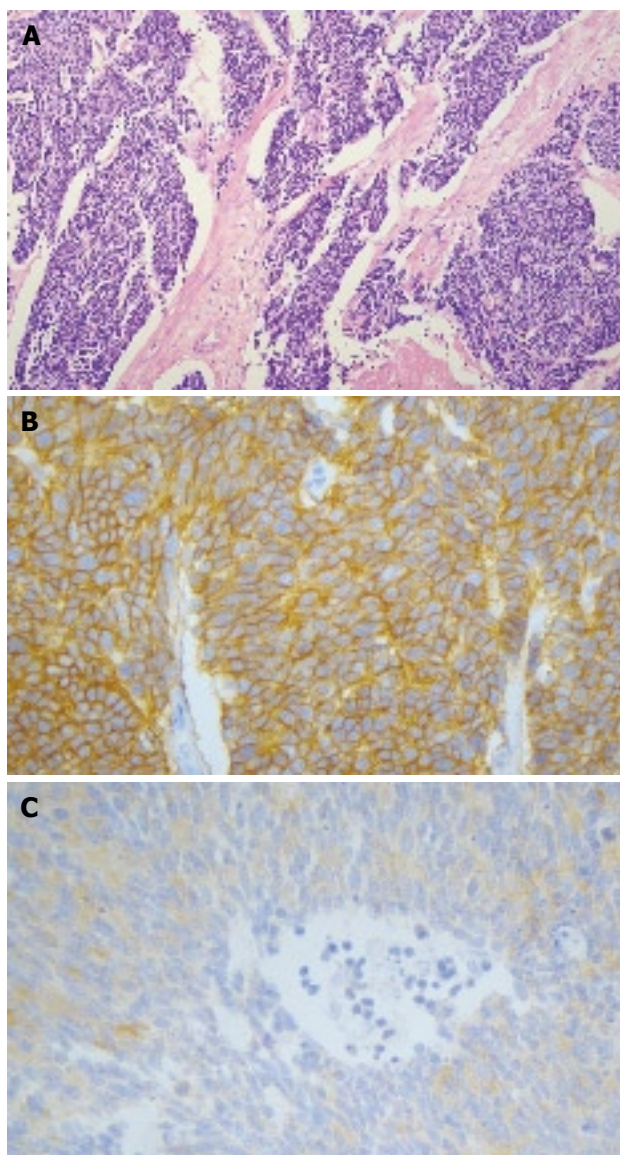


Figure 4 A: The tumor consisted of small round or oval cells with hyperchromatic nuclei and scant cytoplasm; B and C: Immunohistochemical staining for CD56 (B) and synaptophysin (C) reveals a positive reaction.

and pathologic findings in 1973^[9], only a few reports have been published with fewer than 40 cases^[3,10-12]. According to the previous studies, SCC of the pancreas is likely to occur in the head of pancreas in patients with average age of 60 and common clinical manifestations are abdominal pain, weight loss and jaundice. Many of the cases reported were diagnosed at autopsy, since most of patients with SSC of the pancreas are diagnosed at an advanced stage of disease and their survival varied between 2-5 mo^[1,3].

SCC is thought to originate from neuroendocrine cells, which are found in the epithelium of many mucosal surfaces. Despite evidence of neuroendocrine involvement, the origin of EPSCC is still unclear as development from undifferentiated airway epithelium has also been suggested along with the amine precursor uptake and decarboxylation (APUD) system hypothesis which proposes a common ancestral cell derived from the neural crest, migrating to various epithelial tissues

and sites within the body^[13,14]. Since SCC cell has similar properties to the endocrine cells of APUD system, EPSCC may also secrete hormones. But in the case of SCC of the pancreas, there is only one report of ACTH-producing tumor by Corrin *et al* and one case associated with hypercalcemia^[9,15], which is different from frequent paraneoplastic syndromes in pulmonary SCC. There were no abnormal findings suggesting paraneoplastic hormone syndromes in the case of our patient. O'Connor *et al* reported NSE was clearly elevated in SCC of the pancreas. They suggested that serum NSE could be a tumor marker for SCC of the pancreas, but NSE was within the normal range in this case^[16].

EPSCC of the pancreas is histologically indistinguishable from metastatic pulmonary SCC. Therefore, exclusion of pulmonary small cell carcinoma is a prerequisite for the diagnosis of EPSCC. In our case, chest X-ray and PET-CT were negative for the lungs. Since the biopsy from the pancreas is difficult, most of the cases were diagnosed by biopsy from the liver metastasis or lymph node or autopsy and a few cases after surgical removal^[3,11,17,18]. To evaluate dilated CBD, our patient received ERCP before surgery. During the procedure it was possible to obtain tissue from the narrowing portion of the CBD. We could confirm the tumor as EPSCC before the surgery.

On gross findings, SCC of the pancreas appears as a poorly demarcated white-gray mass with areas of necrosis and hemorrhage. It usually involves the head of the pancreas with a mean diameter of 4.2 cm^[3]. The histopathologic appearance of the tumor consists of nest of small to medium sized round to oval shaped cells with a finely granular and hyperchromatic nucleus, inconspicuous nucleoli and scanty cytoplasm. Primary small cell carcinoma of the pancreas has a varied immunohistochemical profile.

Neuroendocrine markers such as CD56, chromogranin, TTF-1 and CEA were positive but NSE and synaptophysin was negative in current case.

Unfortunately clinical presentation of EPSCC is usually at an advanced stage due to the aggressive nature of the disease. Therapeutic modalities are determined by the location and extent of disease. Usually chemotherapy remains the treatment of choice and local modalities such as surgery and radiotherapy remain limited in localized disease^[19].

In our case, the tumor was localized in the pancreas with regional lymph node involvement. Since there was no extension beyond the locoregional boundaries (limited disease), we could perform curative surgery.

Because of the high incidence of metastasis, chemotherapy should be given after a successful resection of the tumor. Only one case was reported long survival after curative resection of SCC of the pancreas without adjuvant chemotherapy^[18]. There are no definite chemotherapeutic regimens for SCC of the pancreas due to the small patient numbers. But the combination cisplatin and etoposide showed best result with response rates reaching 70% in an analysis of the

different patients of EPSCC with chemotherapeutic regimens. Doxorubicin-based regimens appear to be less effective^[20].

Complete response has been observed with cisplatin-etoposide based treatment in a patient with widespread metastatic disease^[11]. The two patients reported by van der Gaast had extensive disease with a survival of 16 and 11 mo after combined chemotherapy with cyclophosphamide, doxorubicin and etoposide^[21]. Another patient survived 14 mo with combined chemotherapy and the radiotherapy^[22]. Our patient received chemotherapy consisting of cisplatin (100 mg/m²), etoposide (60 mg/m², day 1-3) for 4 wk after the surgery. A CT scan of the abdomen after 6 cycles of chemotherapy showed no evidence of metastasis. The patient remains in good health 36 mo after the surgery. The reason for the good prognosis may be associated with an early detection of the tumor and the fact that the tumor was localized and showed no metastasis or dissemination.

Because of the unfavorable prognosis of EPSCC, multimodal therapy was used in most of reported cases with limited disease. In the case of resectable SCC of the pancreas, it is reasonable to perform the extensive surgery followed by chemotherapy. We report a case of primary SCC of the pancreas with unusually long-term survival after multimodal therapy.

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CASE REPORT

Primary malignant melanoma of the liver: A case report

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INTRODUCTION

Malignant melanoma occurs most frequently in skin but also in many organs and tissues of the body. However, primary hepatic malignant melanoma is exceedingly rare. Only 12 cases, including 8 cases from PubMed and 4 cases from Chinese literature^[1-4], have been reported. In PubMed, there are only 3 cases of definite primary melanoma^[5-7]. Microscopically, it may be easily misdiagnosed because of morphological heterogeneity and hypomelanotic appearance. The present case represents the only case encountered in our department. In this report, we describe our pathological observations and review the literature in order to improve our understanding of the disease, avoid misdiagnosis and provide evidence for its clinical treatment and prognosis.

CASE REPORT

A 36-year-old man was admitted to the Department of General Surgery, Tangdu Hospital, Fourth Military Medical University (Xi'an, Shaanxi Province, China) because a mass in his liver was found 4 d ago at a routine health check. He had no history of alcohol abuse or hepatitis. No record of hepatocellular carcinoma (HCC) or any hereditary disease was found in his family members. Routine clinical biochemistry showed normal levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), γ -glutamyltransferase (γ -GTP), α -fetoprotein (AFP) and plasma proteins. Laboratory tests failed to show any positive hepatitis B surface antigen (HBsAg) or anti-hepatitis C virus (HCV) antibody. Contrast-enhanced abdominal computerized tomography (CT) displayed a 10.1 cm \times 12.8 cm well-defined hepatic mass in the right-posterior lobe of the liver without evidence for spread to neighboring lymph nodes or abdominal dropsy

Abstract

Primary malignant melanoma of the liver is an exceedingly rare tumor. Only 12 cases have been reported in the worldwide literature. We present a case of isolated malignant melanoma of the liver occurring in a 36-year-old Chinese male patient. Comprehensive dermatologic and ophthalmologic examinations revealed no evidence of a cutaneous or ocular primary lesion. Other lesions in brain, respiratory tract, lung, gastrointestinal tract and anus, were not demonstrated by serial position emission tomography (PET). Microscopic examination of the resected specimen revealed a malignant melanoma, which was confirmed by immunohistochemical staining for HMB-45, S-100 protein, melanoma-pan and vimentin. Moreover, electron microscopy demonstrated melanosomes in tumor cell cytoplasm. Our case shows that primary malignant melanoma may occur in the liver and should be considered when the histopathological appearance is not typical for other hepatic neoplasm.

Key words: Primary malignant melanoma; Liver; Diagnosis; Histopathology; Immunohistochemistry

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Figure 1 Contrast-enhanced abdominal CT scan showing an oval, low-dense, well-defined mass measuring 10.1 cm × 12.8 cm in the right posterior lobe of the liver.

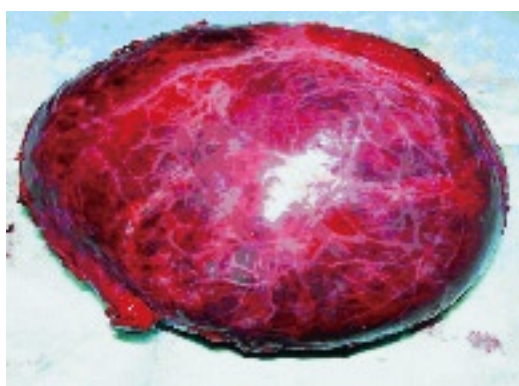


Figure 2 Gross appearance of the resected specimen showing the oval outline of tumor adjacent to normal liver tissue.

(Figure 1). The resected fresh tissue was fixed in 40 g/L formaldehyde solution, embedded in paraffin. Sections of 4 μ m in thickness were prepared and stained with hematoxylin and eosin (HE). Immunostaining was carried out using a streptavidin-labeled peroxidase (S-P) kit (KIT9730) according to its manufacturer's instructions. Primary antibodies used in this study included those against HMB-45, melanoma-pan, epithelial membrane antigen (EMA), carcinoembryonic antigen (CEA), cytokeratins (18, 19, 7, 8), high-MW-CK, chromogranin A (CgA), synaptophysin (syn), sesmin, nerve specificity enolase (NSE), smooth muscle actin (SM-actin), vimentin, CD34, AFP, S-100 protein, CD117, human chorionic gonadotrophin (HCG), leucocyte common antigen (LCA), HBsAg, hepatitis B core antigen (HBcAg), as well as anti-HCV antibody. All of the reagents for immunostaining were supplied by Maxim Biotechnology Corporation Ltd, Fuzhou, China.

Grossly, the resected mass measured 12 cm × 11 cm × 6 cm (Figure 2). There was a clear differentiation between the mass and its surrounding normal tissue, and the cut surface of the tumor was grayish-yellow in color. Microscopically, the tumor cells showed a diffuse infiltration, and fibrous tissue could be observed between the lesion and normal hepatocytes. A few tumor cells breaking through the capsule infiltrated the adjacent liver tissue (Figure 3A and B). The tumor cells were

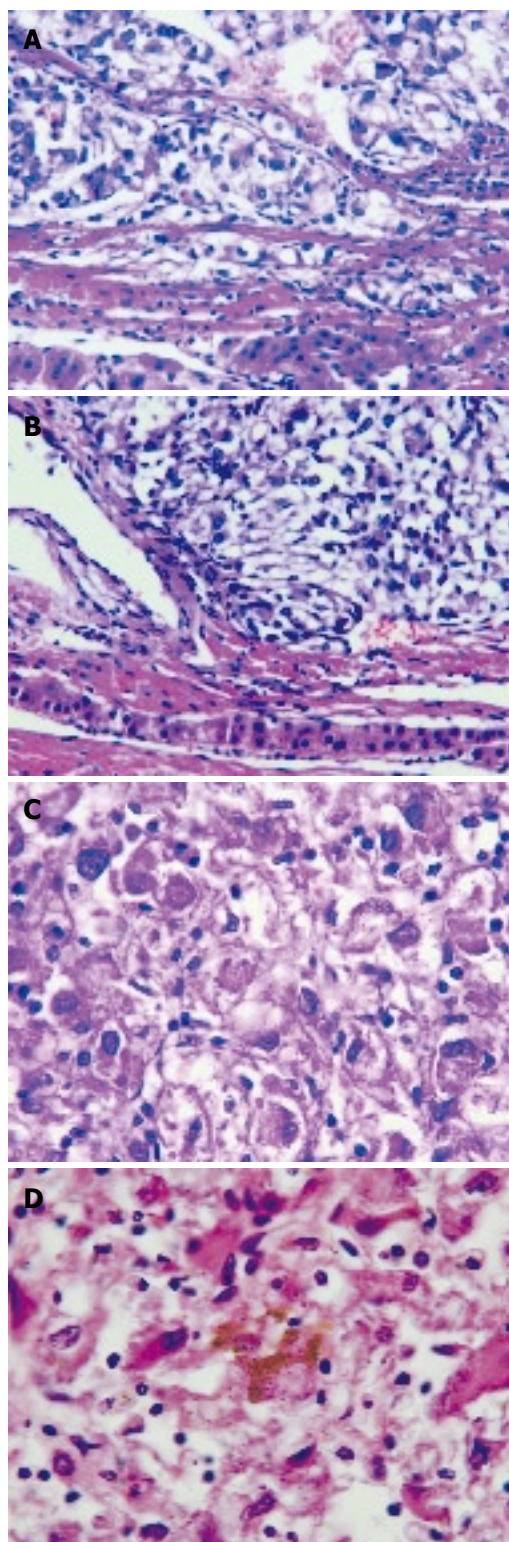


Figure 3 Microscopy showing diffusely infiltrating tumor cells (A) and fibrous tissues (B) between the lesion and normal hepatocytes with a few broken tumor cells through the capsule infiltrating the adjacent liver tissue ($\times 200$), pleomorphic tumor cells with round, spindle-shaped and irregular morphologies ($\times 400$) (C), and some tumor cells containing melanin deposition ($\times 200$) (D).

pleomorphic, with round, spindle-shaped and irregular morphologies. The tumor cell nuclei were round or oval, and the nucleoli were prominent, with both eosinophilic and basophilic varieties observed (Figure 3C). Some

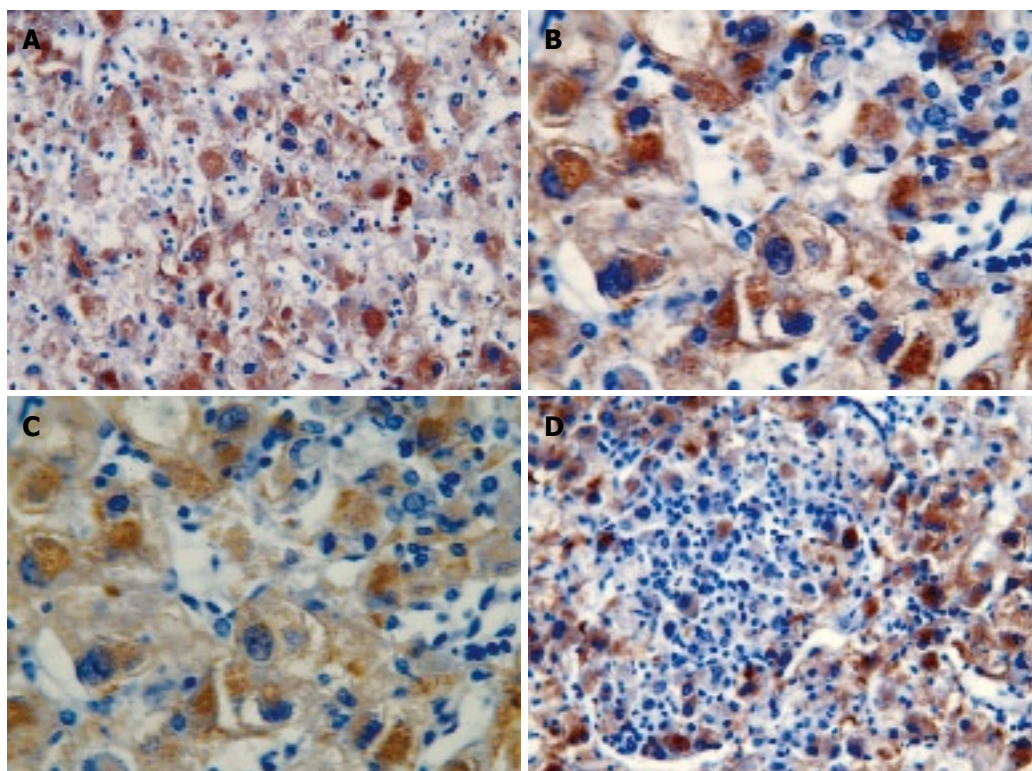


Figure 4 Immunohistochemistry revealing tumor cells positive for vimentin ($\times 200$) (A), melanoma-pan ($\times 400$) (B), HMB45 ($\times 400$) (C) and S-100 protein ($\times 200$) (D).

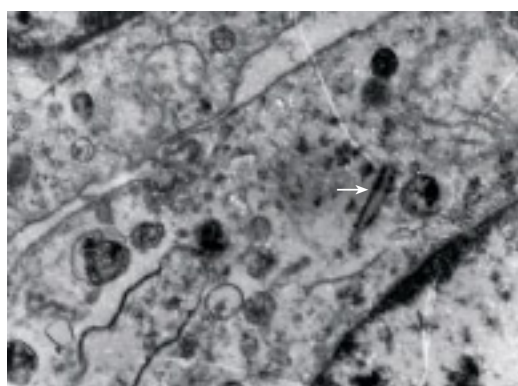


Figure 5 Electron microscopy displaying occasional melanosomes in the cytoplasm (arrow).

tumor cells contained melanin deposition (Figure 3D). Immunohistochemically, the tumor cells were positive for vimentin (Figure 4A), melanoma-pan (Figure 4B), HMB-45 (Figure 4C) and S-100 protein (Figure 4D), but did not display AFP, high-MW-CK, CK18, CEA, CK19, CD34, CD117, NSE, LCA, CgA, HCG, EMA, desmin, SM-actin, SC-actin, myoglobin and immunoreactivity. Electron microscopy revealed occasional melanosomes in cytoplasm (Figure 5).

The complete absence of evidence for any cutaneous, ocular, mucosal lesions in all organs examined by serial position emission tomography (PET) supported the final diagnosis of primary melanoma.

After a wide local surgical resection, the patient received a 4-wk immunomodulatory therapy at a high dose of IV interferon alpha-2b, once a day. He then received a lower dose subcutaneous regimen, three times a week for a further 9 wk. This regimen was

well-tolerated. He was regularly followed up. Three months after operation, he had no recurrence of the disease. However, recurrent foci were found 5 mo after operation.

DISCUSSION

Melanoma is a melanin-induced malignant tumor, most prevalent in patients over the age of 30 years. It mainly occurs in skin, but may be found in retina, anorectal canal, genital tract, gastrointestinal (GI) tract, accessory nasal cavity and parotid. Non-cutaneous primary melanomas carry a particularly high mortality because of their propensity for dissemination and invasion, often before they are clinically apparent.

Primary malignant melanoma of the liver is an extremely rare non-epithelial neoplasm, and few cases have been reported. We thus summarize, in this paper, the clinical characteristics and histopathologic manifestations of this unusual tumor.

Malignant melanomas of the skin originate from epidermal melanocytes or neural cells, both are derived from neural crest precursors^[8]. Several factors, including race, heredity, tissue injury, stimulation, viral infection, sun-exposure and immunization, can lead to malignant transformation^[9]. The origin of mucosal malignant melanoma is unclear. Most experts hold that malignant melanomas of non sun-exposed tissues are linked to stimulation by the blood-borne sunlight circulation factor, expressed in sun-exposed melanoblasts^[10]. Based on the origin of primary melanomas of the esophagus and stomach, some authors believe that these alimentary tract neoplasms originate from migrating melanocytes invaginated by digestive tract epithelial cells during

embryogenesis^[11,12], which is supported by the distribution of melanocytes in other typically-occurring mucosal cells of the rectum^[13]. However, the origin and pathogenesis of primary melanomas arising in parenchymal organs are still unclear.

It is difficult to show the clinical characteristics of primary hepatic malignant melanoma because case reports are available prior to the 1970s, except for 5 Chinese patients (2 males and 3 females, mean age 42.2 years, range 27-60 years). Previously reported patients had no readily identifiable risk factors when compared to patients with primary HCC.

Pathologically, hepatic malignant melanoma resembles that of the skin or mucosa, exhibiting morphologic variability within the tumor sample. Microscopically, the tumor mass is comprised of epithelioid cells arranged in nests, or spindle cells arranged in fascicles, with or without melanin pigment deposition. Mitotic figures are readily apparent. However, our case suggests that it may be difficult to identify malignant melanoma from a biopsy sample in some cases, because portions of the tumor may be amelanotic. In these cases, ancillary immunohistochemical staining may be extremely valuable^[14]. In our case, when specimens of HCC, haemangioma, large-B cell lymphoma, smooth muscle tumor and rhabdomyosarcoma were all considered in the differential diagnosis, all of which were potentially consistent with the intact capsule, large size, and well-circumscribed boundaries. However, all the above tumors could be ultimately excluded based on their histopathologic characteristics and immunohistochemical staining. After reviewing a number of additional sections and detecting sporadic pigment granules in the tumor cytoplasm, we considered a diagnosis of malignant melanoma. The tumor cells expressed HMB45, S-100 protein, vimentin and melanoma-pan, all of which were consistent with hepatic melanoma. Our preliminary diagnosis was then confirmed by electron microscopy.

Once the pathologic diagnosis was established, whether the tumor was a primary or secondary lesion should be considered. An extensive investigation of potential primary sites demonstrated no evidence for hepatic melanoma, suggesting that the tumor is a primary melanoma of the liver.

Given the rarity of this tumor, the optimal therapeutic regimen is not known. In fact, the natural

history of these cases also remains unclear. Since surgical therapy is usually palliative, a more aggressive oncologic regimen consisting of chemotherapy, immunotherapy and radiotherapy may be required. With the natural history largely unknown, it is necessary to find treatment modalities for other deep primary melanomas,

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Meetings

Events Calendar 2008-2009

FALK SYMPOSIA 2008
 January 24-25, Frankfurt, Germany
 Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008
 February 14-16, Paris, France
 EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
 8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
 3rd Congress of ECCO - the European Crohn's and Colitis Organisation
 Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
 Canadian Association of Gastroenterology
 E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
 British Society of Gastroenterology Annual Meeting
 E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
 Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
 Asian Pacific Association for the Study of the Liver
 18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
 Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
 OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
 E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
 SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
 9th World Congress of the International Hepato-Pancreato Biliary Association
 Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
 43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
 Falk Symposium 164: Intestinal

Disorders

May 18-21, San Diego, California, USA
 Digestive Disease Week 2008

May 21-22, California, USA
 ASGE Annual Postgraduate Course
 Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
 E-mail: education@asge.org

June 4-7, Helsinki, Finland
 The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
 Semana de las Enfermedades Digestivas
 E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
 3rd Annual European Meeting: Perspectives in Inflammatory Bowel Diseases
 E-mail: meetings@imedex.com

June 10-13, Istanbul, Turkey
 ESGAR 2008 19th Annual Meeting and Postgraduate Course
 E-mail: fca@netvisao.pt

June 11-13, Stockholm, Sweden
 16th International Congress of the European Association for Endoscopic Surgery
 E-mail: info@aes-eur.org

June 13-14, Amsterdam, Netherlands
 Falk Symposium 165: XX International Bile Acid Meeting. Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
 Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
 E-mail: idca2008@guarant.cz

June 25-28, Barcelona, Spain
 10th World Congress on Gastrointestinal Cancer
 Imedex and ESMO
 E-mail: meetings@imedex.com

June 25-28, Lodz, Poland
 Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatologists (IAP)
 E-mail: office@epc-iap2008.org
www.e-p-c.org
www.pancreatology.org

June 26-28, Bratislava, Slovakia
 5th Central European Gastroenterology Meeting
www.ceurgem2008.cz

July 9-12, Paris, France
 ILTS 14th Annual International Congress
www.ilt.s.org

September 10-13, Budapest, Hungary
 11th World Congress of the International Society for Diseases of the Esophagus
 E-mail: isde@isde.net

September 13-16, New Delhi, India
 Asia Pacific Digestive Week
 E-mail: apdw@apdw2008.net

APDW 2008
 September 13-16, New Delhi, India
 Organized: Indian Society of Gastroenterology

III FALK GASTRO-CONFERENCE

September 17, Mainz, Germany
 Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany
 Falk Symposium 166:
 GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic
 Prague Hepatology Meeting 2008
www.czech-hepatology.cz/phm2008

September 20-21, Mainz, Germany
 Falk Symposium 167:
 Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France
 Third Annual Meeting
 European Society of Coloproctology
www.escp.eu.com



October 8-11, Istanbul, Turkey
 18th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists
 E-mail: orkun.sahin@serenas.com.tr

October 18-22, Vienna, Austria
 16th United European Gastroenterology Week
www.negf.org
www.acv.at

October 22-25, Minnesota, USA
 Anstralian Gastroenterology Week 2008
 E-mail: gesa@gesa.org.au

October 22-25, Brisbane, Australia
 71st Annual Colon and Rectal Surgery Conference
 E-mail: info@colonrectalcourse.org

October 31-November 4, Moscone West Convention Center, San Francisco, CA
 59th AASLD Annual Meeting and Postgraduate Course
 The Liver Meeting
 Information: www.aasld.org

November 6-9, Lucerne, Switzerland
 Neurogastroenterology & Motility Joint International Meeting 2008
 E-mail: ngm2008@mci-group.com
www.ngm2008.com

November 12, Santiago de Chile, Chile
 Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt
 1st Hepatology and Gastroenterology Post Graduate Course
www.egyptgastrohep.com

December 7-9, Seoul, Korea
 6th International Meeting
 Hepatocellular Carcinoma: Eastern and Western Experiences
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International Gastroenterological Congresses 2009
 March 23-26, Glasgow, Scotland
 Meeting of the British Society of Gastroenterology (BSG)
 E-mail: bsg@mailbox.ulcc.ac.uk

May 17-20, Denver, Colorado, USA
 Digestive Disease Week 2009

November 21-25, London, UK
 Gastro 2009 UEGW/World Congress of Gastroenterology
www.gastro2009.org



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For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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Acknowledgments

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Format

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- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; 42 Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (401): 230-238 [PMID: 12151900]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS/A Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorffheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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