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Mycotoxins are conventional and novel risk biomarkers for hepatocellular carcinoma

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Core tip: Mycotoxins are one of the possible important carcinogens of hepatocellular carcinoma (HCC). Recently, a chromatographic separation technique based on high-performance liquid chromatography (HPLC) has been recognized as a useful method for the quantitative analyses of mycotoxins in the sera of individuals. HPLC-based analysis of mycotoxins in the clinical samples would provide some new epidemiological information about non-viral HCC.

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Abstract

Hepatocellular carcinoma (HCC) is a common malignant disease with poor prognosis. To improve the clinical outcome, early diagnosis of HCC arising from nonviral agents and hepatitis virus is important. Among several etiological factors, mycotoxins defined as carcinogens by the International Agency for Research in Cancer might be one of the critical risk factors for nonviral HCC. Aflatoxin B1 is the most well-known carcinogenic mycotoxin for HCC, but the role of the other types of mycotoxin remains unclear. Several studies have reported that a chromatographic separation technique based on high-performance liquid chromatography can successfully detect the concentration of mycotoxins in plasma. In this article, we review recent studies of mycotoxin, and discuss its possible significance as a biomarker of HCC.

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COMMENTARY ON HOT TOPICS

Hepatocellular carcinoma (HCC) is a common malignancy, with a high prevalence worldwide^[1,2]. The prognosis of HCC has remained poor, because many of the patients also have chronic liver diseases and are not suitable for radical surgical treatment^[1-3]. Another obstacle to HCC treatment is that hepatoma cells display strong resistance against standard chemotherapeutic drugs^[2,3]. To enable early diagnosis and treatment, understanding the etiological risk factors of HCC is desirable. Currently, approximately half of all HCC patients suffer from chronic hepatitis B and C virus (HBV and HCV) infection, and remaining cases are affected by various types of etiological factors including alcohol abuse, cigarette smoking, mycotoxins, obesity, and oral contraceptive drugs^[4]. Intriguingly, growing evidence has suggested that the types

of etiological risk factors for HCC might differ between geographic areas. For example, obesity-associated HCC has become one of the most important medical issues in developed countries^[5], while food contamination with mycotoxins remains a critical risk factor for HCC in developing countries, including South and East Africa, India and China^[6].

Mycotoxins as a major dietary risk factor for liver cancer

In recent years, the relationship between cancer risk and mycotoxin has been universally publicized by global surveillance of food contamination. Mycotoxins are secondary metabolites produced by fungi and are present in various types of stored grains, and most of them are resistant against cooking, freezing and digestion after intake of contaminated food. Several food-contaminating mycotoxins have been defined as harmful carcinogens by the International Agency for Research in Cancer (IARC) (Table 1), that is, deoxynivalenol/nivalenol, zearalenone, ochratoxin, fumonisins and aflatoxins. Of these, aflatoxin B1 (AFB1) is the most well-known bioaccumulative toxin involved in the development of HCC^[7]. AFB1 is produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, and mainly contaminates improperly stored cereals and peanuts. When individuals are exposed to AFB1 for a long time, mono-oxygenases produce reactive epoxide in the liver, leading to formation of toxic derivatives with nucleic acids and proteins^[8]. AFB1 has a strong mutagenic effect, and induces G to T transversion within codon 249 of the tumor suppressor *p53* gene. Point mutation of *p53* has been observed in approximately half of all patients with AFB1-associated HCC^[9-11]. It has also been reported that geographical distribution of aflatoxin exposure and HBV infection overlap, leading to a synergistic effect on the genetic mutation of *p53*^[10,11]. AFB1 is now regarded as the representative of orally ingested carcinogens, and has been classified as a Group 1 carcinogen by IARC (IARC 7th Annual Report on Carcinogens, 1987).

Ochratoxin A: A possible diagnostic marker of HCC?

Based on the clinical evidence of AFB1 in HCC, several researchers have turned their attention to the other type of mycotoxins. Ochratoxin A (OTA), which has been classified as a possible human carcinogen (Group 2B) by the IARC, is a secondary metabolite of *Aspergillus* and *Penicillium* fungi. OTA is widely spread in cereals such as barley, wheat, coffee and bread^[12], and is well known for its possible contribution to nephritic diseases. Although the evidence is still open to debate, several studies have reported that OTA might be a causative agent of Balkan endemic nephropathy^[13]. Moreover, OTA is increased to high levels in the plasma of patients with nephropathy in specific regions such as Tunisia^[14], suggesting that OTA plays a critical role in the development of nephritic diseases.

Unfortunately, the role of OTA in hepatocarcinogenesis remains unclear. Although the results of relevant studies are controversial, several have suggested that the

Table 1 Classification of food mycotoxins as human carcinogens or potential human carcinogens

Group	Classification of food mycotoxins
1	Aflatoxin B1, B2, G1, G2
2A	-
2B	Aflatoxin M1, ochratoxin A, sterigmatocystin
3	Citrinin, patulin, luteoskyrin, cyclochlorotine, deoxynivalenol
4	-

Group 1: Carcinogenic to humans; Group 2A: Probably carcinogenic to humans; Group 2B: Possibly carcinogenic to humans; Group 3: Not classifiable as to its carcinogenicity to humans; Group 4: Probably not carcinogenic to humans (classified by the International Agency for Research in Cancer).

carcinogenic effect of OTA is due to increased hepatotoxicity and DNA damage. Ehrlich *et al.*^[15] have tested the genotoxic effect of OTA in human hepatoma HepG2 cells using both micronucleus and single-cell gel electrophoresis assays, and have found that it causes pronounced dose-dependent effects on DNA damage. Renzulli *et al.*^[16] have reported that OTA induces DNA damage through oxidative stress, and this could be prevented by rosmarinic acid, a natural phenolic compound contained in many Lamiaceae herbs. Bouaziz *et al.*^[17] have reported that OTA triggers a p53- and caspase-dependent mitochondrial apoptotic pathway in HepG2 cells. In contrast, El Golli Bennour *et al.*^[18] have reported that OTA does not induce significant reactive oxygen species generation in cultured HepG2 cells, but induces mitochondrial and caspase-dependent apoptotic cell death mediated by p53 transcription-independent activities. The aforementioned different and controversial studies suggest that etiological analysis of patients with HCC is indispensable for assessing the relationship between OTA and HCC.

Until recent decades, meta-analysis of etiological risk factors for carcinogenesis has been complicated because traditional methods for assessing toxin exposure were mainly performed by questionnaires or standard enzyme linked immunosorbent assay and these cannot assess minute quantities of environmental toxins. During the last decades, however, a chromatographic separation technique based on high-performance liquid chromatography (HPLC) has enabled us to assess the concentration of mycotoxins in clinical samples such as urine and plasma^[19]. HPLC enables us to detect OTA at a low level of 0.005 ng/mL in plasma^[20], which is often less than the mean level in healthy individuals; therefore, this analytical method is preferable for evaluation of the etiological significance of OTA. For example, Grosso *et al.*^[21] and Aslam *et al.*^[22] have examined the levels of OTA in the serum of bladder cancer patients by HPLC, and have reported that OTA is unlikely to be a risk factor for bladder cancer in Tunisia and Karachi. di Giuseppe *et al.*^[23] have examined serum OTA in patients in the Molise region in Italy, and have reported that the levels of OTA are significantly associated with C-reactive protein and cardiovascular risk score in men. These lines of evidence

strongly suggest that examining mycotoxin such as AFB1 and OTA might be useful for investigating the molecular mechanism of HCC development.

Mycotoxin and hepatitis virus: Another mechanism of HCC development?

Of interest, basic studies have suggested that oncogenic role of mycotoxin might be significantly enhanced when the host cells are affected by hepatitis viruses. The most well-known story is the combination effect of hepatitis B virus X (HBX) protein with AFB1. Groisman *et al.*^[24] reported that expression of HBX impaired the repair of the DNA damage induced by AFB1 in cultured hepatoma cells, suggesting that the combination of HBX and AFB1 leads to enhanced DNA mutation. Madden *et al.*^[25] examined the rate of DNA mutation in HBX-transgenic mice, and found that the incidence of DNA mutations following AFB1 was increased to two fold in these animal models. More intriguingly, Li *et al.*^[26] implanted the HBX-transfected oval cells into nude mice with AFB1 treatment, and found that combination of HBx gene and treatment with AFB1 produces tumors *in vivo*. These lines of evidence strongly suggest that the long exposure to mycotoxin may cause unexpectedly enhanced tumorigenesis in the individuals with chronic hepatitis virus infection. Unfortunately, there have been no studies investigating the mechanism of combination of hepatitis viruses and mycotoxins. Further studies of the relationship between food contamination and hepatitis viruses might help to understand the molecular mechanism of hepatocarcinogenesis.

Summary

Recently, progress in the diagnosis and treatment of hepatitis virus infection has significantly improved the outcome of patients with HBV- and HCV-associated HCC. Early diagnosis of HCC arising in patients with nonviral disease, however, has been remained difficult. HPLC-based analysis of clinical samples might offer a useful tool for detecting minute concentrations of environmental toxins that have accumulated in the host. Although food contamination with mycotoxins has so far been recorded in specific geographic regions, it is conceivable that current advances in the transportation system could cause unexpected food contamination in a wide area. Therefore, to address whether mycotoxin would be a real causal agent for HCC, assessment of the level of mycotoxins, including OTA, in clinical samples would be of value.

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Tumor necrosis factor- α inhibitor therapy and fetal risk: A systematic literature review

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Abstract

Tumor necrosis factor- α inhibitors (anti-TNFs) are effective in the treatment of inflammatory bowel disease (IBD) recalcitrant to conventional medical therapy. As the peak incidence of IBD overlaps with the prime reproductive years, it is crucial to establish pharmacologic regimens for women of childbearing age that achieve effective disease control without posing significant fetal harm. A systematic literature review was performed to identify all human studies with birth outcomes data after maternal exposure to infliximab, adalimumab, or certolizumab pegol within 3 mo of conception or during any trimester of pregnancy. Live births, spontaneous abortions or stillbirths, preterm or premature births, low birth weight or small for gestational age infants, and congenital abnormalities were recorded. Fifty selected references identified 472 pregnancy exposures. The subsequent review includes general information regarding anti-TNF therapy in pregnancy followed by a summary of our findings. The benefits of biologic modalities in optimizing disease control during pregnancy

must be weighed against the potential toxicity of drug exposure on the developing fetus. Although promising overall, there is insufficient evidence to prove absolute safety for use of anti-TNFs during pregnancy given the limitations of available data and lack of controlled trials.

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Key words: Tumor necrosis factor- α inhibitors; Pregnancy; Congenital abnormalities; Safety; Infliximab; Adalimumab; Certolizumab

Core tip: A systematic literature review was performed to identify all human studies with birth outcomes data after maternal exposure to infliximab, adalimumab, or certolizumab pegol within 3 mo of conception or during any trimester of pregnancy. After systematic literature review investigating tumor necrosis factor- α inhibitor therapy and fetal risk, there is insufficient evidence to prove absolute safety for the use of biologics (specifically infliximab, adalimumab, and certolizumab pegol) during pregnancy.

Marchioni RM, Lichtenstein GR. Tumor necrosis factor- α inhibitor therapy and fetal risk: A systematic literature review. *World J Gastroenterol* 2013; 19(17): 2591-2602 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2591.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2591>

INTRODUCTION

Inflammatory bowel disease (IBD) encompasses the diagnoses of Crohn's disease (CD) and ulcerative colitis (UC). These are chronic relapsing gastrointestinal illnesses that involve proinflammatory molecules. The onset of IBD has a bimodal distribution with a higher peak in the younger population aged 15-30 years; fifty percent of patients afflicted by IBD are diagnosed before the age

of 35^[1]. Hence, the peak incidence for developing these conditions overlaps with the prime reproductive years^[2,3].

Effective control of IBD is essential during pregnancy. Active disease or disease flares have been associated with adverse obstetrical outcomes^[4]. About 50% of the pregnancies in North America are unplanned, and less than half of females realize their pregnancy status by week four of gestation^[5]. Inadvertent fetal exposure to medications during the crucial stages of organogenesis is thus possible and common. For these reasons, preconception discussions addressing risks and benefits of pharmacologic therapy during pregnancy are clinically warranted for all patients of childbearing potential.

The decision to pursue or maintain certain drug regimens throughout the prenatal and pregnancy periods may pose a significant challenge; the risks of disease activity must be weighed against the potential side effects of medical therapy. Untreated disease may create greater risks to a pregnancy than the drugs themselves^[2]. Identifying the safest management strategy is crucial, as medication use during pregnancy impacts maternal disease activity, fetal development, and pregnancy outcomes.

Tumor necrosis factor- α (TNF- α) is a pleiotropic cytokine that plays a role both in pregnancy and in the pathophysiology of inflammatory conditions including IBD. Mouse models have demonstrated that TNF- α is one of several cytokines bearing a potent regulatory effect on early development^[6]. It controls cyclooxygenases that affect blastocyst implantation, vascular permeability of the endometrium, and uterine decidualization^[7]. TNF- α also contributes to the process of labor by stimulating uterine contractions in conjunction with other inflammatory cytokines^[8]. The production of TNF- α increases throughout pregnancy and reaches a peak at the onset of labor. High levels of TNF- α have been implicated in such pregnancy complications as infection and fetal growth retardation and have even been linked to early and unexplained spontaneous abortions^[8,9].

There is a characteristic abundance of gut inflammation in IBD originating *via* various mechanisms at the cellular and subcellular levels. TNF- α is a key cytokine in the development and perpetuation of this abnormal immune response^[10]. Several studies support the heightened production of TNF- α in the intestinal mucosa of patients with CD, and the levels are increased in both inflamed and histologically normal mucosa^[11-13]. Increased TNF- α has also been linked to such rheumatologic and dermatologic conditions as rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, and psoriasis.

TNF- α inhibitors (anti-TNFs) are drugs that block the action of TNF- α and neutralize its biologic effect. This class has demonstrated efficacy in controlling disease activity associated with various inflammatory conditions. Infliximab (IFX), adalimumab (ADA), and certolizumab pegol (CTZ) are three such synthetic antibodies available in the United States for the treatment of IBD. Of these, infliximab has been the most highly studied.

Recognizing the effects of maternal drug use on fetal

development is an important aspect of providing care to pregnant patients and women of childbearing age with IBD. There is limited data, though, pertaining to the safety of biologic agents when used during pregnancy. The United States Food and Drug Administration (FDA) lists anti-TNF agents as category B drugs^[14-16] (category B specifies that animal studies do not indicate fetal risk and there are no controlled studies in women or that animal studies have demonstrated adverse effects but controlled studies in women have failed to demonstrate risk). A recent consensus statement declared anti-TNF agents to be low risk during certain stages of pregnancy^[17]. Some case reports and small case series reporting anti-TNF exposure and pregnancy outcomes have been published. However, large population-based studies are sparse, and there is a lack of prospective data in pregnant women. In addition, there is a relatively short number of post-marketing years since the advent of biologics, thus narrowing the safety information pool even further. The increasing use of antibody-based therapeutics fosters the need for further study in this group of patients.

A systematic literature review was performed to investigate fetal risks associated with maternal exposure to TNF- α inhibitors (IFX, ADA, and CTZ) during pregnancy.

SEARCH STRATEGY

The search strategy was developed with the assistance of a medical librarian. Databases searched included MEDLINE, EMBASE, SCOPUS, and BIOSIS Previews through November 2011 and were restricted to studies published in English and performed in humans. Structured searches were conducted using both medical subject heading terms and keyword/exploded terms as follows: (“congenital abnormalities” OR “congenital disorders” OR “pregnancy” OR “safety”) AND (“infliximab” OR “adalimumab” OR “certolizumab”). Titles and abstracts were screened for relevance; reference lists of the applicable publications were hand-searched to identify additional studies.

ELIGIBILITY CRITERIA

Case reports, case series, or observational studies published in article or abstract form were eligible for inclusion if there was documented female exposure to IFX, ADA, or CTZ within three months of conception or during any trimester of pregnancy and if > one of the following birth outcomes was assessed: live births, spontaneous abortions (SA), stillbirths (SB), preterm or premature births (PTB/PMB), low birth weight (LBW)/small for gestational age (SGA), or congenital abnormalities (CA). Studies were excluded if there was insufficient detail to link specific anti-TNF exposure with birth outcomes. One investigator independently performed the searches described above and reviewed the citations (titles and abstracts) to determine eligibility. Discrepancies were

resolved by the second investigator.

DATA EXTRACTION

A standardized form was used to abstract the following data points from each study: anti-TNF drug exposure, indication for anti-TNF agent, pregnancy stage(s) of exposure by trimester, live births, and birth outcomes as aforementioned. Spontaneous abortions were defined as fetal death at < 20 wk, stillbirths as fetal death at > 20 wk or at weight > 350-500 g if gestational age unknown, preterm deliveries as < 37 wk gestation, premature deliveries as < 37 wk gestation and prior to completion of organ development, and low birth weight newborns as < 2500 g. Small for gestational age infants were described by authors as smaller than average size given the number of pregnancy weeks.

SEARCH RESULTS

The initial search yielded 11452 citations. Fifty studies (Table 1)^[18-68] met inclusion criteria for full review, including 13 case series, 36 case reports, and 2 prospective studies with control groups. Reports in Table 1 are categorized by biologic agent and study type, and details of maternal anti-TNF exposures and pregnancy outcomes are presented.

The total number of patients exposed to anti-TNFs was 472 (IFX 194/ADA 261/CTZ 17). Table 2^[69-73] displays anti-TNF exposures and birth outcomes for the following categories: live births, spontaneous abortions, stillbirths, preterm/premature births, low birth weight/small for gestational age, and congenital abnormalities. Outcomes in Table 2 have been listed by anti-TNF exposure (IFX, ADA, and CTZ) and indication (for all medical conditions and for IBD patients alone), and results are compared to the general United States population.

Table 3 summarizes the reported congenital abnormalities associated with live births (4.1%). Among 19 congenital anomalies (IFX 9/ADA 10/CTZ 0), no specific pattern of birth defects was identified^[74-76].

DISCUSSION

We performed a systematic literature review to assess the risk of adverse birth outcomes after maternal exposure to IFX, ADA, or CTZ and identified 50 references with a total of 472 fetal exposures.

The subsequent discussion highlights each biologic agent in the context of pregnancy and provides a summary of our data.

Infliximab

Infliximab (Remicade) is a human-murine chimeric monoclonal antibody that neutralizes the activity of TNF- α . It is composed of a human immunoglobulin G1 (IgG1) constant region and a murine variable region. Its efficacy in IBD has been documented in randomized

controlled trials in the treatment of moderate to severe CD refractory to conventional therapy as well as enterocutaneous fistulae^[77,78]. The drug can reduce the need for corticosteroids and, in patients who respond to initial dosing, IFX is effective for the maintenance of response and prolonged remission in CD^[79,80].

IFX is classified by the United States FDA as pregnancy category B. Murine models show no evidence of teratogenicity or embryotoxicity. However, anti-TNF- α antibodies vary among species; data cannot simply be paralleled to human pregnancy outcomes. Infliximab does not cross-react with TNF- α in species other than humans and chimpanzees, and it has not been tested in animal reproduction studies^[14].

IFX is not thought to cross the placenta in the first trimester due to its human IgG1 constant region^[81], but this subclass is known to efficiently cross in the late second and third trimesters^[26]. Given this timing, the infant is somewhat shielded from drug exposure during the critical period of organogenesis. IFX levels can be detected in newborns of exposed mothers, and the drug remains in the system for up to six months after delivery^[19,50]. This bears important consequences in terms of newborn infection risks and vaccination responses^[17]. Discontinuing infliximab in the third trimester is an option to decrease late placental transport to the newborn.

Adalimumab

Adalimumab (Humira) is a fully human monoclonal IgG1 antibody against TNF- α . It has proven effective for inducing and maintaining remission in CD^[82,83], especially in those who have lost response to or have become intolerant of infliximab^[84].

ADA is classified as an FDA pregnancy category B drug. In an embryo-fetal perinatal developmental toxicity study, cynomolgus monkeys were administered ADA at extreme dosages of up to 100 mg/kg [266 times human area under the curve (AUC) when dispensed as 40 mg subcutaneously with methotrexate weekly or 373 times human AUC when dispensed as 40 mg subcutaneously without methotrexate]. No evidence of fetal harm due to ADA was recorded. Adequate and well-controlled studies have not been conducted in pregnant women. Again, animal reproduction and developmental studies are not always indicative of human response, and ADA must be used with caution in pregnancy^[15]. There is no long-term data regarding effects of adalimumab on the developing fetus.

Less information exists on the transplacental diffusion of ADA throughout the trimesters compared to infliximab. Determining the time course of drug administration and when to potentially discontinue ADA during pregnancy is not well-defined due to shorter dosing intervals and limited ability to commercially measure ADA levels. Withholding the drug in the third trimester may be considered to reduce late placental transport to the newborn. Mahadevan *et al*^[17] suggests discontinuation 8-10 wk prior to estimated date of delivery.

Table 1 Summary of reports of maternal exposure to anti-tumor necrosis factor agents during pregnancy

Ref.	Study type	Disease	Anti-TNF- α agent	Exposure to other drugs	Exposures in pregnancies with documented outcome	Maternal exposure: Pregnancy stage	Live births (n)	SA/SB (n)	PTB/ PMB (n)	LBW/ SGA (n)	CA (n)	Pregnancy outcomes: Details/complications
Chambers <i>et al</i> ^[18]	Prospective	RA	IFX	NS	4	T1	3	1 SA	2			
Mahadevan <i>et al</i> ^[19]	Prospective	CD: (4) UC: (1)	IFX	NS	5	T2/T3 other exposure details	5					
Berthelot <i>et al</i> ^[20]	Case series	Rheumatologic disease	IFX	- ¹	3	NS C/T1: 1 C/T1/T2: 2	3					
Chakravarty <i>et al</i> ^[21]	Case series	RA	IFX	Some pts	1	Pregnancy, not otherwise specified	1					
Correia <i>et al</i> ^[22]	Case series	CD	IFX	Yes: 1 No: 1	2	C/T1/T2/T3	2	1	1	1	1	1 preterm/premature birth due to placental detachment (31 wk, 1.6 kg with acute respiratory failure requiring mechanical ventilation \times 24 h and intensive care \times 40 d; healthy at 8 mo follow-up)
Hyrich <i>et al</i> ^[23]	Case series	Rheumatologic disease	IFX	Some pts	3	C/T1	2	1 SA				
Kane <i>et al</i> ^[24]	Case series	CD	IFX	Some pts	3	T1/T2/T3: 2 T2/T3: 1	3		1			
Katz <i>et al</i> ^[25]	Case series	CD: (82) UC: (1) RA: (8) JRA: (2)	IFX	Some pts	100	C: 53 T1: 30 > 3 mo prior to C: 7	68	10 SA	1	1	3	CA (3): 1 full-term with tetralogy of Fallot 1 intestinal malrotation 1 developmental delay and hypothyroidism
		Unknown: (3)				Unknown: 6		1 SB				1 complicated neonatal course: Respiratory distress/jaundice/seizure. Mother was also exposed to several antibiotics for pulmonary and urinary infections, azathioprine, hydrocortisone, and total parental nutrition early in pregnancy
Mahadevan <i>et al</i> ^[26]	Case series	CD	IFX	Some pts	10	T1: 1 T3: 1 C/T1/T2/T3: 8	10		3	1		Miscarriages (14): 10 SA 1 SB (mother exposed to leflunomide) 3 unknown type 1 neonatal jaundice (resolved) 1 complicated neonatal course: term delivery at 39 wk with respiratory distress/desaturation/gastric ulcer day 5; healthy at 6 mo follow-up
Rosner <i>et al</i> ^[27]	Case series	Rheumatologic disease	IFX	Yes	3	C/T1/T2/T3	3		1			1 premature rupture of membranes
Schnitzler <i>et al</i> ^[28]	Case series	CD/UC/IC	IFX	NS	10	C/T1/T2	9	1 SB	2			

Webster-Schoenderfer <i>et al.</i> ^[29]	Case series	NS	IFX	NS	25	T1	22	2	4	2	CA (2): 1 ventricular septal defect 1 growing hemangioma requiring therapy CA (1): L hand polydactyly (Infant also had respiratory depression after anesthetics that resolved spontaneously. Mother was taking methotrexate 2 mo prior to conception without folic acid supplement.)
Zelinkova <i>et al.</i> ^[30]	Case series	CD: (3) UC: (1)	IFX	Some pts	4	C/T1/T2: 3 C/T1/T2/T3: 1	4	SA	1	1	
Akinci <i>et al.</i> ^[31]	Case report	Rheumatologic disease	IFX	Yes	1	C/T1/T2/T3	1				
Angelucci <i>et al.</i> ^[32]	Case report	CD	IFX	Yes	1	C/T1	1		1	1	LBW
Angelucci <i>et al.</i> ^[33]	Case report	CD	IFX	Yes	1	T1	1				
Antoni <i>et al.</i> ^[34]	Case report	Psoriatic Arthritis	IFX	NS	1	C/T1	1				
Arai <i>et al.</i> ^[35]	Case report	CD	IFX	Yes	1	C/T1/T2	1				
Aratani <i>et al.</i> ^[36]	Case report	CD	IFX	Yes	1	T2	1			1	SGA
Burt <i>et al.</i> ^[37]	Case report	CD	IFX	Yes	1	C/T1	1		1		
Chaparro <i>et al.</i> ^[38]	Case report	CD	IFX	NS	1	C/T1/T2/T3	1		1		
Cheent <i>et al.</i> ^[39]	Case report	CD	IFX	NS	1	C/T1/T2/T3	1		1		Infant developed disseminated BCG after vaccination at 3 mo and died at 4.5 mo
Epping <i>et al.</i> ^[40]	Case report	CD	IFX	Yes	1	C/T1/T2/T3	1				
Hou <i>et al.</i> ^[41]	Case report	CD	IFX	NS	1	C/T1/T2/T3	1				
James <i>et al.</i> ^[42]	Case report	CD	IFX	Yes	1	T2	1				
Kinder <i>et al.</i> ^[43]	Case report	RA	IFX	Yes	1	C/T1	0	1			
Østensen <i>et al.</i> ^[44]	Case report	RA	IFX	Yes	1	C/T1	1	SA			Oligohydramnios detected on 18 wk ultrasound that resolved with discontinuation of Nimesulide
Puig <i>et al.</i> ^[45]	Case report	Psoriasis	IFX	Yes	1	C/T1/T2/T3	1				
Srinivasan <i>et al.</i> ^[46]	Case report	CD	IFX	Yes	1 ²	C/T1	1		1		Preterm premature birth (24 wk) complicated by intracerebral and intrapulmonary hemorrhages and neonate died at 3 d Mother was also exposed to metronidazole, azathioprine, and mesalamine for fistulizing CD
Steenholdt <i>et al.</i> ^[47]	Case report	UC	IFX	Yes	1	C/T1/T2/T3	1				
Stengel <i>et al.</i> ^[48]	Case report	CD	IFX	Yes	1	C/T1/T2/T3	1				
Tursi <i>et al.</i> ^[49]	Case report	CD	IFX	NS	1	C/T1/T2/T3	1		1		
Vasiltauskas <i>et al.</i> ^[50]	Case report	CD	IFX	NS	1	C/T1/T2/T3	1				
Wilbaux <i>et al.</i> ^[51]	Case report	AS	IFX	NS	1	C/T1	1				
Xirouchakis <i>et al.</i> ^[52]	Case report	CD	IFX	Yes	1	C/T1	1		1		Preterm (29 wk) birth with neonatal hospitalization \times 30 d post-delivery. Baby in "good condition" at follow-up
Johnson <i>et al.</i> ^[53,54]	Prospective	CD and RA	ADA	NS	94	T1 Other exposure details NS	80	13 SA	12	7 (among live births)	CA (7) (live births): 1 undescended testicle 1 microcephaly 1 congenital hip dysplasia with inguinal hernia 1 congenital hypothyroidism 1 ventricular septal defect

Berthelot <i>et al</i> ^[20]	Case series	Rheumatologic disease	ADA	3	2	C/T1: 1 C/T1/T2/T3: 1	2	1 bicuspid aortic valve and agenesis of corpus callosum (twin sibling had patent ductus arteriosus) 1 congenital hydronephrosis
Hyrich <i>et al</i> ^[23]	Case series	Rheumatologic disease	ADA	Some pts	3	C/T1	2	CA (9) (all pregnancies): In addition to above 7 defects were: 1 spina bifida and hydrocephalus (resulted in elective termination) 1 ectopia cordis and caudal regression (twin pregnancy resulting in a spontaneous abortion)
Johnson <i>et al</i> ^[53,54]	Case series	CD and RA	ADA	NS	122	T1	122	5 CA (5): 2 chromosomal abnormalities 1 atrial septal defect and peripheral pulmonic stenosis 1 ventricular septal defect 1 congenital hip dysplasia 1 infant with autosomal dominant disease (not otherwise specified); paternal inheritance Twin-to-twin transfusion syndrome (1 small due to discordance)
Weber-Schoenderfer <i>et al</i> ^[29]	Case series	NS	ADA	NS	28	T1	24	1 infant with autosomal dominant disease (not otherwise specified); paternal inheritance Twin-to-twin transfusion syndrome (1 small due to discordance)
Abdul Wahab <i>et al</i> ^[55]	Case report	CD	ADA	Yes	1	C/T1/T2/T3	2 (twins)	1 SGA
Ben-Horin <i>et al</i> ^[56]	Case report	CD	ADA	NS	1	C/T1/T2/T3	1	1
Bosworth <i>et al</i> ^[57]	Case report	CD	ADA	Yes	1	C/T1/T2/T3	1	1
Coburn <i>et al</i> ^[58]	Case report	CD	ADA	Yes	1	T2/T3	1	1
Dessinioti <i>et al</i> ^[59]	Case report	Psoriasis	ADA	NS	1	C/T1	1	1
Jurgens <i>et al</i> ^[60]	Case report	CD	ADA	NS	1	C/T1	1	1
Kraemer <i>et al</i> ^[61]	Case report	Takayasu's Arteritis	ADA	Yes	1	C/T1/T2/T3	1	LBW
Mishkin <i>et al</i> ^[62]	Case report	CD	ADA	Yes	1	C/T1/T2/T3	1	1
Roux <i>et al</i> ^[63]	Case report	RA	ADA	NS	1	C/T1	1	1
Vesga <i>et al</i> ^[64]	Case report	CD	ADA	Yes	1	C/T1/T2/T3	1	1
Witbaux <i>et al</i> ^[51]	Case report	AS	ADA	Yes	1	C/T1/T2	1	1
Kane <i>et al</i> ^[65]	Case series	CD	CTZ	NS	14	NS	5	1
Mahadevan <i>et al</i> ^[66]	Case report	CD	CTZ	Yes	1	T2/T3	1	1
Ousallah <i>et al</i> ^[67]	Case report	CD	CTZ	NS	1	C/T1/T3	1	1
Steinberg <i>et al</i> ^[68]	Case report	CD	CTZ	Yes	1	T2	1	1

¹Specified no exposure to disease-modifying antirheumatic drugs, methotrexate, or non-steroidal anti-inflammatory drugs; ²Case reported by Srinivasan *et al*^[61] was also documented by Katz *et al*^[23]; ³Specified no exposure to disease-modifying antirheumatic drugs, methotrexate, or non-steroidal anti-inflammatory drugs. TNF: Tumor necrosis factor; CD: Crohn's disease; UC: Ulcerative colitis; IC: Indeterminate colitis; RA: Rheumatoid arthritis; JRA: Juvenile rheumatoid arthritis; AS: Ankylosing spondylitis; IFX: Infliximab; ADA: Adalimumab; CTZ: Certolizumab pegol; NS: Not specified; Pts: Patients; C: Within < 3 mo prior to conception; T1: First trimester (LMP to 13 wk); T2: Second trimester (14 to 27 wk); T3: Third trimester (28 to 40 wk); SA: Spontaneous abortion; SB: Stillbirth; Preterm birth (< 37 wk gestation); PMB: Premature birth (< 37 wk gestation and prior to organ development); LBW: Low birth weight (< 2500 g); SGA: Small for gestational age (smaller than average size given the number of weeks of pregnancy).

Table 2 Summary of anti-tumor necrosis factor exposures and birth outcomes *n* (%)

Anti-TNF exposure	Birth outcomes, <i>n</i> (with relative percents)						
	Fetal exposures	Live births	SA	SB	PTB/ PMB	LBW/SGA	CA
IFX/ADA/CTZ total	472	405 (85.8)	32 (8.2)	2 (0.6)	41 (19.9)	8 (6.1)	19 (4.1)
IFX ¹	194	155 (79.9)	15 (10.6)	2 (1.1)	21 (26.9)	5 (4.4)	6 (4.0)
IFX in IBD ²	151	117 (77.5)	11 (8.9)	2 (1.4)	16 (36.4)	5 (4.8)	4 (3.5)
ADA ¹	261	242 (92.7)	16 (6.9)	0 (0.0)	20 (15.9)	2 (28.6)	13 (5.4)
ADA in IBD ²	224	210 (93.8)	13 (5.8)	0 (0.0)	15 (17.0)	2 (28.6)	12 (5.7)
CTZ ¹	17	8 (47.1)	1 (5.9)	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)
CTZ in IBD ²	17	8 (47.1)	1 (5.9)	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)
Outcome percents in general US population ^[69-73]		64.60%	16.50%	0.60%	12.30%	8.20%	3.00%-5.00%

¹Exposure in all reported medical conditions; ²Exposure in inflammatory bowel disease (IBD) patients. TNF: Tumor necrosis factor; IFX: Infliximab; ADA: Adalimumab; CTZ: Certolizumab pegol; SA: Spontaneous abortion (fetal death at < 20 wk); SB: Stillbirth (fetal death at > 20 wk or > 350 g if gestational age unknown); PTB: Preterm birth (< 37 wk gestation); PMB: Premature birth (< 37 wk gestation and prior to completion of organ development); LBW: Low birth weight (< 2500 g); SGA: Small for gestational age (smaller than average size given number of pregnancy weeks).

Table 3 Summary of congenital abnormalities reported

Congenital abnormalities (<i>n</i> = 19)	Affected (<i>n</i>)	Anti-TNF exposure
Ventricular septal defect	3	IFX (1), ADA (2)
Chromosomal abnormalities	2	IFX
Congenital hip dysplasia	2	IFX (1), ADA (1)
Intestinal malrotation	1	IFX
Congenital hypothyroidism	1	IFX
Hemangiomas	1	IFX
L hand polydactyly	1	IFX
Tetralogy of Fallot	1	IFX
Patent ductus arteriosus	1	ADA
Atrial septal defect and peripheral pulmonic stenosis	1	ADA
Bicuspid aortic valve and agenesis of corpus callosum	1	ADA
Primary craniosynostosis	1	ADA
Microcephaly	1	ADA
Congenital hydronephrosis	1	ADA
Undescended testes	1	ADA

IFX: Infliximab; ADA: Adalimumab; TNF: Tumor necrosis factor.

Certolizumab pegol

Certolizumab pegol (Cimzia) is a recombinant humanized anti-TNF- α fragment antigen binding (Fab') fragment. The antibody fragment is bound to a polyethylene glycol molecule that extends the drug's half-life to approximately two weeks in the plasma, thereby reducing dosing frequency^[85]. Studies have demonstrated the efficacy of CTZ for induction and maintenance of remission in CD^[86].

CTZ is a pregnancy category B drug. It does not cross-react with mouse or rat TNF- α . Reproduction studies in rats have thus been performed using a rodent anti-murine TNF- α pegylated Fab' fragment (cTN3 PF) that is similar in function to CTZ. These studies have been conducted using doses up to 100 mg/kg and have revealed no evidence of impaired fertility or fetal adversities due to cTN3 PF. Adequate and well-controlled studies have not been performed in pregnant women. As animal reproduction studies are not always indicative of human response, this drug must be used with caution in pregnancy^[16].

The molecular structure of CTZ lacks an Fc portion, so its cross-placental transfer is different from that of IFX and ADA. The Fab' fragment may passively cross the placenta in low levels during the first trimester, an event that is not expected with the IgG1 antibody. Although CTZ therapy would likely not need to be discontinued in the third trimester, it is important to recognize that the transplacental transfer of this drug occurs during a critical period of organogenesis in the first trimester.

In an animal model, pregnant rats received a murinized IgG1 TNF- α antibody and a PEGylated Fab' fragment of the antibody. Lower levels of the drug were detected in the infant and in breast milk with the Fab' fragment versus the full antibody^[87]. Mahadevan *et al*^[66] demonstrated these findings in two human patients receiving certolizumab during pregnancy. The drug was administered to both women two weeks prior to delivery. Although the mothers' drug levels were higher on the date of delivery, newborn cord blood levels were low.

There are few published reports on the use of CTZ during pregnancy. As with the other anti-TNF agents, it is possible that the Fab' fragment passively crosses the placenta at low levels in the first trimester. The drug must be further studied in humans to fully appreciate the course of drug transfer during gestation and subsequent effects on fetal development and pregnancy outcomes.

SUMMARY OF DATA

Our review indicates that rates of SA and CA in anti-TNF-exposed patients are similar to rates in the general United States population^[69-73] and in women with IBD unexposed to anti-TNF agents^[74-76]. The live birth rate in the anti-TNF-exposed group (85.8%) is higher than that of the general United States population (64.6%); this holds true for all patients exposed to IFX or ADA regardless of underlying inflammatory disease and perhaps reflects a state of controlled disease activity. The live birth rate for patients exposed to CTZ (47.1%) is lower than that of the general population, although there is a very small collective sample size. The rates of SA and

SB for all groups are similar to the general United States population^[72] with the exception of IFX-exposed patients, in whom the rate of SB is just slightly higher. The PTB/PMB rate in the anti-TNF-exposed group (19.9%) is higher than in the United States population (12.3%)^[72], perhaps due to an underlying predisposition as in the setting of IBD^[76]. LBW/SGA infants are more common in ADA- and CTZ-exposed patients than in the general United States population^[73], again possibly reflecting the underlying disease itself or the severity of disease activity.

In general, pregnancy does not increase the risk of disease exacerbation in CD or UC^[88,89]. Approximately one-third of women with inactive IBD at the time of conception are expected to flare during pregnancy and the puerperium^[90]. Alternatively, if pregnancy overlaps with a period of active IBD, the disease may be difficult to control^[91]. Active disease at the time of conception has been associated with increased rates of PTB^[89] and fetal loss^[92], and disease flares during pregnancy have been associated with PTB and LBW^[4,93]. Studies are mixed regarding the risk of congenital malformations among IBD progeny, with some data showing an increased risk for both CD and UC patients^[94] or for UC patients alone^[95,96] and other data showing no increased risk in CD or UC^[97,98]. Regardless of disease activity, women with IBD have an increased risk for such adverse pregnancy outcomes as PTB, SB, LBW, SGA, and delivery complications such as cesarean sections compared to the general population^[97-101]. In our study, no discernible increased risks for SA or CA were identified. Overall, unless there is a clear risk of fetal harm (*i.e.*, an FDA category X drug) that dictates otherwise, maintenance therapy is conventionally continued throughout pregnancy to optimize maternal disease control and prevent relapse or progression^[102].

This systematic review has limitations. Pooling data from different studies yields inherent heterogeneity based on study designs, study populations, and recording of birth outcomes data. As evidenced, there are a limited number of reported pregnancy exposures to anti-TNF agents, many published as case reports or case series with small sample sizes; these do not necessarily reflect outcomes that can be extracted to the general population. Our review is affected by the limitations of the individual studies, including the inability to adjust for maternal disease activity and severity, concomitant medication or substance use, comorbidities, or other maternal characteristics. Additionally, there exist potential publication bias against negative outcomes and recall bias involving drug exposure and timing of administration during conception and pregnancy. The decision to exclude studies based on the English language and on the inability to link specific anti-TNF exposure with birth outcomes may have discounted pertinent publications. Although care was taken to account for evident overlap, it is possible that repeated data exists given the nature of our information (for example, a case report that has also been reported within drug registry data).

A growing body of evidence supports that IFX, ADA, and CTZ are low risk in pregnancy^[17], and studies beyond those included in our data set are underway to further elucidate fetal risk and optimal timing of biologic administration during pregnancy^[103,104]. Thus far, it is believed that IFX and ADA are most compatible for use during conception and at least the first and second trimesters considering mechanisms of placental transport^[17,102]; further human data are needed to generate safety guidelines for the use of CTZ. In a recent study of pregnant women receiving biologic therapy, IFX and ADA were shown to be transplacentally transferred to infants at birth, with high levels of drug in cord blood and detectable drug levels up to six months after birth. CTZ was found to be least detectable in both cord blood and infant serum after birth. Of note, no CA or significant fetal complications were reported in this study^[104].

Future efforts are promising and include the expansion of drug safety data registries and the development of larger prospective trials to help definitively quantify fetal risk and to facilitate clinical decision-making in treating women with IBD during their childbearing years. One such project is the highly anticipated Pregnancy IBD and Neonatal Outcomes study, a prospective data collection from multiple IBD centers in the United States^[105]. This large cohort registry not only accounts for maternal factors including IBD activity, medication use, delivery methods, and pregnancy complications but also tracks data over time from the neonatal period through children's first year of life. Similarly, post-marketing surveillance data may uncover additional consequences of fetal exposure to biologic agents over time.

While evidence in the field is mounting, caution should indefinitely be exercised. Given the limitations of the available data and lack of controlled trials, there is insufficient evidence to prove absolute safety for use of anti-TNFs during pregnancy. Although the benefits of therapy in optimizing disease activity during gestation may lend to more favorable pregnancy outcomes based on a controlled disease state, definitive safety of drug exposure on the developing fetus has not been confirmed.

Medical management decisions during the preconception and pregnancy periods will inevitably vary by case based on respective risk-to-benefit ratios, details of disease activity, response to alternative therapies, and individual preferences. Women and men of childbearing age should be educated about the effects of IBD on pregnancy and the potential implications of treatment on fetal development. In addition, patients should be encouraged to discuss reproductive plans with their physicians in order to achieve remission prior to conceiving. Ideally, the primary preconception goal should be quiescent disease, as this lends to the most favorable pregnancy outcomes.

CONCLUSION

After systematic literature review investigating TNF- α inhibitor therapy and fetal risk, there is insufficient evi-

dence to prove absolute safety for the use of biologics (specifically infliximab, adalimumab, and certolizumab pegol) during pregnancy.

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CD133: A cancer stem cells marker, is used in colorectal cancers

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Abstract

Colorectal cancer is one of the most common malignant tumors worldwide. A model of cancer development involving cancer stem cells has been put forward because it provides a possible explanation of tumor hierarchy. Cancer stem cells are characterized by their proliferation, tumorigenesis, differentiation, and self-renewal capacities, and chemoradiotherapy resistance. Due to the role of cancer stem cells in tumor initiation and treatment failure, studies of cancer stem cell markers, such as CD133, have been of great interest. CD133, a five-transmembrane glycoprotein, is widely used as a marker to identify and isolate colorectal cancer stem cells. This marker has been investigated to better understand the characteristics and functions of cancer stem cells. Moreover, it can also be used to predict tumor progression, patient survival, chemoradiotherapy resistance and other clinical parameters. In this review, we discuss the use of CD133 in the identification of colorectal cancer stem cell, which is currently controversial. Although the function of CD133 is as yet

unclear, we have discussed several possible functions and associated mechanisms that may partially explain the role of CD133 in colorectal cancers. In addition, we focus on the prognostic value of CD133 in colorectal cancers. Finally, we predict that CD133 may be used as a possible target for colorectal cancer treatment.

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Key words: CD133; Colorectal cancer; Cancer stem cells; Prognosis; Chemoradiotherapy resistance

Core tip: CD133 is not a reliable marker to identify the entire population of cancer stem cells (CSCs). However, the abundance of CD133 may be a good indicator of CSC identity and consistent with the biological characteristics of CSCs; The expression of CD133 is correlated to the poor survival; CD133(+) cells exhibit more chemoresistant behavior than CD133(-) cells; Whether CD133-targeting therapies can be a specific or efficient treatment for colorectal cancer has not been confirmed.

Ren F, Sheng WQ, Du X. CD133: A cancer stem cells marker, is used in colorectal cancers. *World J Gastroenterol* 2013; 19(17): 2603-2611 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2603.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2603>

INTRODUCTION

Colorectal cancer is regarded as one of the most common cancers in the world, and a main cause of cancer-related death in western countries. In spite of progressing treatments, a large percentage of advanced tumors have poor prognosis. Recent studies have shown that a small population of tumor cells, known as cancer stem cells (CSCs), may be considered the main initiators of recurrence and metastasis. It is critical to find specific bio-

markers to identify and isolate CSCs as well as to predict patient prognosis. CD133 is one of the best-characterized markers of CSCs. However, its role in colorectal cancer needs further study. Here, we have attempted to elucidate the relationship between CD133 and colorectal cancer based on the results of previous studies.

INTRODUCTION OF CD133

CD133, a five-transmembrane glycoprotein, was first found to be expressed in hematopoietic stem and progenitor cells by Yin *et al.*^[1]. The protein has a molecular weight of 120 kDa^[1] and localizes to membrane protrusions. The protein may be expressed as one of two isoforms, CD133-1 and CD133-2. CD133-1 was the first to be discovered, by Yin *et al.*^[1], and it is mainly expressed in human fetal liver, bone marrow and blood. CD133-2, first cloned and identified by Yu *et al.*^[2], is another cell surface antigen that is recognized by anti-AC133 monoclonal antibodies. Relative to AC133-1 cDNA, a small exon of 27 nucleotides is deleted in AC133-2 by alternative mRNA splicing^[2]. CD133-2 mRNA is prominently expressed in human fetal tissue, adult tissues and several carcinomas^[2]. It has also been suggested that CD133-2 is expressed in multiple stem cell niches^[2]. Based on these biological characteristics, CD133 is widely used to identify and isolate stem cells and cancer stem cells. However, its function is still unclear. It is hypothesized to be associated with the cell-cell interaction or signal transduction.

CANCER STEM CELLS AND CD133

Tumor cells show heterogeneity in their morphology, inheritance, functions and other characteristics. However, some tumor cells present not only heterogeneity but also hierarchy. The increasing CSCs model represents a breakthrough in explaining this phenomenon. In this model, CSCs, despite being only a small subset of cancer cells, have the capability to self-renew and sustain the tumor. These CSCs also have the ability to proliferate, resulting in expansion of the CSC pool, and to differentiate into the heterogeneous cancer cell subgroups that may not themselves be tumorigenic but usually constitute the majority of the tumor^[3].

More and more studies have indicated that CD133 is a surface marker of CSCs. CD133 has been found in many tumors, including cancers of the brain^[4], colon^[5,6], liver^[7], pancreas^[8], kidney^[9], lung^[10], endometrium^[11], ovary^[12] and bone^[13]. The exploration of CD133 as a surface marker of colon cancer stem cells (Co-CSCs) is still in progress. In 2007, both O'Brien *et al.*^[14] and Ricci *et al.*^[15] found that CD133(+) cells in colon cancers had the ability to initiate tumor growth. The colon cancer-initiating cells (CC-ICs) represented enrichment in CD133(+) populations. These two studies strongly support CD133 as a marker of Co-CSCs based on the evidence that CD133(+) cells could produce tumors with preserved self-renewal and differentiation capabilities and without phenotypic alterations

after serial transplantation. The long-term tumorigenic potential of CD133(+) colon cancer cells has also been confirmed *in vitro*^[15]. More importantly, CD133(-) colon cancer cells have no ability to form tumors. However, Shmelkov *et al.*^[16] discovered that CD133 was ubiquitously expressed in differentiated colonic epithelium rather than restricted to stem cells. Furthermore, *in vitro*, both CD133(+) and CD133(-) metastatic tumor subpopulations formed colonospheres. Both subpopulations maintained long-term tumorigenesis in a NOD/SCID serial xenotransplantation model. It should be noted that Shmelkov *et al.*^[16] used subpopulations from metastatic tumors. However, the distinction of CSCs and the function of CD133 in primary and metastatic tumors are still unknown. Despite this fact, the discovery challenged the view of CD133 as a marker of Co-CSCs, and further studies were performed to investigate the discrepancy.

Kawamoto *et al.*^[17] showed that although both CD133(+) and CD133(-) cells could form tumors after injection into NOD/SCID mice, the CD133(+) cells formed larger tumors. There remained a difference in these tumors. CD133(+) cells could not be found in tumors generated by the injection of CD133(-) cells but were observed in tumors from injections of CD133(+) cells, suggesting that CD133(+) cells had self-renewing capability whereas CD133(-) cells did not. The investigation is consistent with Shmelkov's finding that CD133(-) cells also have the ability to form tumors, although it seems that the CD133(+) cells were associated with stronger tumorigenesis than CD133(-) cells. Thus, the presence of CD133 may not be a reliable indicator of CSC *vs* non-CSC identity. A more appropriate distinction is the relative abundance of the CD133 protein^[18]. Liao *et al.*^[19] attempted to confirm this hypothesis by sorting cancer cells according to the abundance of CD133. CD133 (High), CD133 (Mid), and CD133 (Low) subgroups of SW620 cells (a colorectal cancer cell line) were distinguished, and their biological characteristics were analyzed. The CD133 (High) subgroup exhibited a higher growth rate than the CD133 (Mid) and CD133 (Low) subgroups did. However, despite its much slower growth, the CD133 (Low) subgroup retained its tumorigenicity.

One likely explanation is that CD133 is expressed on not only CSCs but also differentiated tumor cells. However, during CSC differentiation, the specific epitopes recognized by AC133 are masked due to differential glycosylation^[20]. The expression of CD133 could be modulated by factors in the microenvironment, such as energy supply^[21]. In addition, the inactivation of CD133 during the progression of colorectal cancer can be considered a result of transcriptional repression, due to promoter hypermethylation of the CD133 CpG islands^[22]. CD133(-) cells likely lack the AC133 epitopes, the expression of which is influenced by posttranslational modification under certain conditions. It appears that CD133 is not a reliable marker to identify the entire population of CSCs. However, the abundance of CD133 may be a good indicator of CSC identity.

Although some studies suggest that CD133(+) cells have characteristics consistent with those of CSCs, such as tumor initiation, proliferation, invasion, differentiation and self-renewing capacities^[23-26], CD133 should be used as the sole marker of Co-CSCs with caution. Thus, additional markers for detecting CSCs and evaluating their clinical significance in colorectal cancers have been proposed. These markers include CD44^[27-31], CD166^[25,32], CD29^[25,32,33], CD24^[5,32,34], Lgr5^[6,32], nuclear beta-catenin^[32,35], EpCam^[33,36], ALDH1^[33,37], CDCEP1^[5], CXCR4^[5] and CC188^[38]. The use of the combination of these markers to identify CSCs in colorectal cancers will uncover more about the function of CSCs and will also play a significant role in clinical usage.

Some pathways, including the wingless related (Wnt), transforming growth factor-beta (TGF- β), Notch and Hedgehog signaling pathways^[39], and other mechanisms have been found to be associated with CSCs and CD133 expression in colorectal cancers. The Wnt pathway plays an essential role in the growth and maintenance of CSCs^[40]. This pathway is regulated at the level of β -catenin, which is degraded by adenomatous polyposis coli (APC). Mutations in the *APC* gene are found in most colorectal tumors^[41]. As a result, β -catenin is accumulated in the nucleus, where it activates target genes with important functions in colorectal cancer development^[42]. Some studies have confirmed the activation of the Wnt pathway in CD133(+) cells^[43,44]. The TGF- β pathway acts as a tumor suppressor pathway in healthy tissues but as a promoter in colorectal cancers^[45]. Mutations in the type II receptor gene^[46], type I receptor gene^[45], Smad family member 4^[47] and other Smads are observed in colorectal cancer specimens. Notch signaling is active in CC-ICs and is essential for the intrinsic maintenance of CC-ICs self-renewal and the repression of secretory cell lineage differentiation gene^[48]. It has also been reported that the Hedgehog signaling, which is active in both colon cancer epithelial cells and, strikingly, CD133(+) cancer stem cells, promotes colon cancer growth, stem cell self-renewal and metastatic behavior in advanced cancers^[49,50]. In addition, the CD133(+) CSCs may be relevant to the Ras-Raf^[51,52], STAT3^[53], Akt, mitogen-activated protein kinase^[54], hypoxia-inducible factor-1 α ^[55] and microRNAs^[56]. Although CD133 was observed to be associated with actively proliferating cells, few studies have investigated the role of CD133 in the cell cycle. However, these studies could not explain the function of CD133 directly.

In colorectal cancer tissues, CD133 is localized to apical/endoluminal surfaces, the cytoplasm and to luminal contents^[57-60]. CD133 is concentrated in plasma membrane protrusions^[61], suggesting that CD133 may play a role in cell-cell and cell-matrix contact formation. CD133(+) cells have an enhanced ability to interact with adjacent carcinoma-associated fibroblasts^[26,62], indicating that CD133(+) cells are more interactive with the stromal microenvironment, and thus more tumorigenic and invasive, than CD133(-) cells. Furthermore, CD133 contains a ganglioside-binding domain at its N-terminus. Through

this epitope, certain gangliosides could modulate the accessibility of CD133 and regulate cell-cell contacts^[63].

However, knocking down the CD133 did not affect the biological characteristics of the colon cancers, which indicated that CD133 has no obvious functions in tumor malignancy^[64]. Whether CD133 has biological function remains a question, but the use of CD133 as one of the CSC markers in colorectal cancers is widely accepted.

THE PROGNOSTIC VALUE OF CD133 IN COLORECTAL CANCERS

As CD133 is a notable marker of CSC identity, it is thought to be a predictive indicator for colorectal cancer. A number of studies have demonstrated that CD133 expression was correlated with survival, recurrence, metastasis and chemotherapy resistance. Horst *et al.*^[60] analyzed tissues from 57 colorectal cancer patients (T2/T3, N0, and M0) using immunohistochemistry (IHC). The CD133-high patients had a worse 5- and 10-survival than CD133-low patients. Further investigations have been performed with larger sample sizes, specific tumor stage, different IHC evaluations, combinations with other markers, the use of polymerase chain reaction (PCR) and preoperative chemotherapy conditions. The majority of these results support the hypothesis that CD133 expression is predictive of survival^[35,51,55,57,59,65-68]. However, Choi *et al.*^[34] investigated 523 colorectal cancer patients with various tumor stages using the IHC approach and reported that survival was not significantly related to CD133 expression. In addition, Kijima *et al.*^[69] analyzed samples from 189 patients with different stages of colorectal cancer by IHC and found that patients with and without CD133 overexpression exhibited no differences in recurrence-free survival but had significantly poorer overall survival. A summary of related studies published is presented in Table 1. Different patient patterns, study designs and the use of commercial antibodies for IHC, which may lead to high background noise, could cause the discrepancies among these studies. In studies involving IHC, different researchers used different valuation criteria. Some studies evaluated IHC results as positive or negative. Others evaluated the results according to the positivity extent. As discussed above, the abundance of CD133 may be a better indicator of CSCs than the presence *vs* absence of CD133. Despite a large sample size, Choi evaluated the presence or absence of CD133, which might not reliably indicate CSC identity. In addition, that study included patients with various stages of colorectal cancers. Those who had poorly-differentiated tumors or higher stage, especially stage IV, but were CD133 negative, could introduce significant statistical confounding. Because no biologically relevant IHC cut-off point has been established to date, most studies set the cut-off arbitrarily. The use of a receiver operating characteristic curve to determine the cut-off point has been proposed to improve the clinical utility of IHC findings^[70]. All studies^[51,55,59,65,67] that used PCR to measure CD133 expres-

Table 1 Studies of the relationship between CD133 expression and survival

Ref.	n	Cancer category	Tumor stage	Method	Did CD133 expression predict poor survival?
Choi <i>et al</i> ^[34]	523	Colorectal adenocarcinomas	Stages I-IV	IHC	No: overall survival
Kemper <i>et al</i> ^[51]	90	Colorectal cancers	Stage II	RT-PCR	Yes: relapse-free survival
Saigusa <i>et al</i> ^[55]	52	Rectal cancers (post-CRT)	Unclear	RT-PCR	Yes: recurrence-free survival
Jao <i>et al</i> ^[57]	233	Colorectal adenocarcinomas (post-CRT)	Stages I-IV	IHC	Yes: overall survival
Saigusa <i>et al</i> ^[59]	33	Rectal cancers (post-CRT)	Stage II / III	RT-PCR	Yes: disease-free survival
Horst <i>et al</i> ^[60]	77	Colorectal adenocarcinomas	T2/T3, N0, M0	IHC	Yes: cancer-specific survival
Yasuda <i>et al</i> ^[65]	40	Rectal cancers (post-CRT)	Advanced	RT-PCR	Yes: disease-free survival
Li <i>et al</i> ^[66]	104	Colon carcinomas	Stage IIIB	IHC	Yes: overall survival
Artells <i>et al</i> ^[67]	60	Colorectal cancers	Stages I-III	RT-PCR	Yes: overall survival
Kojima <i>et al</i> ^[69]	160	Colorectal cancers	Stages I-IV (well- and moderately-differentiated)	IHC	Yes: overall survival
Kojima <i>et al</i> ^[69]	140	Colorectal cancers	Stages I-IV (well- and moderately-differentiated)	IHC	No: recurrence-free survival

IHC: Immunohistochemistry; RT-PCR: Reverse-transcription polymerase chain reaction; CRT: Chemoradiotherapy.

sion at the mRNA level, concluded that CD133 expression is inversely correlated with survival. The majority of these studies included post-chemoradiotherapy patients. However, all of these studies used small sample sizes. Although some studies had deficiency in their design, majority of them came to the similar conclusion that the expression of CD133 was correlated to the poor survival, and more uniform studies should be performed to demonstrate this confusion.

CD133 can be found in not only primary tumors but also metastatic tumors such as liver metastases. It was reported that the CD133(+)/CD44(+) subpopulation was responsible for this metastasis^[30,71]. Neumann *et al*^[72] reported that cases with MLH1(+), CD133 (high scores) and β -catenin (high scores) tumors were associated with a very high rate of distant metastases (94.4%). Thus, it seems that CD133, in combination with other markers, may be a good predictor of metastasis risk.

Several studies have investigated whether the CD133 mRNA level in peripheral blood is useful for prognosis in colorectal cancers. Lin *et al*^[73] first described that an increased level of CD133 mRNA in the peripheral blood could predict colon cancer recurrence, independent of tumor-node-metastasis stage. Pilati *et al*^[68] investigated patients with liver-confined hepatic metastasis from colorectal cancers. The level of CD133, used as a marker of circulating tumor cells, increased inversely with the survival. Iinuma *et al*^[74] showed that in patients with Dukes' stage B and C cancer, CEA/CK/CD133 demonstrated significant prognostic value. In contrast, no significant differences were seen in patients with Dukes' stage A disease. These studies support the use of CD133 mRNA in peripheral blood as prognostic marker. However, Gazzaniga *et al*^[75] reported that there was no correlation between the expression of CD133 in circulating tumor cells isolated from peripheral blood and outcomes in patients with metastatic colorectal cancers. All these studies used an reverse-transcription polymerase chain reaction approach, but this method may have low sensitivity and lead to a high rate of false positive results in detecting circulating tumor cells^[76]. Due to the limitation of this method and the inclusion of early stage (stage I)

patients with better outcomes as well as advanced stage (stage IV) patients with worse outcomes, current studies may not accurately distinguish between the outcomes in patients with early and advanced stages of colorectal cancer. However, the prognostic value of CD133 mRNA in the peripheral blood of patients with middle-stage disease (stages II and III) has been confirmed by some studies.

Chemoradiotherapy (CRT) resistance is a major problem that affects the survival of patients with colorectal cancer. Furthermore, the acquired resistance has a long-term memory^[77]. Conventional chemotherapy targets rapidly dividing cells, but CSCs divide slowly. Therefore, CSCs are likely to contribute to the resistance of cytotoxic systemic therapies^[78]. Efforts have been made to demonstrate that the role of CD133(+) colorectal tumors are more resistant to 5-fluorouracil-based chemotherapy than CD133(-) tumors. Ong *et al*^[79] conducted a clinical study containing 501 primary colorectal cancer cases that provided evidence supporting this hypothesis. Moreover, recent studies showed that the level of CD133 increased in post-CRT specimens^[65] and that CD133 expression was detected in 27.5% of non-CRT and 70% of CRT specimens^[80]. *In vitro*, CD133 was overexpressed in human colon cancer cell lines that were resistant to 5-fluorouracil and oxaliplatin, such as HT29/5FU-R and HT29/OxR^[81]. These results suggest that CD133 is a good predictor of CRT resistance. However, Hongo *et al*^[82] recently reported that CD133(-) cells are more resistant to 5-fluorouracil than CD133(+) cells, which challenged the previous view. However, these authors isolated CD133(+) and CD133(-) cells from a single colorectal cancer cell line, and the original characteristics may have been altered during long-term culture. Therefore, the clinical studies are more reliable than *in vitro* studies. Meanwhile, Ong's research was conducted in a large clinical sample size, and other clinical^[57,80,83] and *in vitro* studies^[81,84] supported their results. Considering that Hongo's study was based on a single cell line and that no further studies have drawn similar conclusions to date, we feel it is appropriate to conclude that CD133(+) cells exhibit more chemoresistant behavior than CD133(-) cells. However, more studies, especially

clinical studies, should be performed to clarify the role of CD133 in chemoresistance.

The mechanism of chemoresistance is still under investigation. Intrinsic factors such as antiapoptotic proteins and soluble microenvironmental molecules, including growth factors and cytokines, may cause the refractoriness of CSCs. Several studies have demonstrated that the interleukin 4 (IL-4) produced by cancer cells themselves negatively regulated apoptosis^[85-87]. Todaro *et al*^[88] reported that IL-4 protected CD133(+) cells from apoptosis in colon cancer. They later found that treatment with an IL-4 receptor alpha chain antagonist or anti-IL-4 neutralizing antibody strongly enhances the antitumor efficacy of standard chemotherapeutic drugs through selective sensitization of CD133(+) cells^[89]. These studies highlight the importance of IL-4 in chemoresistance. In addition, the secretion of IL-4 induced an immunosuppressive state in the microenvironment of the tumor, which facilitates the tumor progression. These results indicate that IL-4 may be a good target of colorectal cancer treatment.

COLORECTAL CANCER TARGET THERAPIES

There are two major targets for advanced colorectal cancer therapy: epidermal growth factor receptor and vascular endothelial growth factor. The Wnt pathway is an additional potential target. Data has shown that Wnt pathway activity could be responsible for the chemoresistance of CD133(+) cells in colorectal cancer cells. Deng *et al*^[43] demonstrated that 5-fluorouracil upregulated Wnt activity in CD133(+) colon cancer stem-like cells. Dickkopf-1, an inhibitor of Wnt signaling, decreased the expression of CD133 and Lgr5. It also reduced the proliferation, migration, and invasion of colon cancer cells^[90]. This indicates that blocking the Wnt pathway may be one possible solution to the problem of chemoresistance. Furthermore, other pathways, such as the Notch and Hedgehog signaling pathways involved in maintaining CSC identity, and other regulators, such as STAT3^[53,91,92] and microRNAs, could be conceivable targets.

CSCs can be eliminated by targeting membrane proteins such as CD133 and then delivering medicines that can specifically induce to death. Several efforts have been made to utilize CD133 in the treatment of cancers. Damek-Poprawa *et al*^[93] conjugated a CD133 monoclonal antibody (MAB) to a genetically modified cytolethal distending toxin (Cdt). The proliferation of CD133(+) cells in cell lines derived from head and neck squamous cell carcinomas was preferentially inhibited in a rate- and dose-dependent manner by the Cdt-MAB complex. Moreover, Rappa *et al*^[94] decreased the number of CD133 molecules using two different short hairpin RNAs in FEMX-I melanoma cells. The cell growth, cell motility and spheroids-forming capacity were inhibited as a result of downregulation of CD133. In addition, Wang *et al*^[95] investigated glioblastoma (GBM) using single-walled

carbon nanotubes (SWNTs) conjugated with CD133 monoclonal antibodies. GBM cells were exposed to these SWNTs and then irradiated with near-infrared laser light. They found that the CD133(+) cells were eliminated, and the tumorigenic and self-renewal characters of CD133(+) cells were also blocked. However, some have argued that CD133(-) cells can be reversed to CD133(+) cells through microenvironmental factors and that CD133 is not the only marker able to identify CSCs, indicating that targeting CSCs through CD133 alone is not sufficient to cure tumors. Whether CD133-targeting therapies can be a specific or efficient treatment for colorectal cancer requires further exploration.

CONCLUSION

Although CD133 has been used as a CSC marker, it cannot be the only marker used to characterize CSCs in colorectal cancers. It seems that a small population of CD133(-) cells also have the biological characteristics of CSCs. Other markers should be used in combination to identify CSCs. Which combination of these markers is the best to identify CSC or shows consistent biological characters with CSC remains a question. Moreover, little is known about the function of CD133. Whether CD133 participates in the biological behavior of CSC or merely acts as a marker of the CSC phenotype is not clear. A variety of studies have confirmed the prognostic value of CD133 in colorectal cancers. However, these studies lack uniform samples, clinical conditions, methods and evaluations. Although CD133 is believed to be a target of advanced colorectal cancer, few efforts have been made to confirm this hypothesis directly. Regardless, it is desirable to explore new strategies of colorectal cancers by the use of CD133.

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Prevalence of celiac disease in Germany: A prospective follow-up study

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specific to celiac disease. Subjects with positive antibody titers and those with histories positive for celiac disease then underwent biopsy. At the first follow up, antibody titers were again determined in these subjects and subjects were questioned regarding symptoms specific for celiac disease and disorders associated with celiac disease. The second follow up consisted of a telephone interview with subjects positive for celiac disease.

RESULTS: Antibody tests consistent with celiac disease were reported in eight subjects, corresponding to an overall prevalence of 1:270 (8/2157). The prevalence among women was 1:224 and 1:518 in men. Classical symptoms were observed in 62.5% of subjects. Atypical celiac disease was present in 25.0%, and transient celiac disease in 12.5%. False-negative test results were returned in three subjects. This yields a sensitivity and specificity of 62.5% and 50.0%, respectively, for tissue transglutaminase immunoglobulin-A antibody; of 62.5% and 71.4% respectively, for endomysium antibody; and of 62.5% and 71.4%, respectively, for anti-gliadin antibody.

CONCLUSION: The prevalence rate in our collective lies within the middle tertile of comparable studies in Europe. The use of a single antibody test for screening purposes must be called into question.

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Key words: Cross-sectional study; Celiac disease; Screening; Prevalence; Serology

Core tip: Only limited data on the prevalence of celiac disease in the adult European population are available. Aim of the study was to determine the prevalence of celiac disease in a randomly selected population sample in Germany and to assess the sensitivity and specificity of antibody tests. Eight of 2157 (1:270) subjects tested

Abstract

AIM: To determine the prevalence of celiac disease in a randomly selected population sample.

METHODS: A total of 2157 subjects (1036 males; 1121 females) participating in a population-based cross-sectional study underwent laboratory testing for tissue transglutaminase and antibodies to immunoglobulin A, endomysium and antigliadin. In a second step, all subjects who had been examined serologically were surveyed using a questionnaire that included questions

positive for celiac disease. Tissue transglutaminase immunoglobulin-A antibody yielded a sensitivity of 62.5% (specificity 50.0%), endomysium antibody of 62.5% (71.4%) and anti gliadin antibody of 62.5% (71.4%). The prevalence rate lies within comparable European study results. The use of a single antibody test for screening purposes must be questioned.

Kratzer W, Kibele M, Akinli A, Porzner M, Boehm BO, Koenig W, Oeztuerk S, Mason RA, Mao R, Haenle MH. Prevalence of celiac disease in Germany: A prospective follow-up study. *World J Gastroenterol* 2013; 19(17): 2612-2620 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2612.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2612>

INTRODUCTION

The prevalence of celiac disease in population-representative collectives is reported between 1:42 and 1:558^[1-10] (Table 1) depending on the size of the population studied and the nature of the antisera used for screening. Among blood donors, the reported range is from 1:37 to 1:681^[11-19] (Table 1) and, for non-population representative samples, between 1:86 and 1:709^[20-24] (Table 1).

Subjects suffering from celiac disease may present with typical clinical symptoms, such as diarrhea, weight loss and bloating; or, they may be completely asymptomatic or exhibit only unspecific symptoms. For example, anemia or iron deficiency may be the only initial signs of the disease. Age distribution of first onset shows a first peak between nine months and two years, and a second during the fourth decade^[25].

It has only been in recent years that the clinical manifestations of this very heterogeneous disorder have been arranged in subclasses^[26]. The system of subtypes proposed by Holtmeier *et al*^[26] for the first time integrates clinical symptoms, histology and antibody titers, thus facilitating reliable diagnosis and therapeutic management.

Celiac disease is associated with specific, often serious, complications, including the development of gastric ulcers with the risk of hemorrhage, perforation and stricture, as well as T cell lymphoma^[25,27]. In fact, the risk of lymphoma in patients with celiac disease is about three times as high as that of the general population, with a peak age of onset at about 60 years^[25]. The risk of complications and neoplasia is associated with all forms of celiac disease: hence, the recognition of non-classical disease forms is particularly crucial. A strict, gluten-free diet started as early as possible and maintained lifelong significantly reduces the risk of malignancy^[28]. The protective effects of a gluten-free diet on the development of autoimmune disorders associated with celiac disease, such as diabetes mellitus or autoimmune thyroid diseases, however, remains controversial in the literature^[29-33].

Small bowel biopsy has been the conventional gold standard for the diagnosis of celiac disease. Today, however, greater importance is now attached to serological

studies, including antibodies to tissue transglutaminase (tTGA), endomysium (EMA) and gliadin (AGA)^[8].

To date, no prospective data on the prevalence of celiac disease in a representative adult population sample in Germany have been published. Objective of the present study was to determine the prevalence of celiac disease in a randomly selected population sample.

MATERIALS AND METHODS

Study population

The study "Echinococcus Multilocularis and other Internal Diseases in Leutkirch" (EMIL), a cross-sectional survey assessing the prevalence of *Echinococcus multilocularis* infection and other medical disorders, was conducted in Leutkirch, Germany in 2002. Initially, 4000 of the total 12475 residents were randomly selected by the staff of the municipal registry office from the roster of inhabitants. Out of these 4000 persons, 107 were excluded because their address was unknown or they had not given their informed consent. A total of 2445 individuals finally participated in the study, corresponding to a participation rate of 62.8%. Following exclusion of subjects less than 18 years and subjects with incomplete laboratory results, 2157 subjects were finally included in the present analysis (Figure 1).

The study was conducted in accordance with the principles of the Helsinki Declaration and Good Clinical Practice. It was approved by the ethics committee of the Landesärztekammer Baden-Württemberg. All subjects provided their written informed consent.

Initial study

All subjects were interviewed by a trained interviewer using a standardized questionnaire. In order to reduce interviewer bias as much as possible, each interviewer underwent in-depth training by an interviewing specialist of the state health office^[34].

Because the original EMIL questionnaire did not include specific questions regarding celiac disease, in 2003 all subjects of the EMIL study were mailed a separate questionnaire addressing celiac disease. Subjects were questioned regarding celiac disease that had been diagnosed prior to the date of the EMIL study and were asked whether they were currently (*i.e.*, at the time of the study) prescribed a gluten-free diet. The response rate to this survey stood at 50%.

Each subject underwent phlebotomy of the cubital vein to obtain ca. 25 mL of venous blood. Total immunoglobulin A (IgA) concentration was determined by nephelometry (BN II, Dade Behring, Marburg, Germany). IgA to tTGA was measured using an indirect, non-competitive enzyme immunoassay (Pharmacia Diagnostics Freiburg, Germany). Human recombinant tTGA was used as the antigen. Titers of 5-8 U/mL were considered borderline, while titers > 8 U/L were considered positive. AGA was determined using ELISA (Vita Diagnostics Merzhausen, Germany). Titers of 12-16 U/mL were con-

Table 1 Prevalence of celiac disease in different countries

Country	Prevalence	Characteristics of populations studied			Antibody test method		
		<i>n</i>	Age (yr) mean/median (range)	Males	tTGA	AGA	EMA
Population-representative samples							
Germany (present study)	1:270	2157	42.6 (18-65)	48.03%	-	-	-
New Zealand ^[1]	1:82	1064	50.2 (> 18)	39.80%	-	-	-
Iran ^[2]	1:104	2799	33.7 (18-66)	50%	-	-	-
Ireland ^[3]	1:122	1823	NA (15-65)	NA	-	-	-
Sweden ^[4]	1:190	1894	50 (25-74)	50%	-	-	-
Netherlands ^[5]	1:286	1440	40.6 (20-59)	46%	-	-	-
Spain ^[6]	1:390	1170	44.9 (2-89)	44.70%	-	-	-
Italy ^[7]	1:559	2237	44 (20-87)	46.90%	-	-	-
Greece ^[8]	1:558	2230	46 (18-80)	45%	-	-	-
Finland ^[9]	1:47	2815	NA (52-74)	48%	-	-	-
Finland ^[10]	1:42	4846	NA (30-64)	47%	-	-	-
Italy ^[10]	1:145	2759	NA (30-64)	42%	-	-	-
Germany ^[10]	1:344	3098	NA (30-64)	49%	-	-	-
Blood donors							
Mexico ^[11]	1:37	1009	34 (NA)	68%	-	-	-
Italy ^[12]	1:100	1002	33 (13-90)	43.40%	-	-	-
United States ^[13]	1:105	2845	NA	43%	-	-	-
Iceland ^[14]	1:136	813	36 (17-64)	76.30%	-	-	-
Brazil ^[15]	1:214	2045	32.8 (18-61)	87.60%	-	-	-
Netherlands ^[16]	1:333	1000	NA	NA	-	-	-
Norway ^[17]	1:340	2069	3 (18-67)	66.30%	-	-	-
Iran ^[18]	1:400	2000	35.5 (18-65)	79%	-	-	-
Brazil ^[19]	1:681	2045	32.8 (18-61)	87.60%	-	-	-
Non-population representative collectives							
Turkey ^[20]	1:100	906	38.6 (20-59)	50%	-	-	-
Switzerland ^[21]	1:132	1450	NA (12-18)	39.90%	-	-	-
Argentina ^[22]	1:167	2000	29 (16-79)	50%	-	-	-
Tunisia ^[23]	1:709	1418	27.5 (17-57)	73%	-	-	-
England ^[24]	1:83	7550	59 (45-76)	41%	-	-	-

tTGA: Tissue transglutaminase antibody; EMA: Endomysial antibody; AGA: Antigliadin antibody; NA: Not available; -: Positive.

sidered borderline, while titers > 16 U/L were considered positive. EMA was measured by means of an indirect immunofluorescence technique using a monkey esophagus immunofluorescence kit (The Binding Site, Ltd., Birmingham, United Kingdom). Positive samples exhibit an apple green fluorescence. IgG antibodies to tTGA were determined using an indirect, non-competitive enzyme immunoassay (Phadia GmbH, Freiburg, Germany) in which human recombinant tTGA antigen served as the solid phase. Titers of 7-10 U/mL were considered borderline, while titers > 10 U/L were considered positive.

All subjects testing positive for tTGA IgA underwent a confirmation test together with serological testing for antibodies to EMA and AGA. Subjects with low concentrations of total IgA were tested for tTGA IgG. Subjects with suspected celiac disease also underwent human leukocyte antigen (HLA) typing. Additional specific antibody testing was performed in order to assess the presence of other immunological disorders often associated with celiac disease (islet-cell antibody, anti-GAD, thyroid peroxidase antibody, auto-antibodies to adrenal cortex, parietal cell antibody).

Every effort was made to refer subjects with suspected celiac disease for esophagogastroduodenoscopy in order to obtain tissue samples from the duodenum.

The endoscopies and biopsies were performed by gastroenterologists in private practice. Between one and seven biopsy samples were obtained from each subject. The samples were examined histologically and classified according to the modified Marsh criteria^[35].

HLA loci were detected using the reverse sequence specific oligonucleotide (SSO) dot-blot method. The HLA-A, B and C loci were typed using the reverse SSO line-blot assay. Allele assignment was performed using the Helmberg score software with reference to the current nomenclature report and current literature^[36-38]. Ambiguities were resolved following sequencing of amplicons obtained after SSP with appropriate primers.

First follow-up screening

At the first follow-up assessment, conducted in December 2005, all antibody-positive subjects and subjects with histories suspicious for celiac disease underwent comprehensive re-examination. A total of 20 subjects were invited, of whom 14 (70%) participated (Figure 1). Subjects completed a diet and digestive symptoms questionnaire, antibody levels were rechecked and laboratory parameters routinely assessed in the work-up of celiac disease (blood count, coagulation parameters, iron, ferritin, hepatic enzymes, parathyroid hormone, calcium, magnesium, phos-

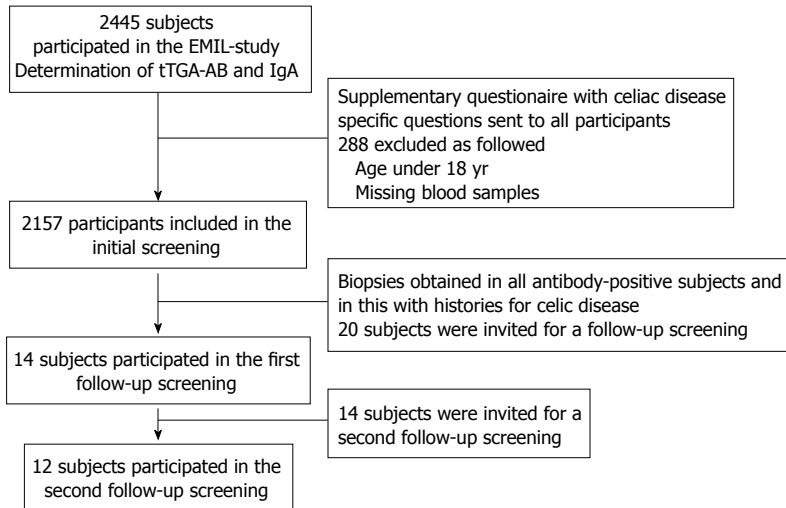


Figure 1 Flow of subjects across the study. tTGA: Tissue transglutaminase antibody; EMIL: Echinococcus Multilocularis and other Internal Diseases in Leutkirch; IgA: Immunoglobulin A.

phate) were obtained.

Second follow-up screening

As a second follow-up screening, a telephone survey was conducted in March 2008. This survey focused on dietary habits, diet compliance and improvement in symptoms as a result of a gluten-free diet. In this follow-up all celiac positives subjects were included. Also included were the questionable celiac positives. Overall, 12 of 14 (86%) subjects participating in the 2005 follow up were contacted by telephone and re interviewed (Figure 1).

Statistical analysis

Statistical calculations were performed with the assistance of the Faculty of Medical Documentation and Informatics of the University of Ulm using the SAS statistical software package (version 9.2; SAS Institute Inc., Cary, NC, United States). Data were analyzed descriptively with regard to absolute and relative frequencies, means and standard deviation. Sensitivity and specificity were calculated for all three antibody test methods. Because of the small number of cases, no statistical tests could be applied.

RESULTS

The study collective available for assessing the prevalence of celiac disease consisted of 2157 subjects (48.0% men, 52.0% women), corresponding to 88% of the total study population. Subjects' age ranged from 18 to 65 years. Their mean age was 42.6 years (standard deviation 12.9 years).

Initial screening

Fifty-five subjects exhibited a reduced total IgA concentration. However, elevated titers for tTGA IgG were not detected in any of these subjects. A total of 14 subjects (0.65%) were positive for tTGA IgA. Histological findings were available for 11 of these subjects. Celiac disease

was confirmed histologically in five cases (Figure 2).

Six subjects were seronegative but reported histories suspicious for celiac disease (Table 2). Histological findings were available for five of these subjects and confirmed the diagnosis of celiac disease in two cases. In two other subjects, the first diagnosis of celiac disease had already been made at a much earlier date and their histological findings were no longer available. Of these, one subject was HLA positive for DQA1 0101, DQB 0501 and was considered positive for celiac disease in our statistical analysis. The second subject was HLA negative for celiac disease and was considered negative for celiac disease in our statistical analysis (Figure 2). Thus, celiac disease was present in eight subjects, confirmed by biopsy in seven cases and by HLA typing in one case.

The sensitivity for tTGA IgA antibodies was 62.5%, with a specificity of 50.0%. EMA was associated with a sensitivity and specificity of 62.5% and 71.4%, respectively; and AGA with sensitivity and specificity of 62.5% and 71.4%, respectively. Confirmed false-negative findings were returned for tTGA IgA antibody in three cases, while false positive findings were documented in four cases. Both EMA and AGA were false negative in two cases and false positive in one case (Table 3).

First follow-up screening

Fourteen of 20 invited subjects participated in the first follow-up screening, corresponding to a participation rate of 70%. tTGA IgA antibodies were detected in three subjects. AGA titers were definitely positive in two subjects. Two subjects were again positive for EMA. Of the six subjects who were initially seronegative but with histories suggestive of celiac disease, three took part in the first follow-up screening: of these, one exhibited positive AGA findings.

Second follow-up screening

The participation rate at the second follow-up screening stood at 86%, 12 of 14 subjects being reached by tele-

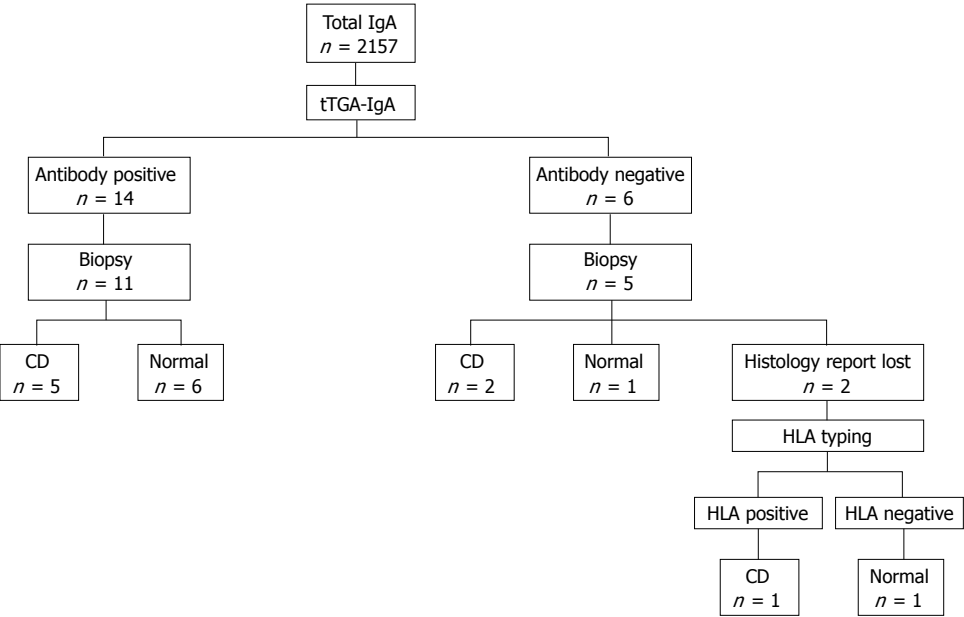


Figure 2 Subjects positive for celiac disease (based on positive antibody tests or by prior history). Total IgA: Total immunoglobulin A; tTGA IgA: Tissue transglutaminase immunoglobulin A; AB positive: Antibody positive; AB negative: Antibody negative; n: Number of subjects; CD: Celiac disease; HLA typing: Human leukocyte antigen typing; HLA: Human leukocyte antigen; HLA positive: Positive for Human leukocyte antigen; HLA negative: Negative for Human leukocyte antigen.

Table 2 Clinical presentation in subjects with seronegative findings but with histories suggestive of celiac disease

Age (yr), sex	Clinical presentation
41 yr, female	Diagnosed in 2000, histology unavailable as report destroyed Diarrhea, weight loss of 43 kg prior to diagnosis, adynamia, flatulence, psychiatric symptoms, paresthesias in the fingers In last six months, new onset of epilepsy with petit-mal seizures
18 yr, female	Diagnosed in 2000, histology unavailable as report destroyed; control biopsies in 1990 and 2005 both without evidence of several signs typical for celiac disease
62 yr, female	Sicca syndrome, lactose intolerance, diarrhea and weight loss Diarrhea, weight loss, fatigue, bone pain, alopecia, osteoporosis, cheilosis. Bronchiectasis known for years
60 yr, female	Diagnosed in 1989, no other data available
40 yr, female	Diagnosed in 1989 Sporadic constipation, meteorism, no diarrhea
56 yr, male	Weight loss, abdominal pain, depressive phases, muscle cramps, eczema on the legs and perianal, meteorism, diarrhea after drinking wheat beer

phone and amenable to participation in the standardized interview. Five of eight subjects in our collective had developed classic celiac disease. All subjects maintained a gluten-free diet and reported significant improvement in their intestinal symptoms (Tables 3 and 4). Two further subjects developed atypical celiac disease, which is characterized by mild, atypical symptoms. One subject was monosymptomatic, exhibiting only psoriasis. Subjects were antibody positive and histology revealed changes consistent with Marsh 0-IIIc disease. Despite a strict gluten-free diet, the subject did not report any improvement in his psoriasis (Tables 3 and 4).

The second, female subject was completely asymptomatic. Lactose intolerance was suspected from the subject's history and she avoided all corresponding foods. The subject was antibody positive and histology was consistent with Marsh IIIc disease. Because she continued to be asymptomatic, the subject refused to maintain a gluten-free diet (Tables 3 and 4).

Transient celiac disease was diagnosed in one of the eight subjects. In this disease form, a gluten-free diet leads to complete remission. In this subject, celiac disease had been first diagnosed when she was nine months of age. The subject maintained a strict gluten-free diet until her sixth year; subsequent gastroscopic monitoring failed to return evidence of celiac disease. Since that time, the subject has returned to a diet containing gluten. The subject was negative for DQ2 and DQ8 at HLA typing, but returned positive findings for DQA1*0101 and DQB1*0501. This HLA pattern is observed in rare cases in patients with celiac disease. Lactose intolerance was diagnosed in 2004. Gastroscopy performed under the impetus of the present study revealed no evidence of celiac disease. Negative antibody titers during gluten exposure and biopsy findings consistently negative for celiac disease both initially and during the patient's subsequent course correspond to the subtype of transient celiac disease. Based on these HLA findings and improvement in

Table 3 Change in celiac-specific antibody titers over time

No.	Age (yr), sex	Initial examination			First follow up		
		tTG-IgA (U/mL)	AGA (U/mL)	EMA	tTG-IgA (U/mL)	AGA (U/mL)	EMA
Classic celiac disease							
2	58, F	5.5	19.4	Weak Pos	Neg	Neg	Neg
4	20, F	> 100	27.6	Pos	Neg	Neg	Neg
5	52, F	44.9	24.7	Pos	Neg	Neg	Neg
17	56, M	Neg	78.1	Pos	Neg	Neg	Neg
18	62, F	Neg	Neg	Neg	7	> 100	Neg
Atypical celiac disease							
1	40, M	> 100	69.6	Pos	29.9	38.1	Pos
3	42, F	41.9	56.2	Pos	27	20.1	Pos
Transient celiac disease							
16	18, F	Neg	Neg	Neg	Neg	Neg	Neg

No.: Subject number; F: Female; M: Male; tTG-IgA: Tissue transglutaminase immunoglobulin A; AGA: Antigliadin antibody immunoglobulin A; EMA: Endomysial antibody; Pos: Positive; Neg: Negative.

symptoms subsequent to starting a gluten-free diet, this patient was considered positive for celiac disease despite lack of initial histological findings.

No other autoimmune disorders were found in our collective of patients with celiac disease. Abnormal laboratory findings suggestive of celiac disease were only moderately pronounced. None of the subjects exhibited iron deficiency anemia. Parathyroid hormone levels were elevated in two subjects with celiac disease.

DISCUSSION

To date, very few studies have prospectively investigated the prevalence of celiac disease in representative, population-based samples (Table 1). In fact, no corresponding data from a population-based study of adults are available for Germany.

The EMIL study was designed as a cross-sectional investigation in order to obtain a collective that most closely represented the general population. By contrast, a large number of recent studies^[11-19] have drawn their collectives from blood donors, who are not necessarily representative of a population. A few other studies have investigated non-population representative collectives^[20-24].

Our findings correspond to a prevalence of 1:270 (0.37%) for adults in an urban population in Germany. Other population-based studies have reported prevalences between 0.18% and 2.4%^[1-10], while, for blood donor collectives, prevalences of 0.15%-2.7%^[11-19] have been reported. Henker *et al*^[39] report a prevalence of 0.19% for asymptomatic celiac disease in children and blood donors. In another study, the same authors report a prevalence of 0.044% in children^[40]. In a study of the incidence of celiac disease in Berlin, Sandforth *et al*^[41] report an incidence rate of 0.05%. Because of the nature of the collectives, however, these data cannot be compared with our findings. In fact, only one study (Monika Project) retrospectively investigated a representative population-based sample in Germany^[10]. The prevalence of 0.3% reported

in that study is comparable to our findings.

Mustalahti *et al*^[10] identified large variability in the prevalence of celiac disease between European nations (Finland, 2.4%; Germany, 0.3%; Italy, 0.7%). Although the precise cause of this difference remains unclear, genetic and environmental factors have been discussed. The study by Mustalahti *et al*^[10] must be assessed critically due to its retrospective nature and the quality of the blood samples.

The antibody test methods utilized in the present study to assess for celiac disease have been shown in a comparison of 34 studies to possess both high sensitivity and quite good specificity (tTG-IgA: 93% *vs* 98%, EMA 93% *vs* 99%)^[42]. The test method for AGA was associated with a lower sensitivity and specificity (80% *vs* 80%-90%)^[43]. In contrast to these results, Dickey *et al*^[44] and Rostami *et al*^[45] report a lower sensitivity for AGA and EMA. The results of these tests depend on the severity of mucosal damage. If the damage is slight, the test results may be negative^[45]. As a consequence, the prevalence of celiac disease is not only underestimated but treatment of affected individuals is delayed, which may be associated with an increased risk of malignancy^[46]. Compared with data published by Lewis *et al*^[42], the present study found a lower sensitivity (62.50%) and specificity (50%) for tTGA IgA antibody. In the present study, EMA and AGA showed comparably a high sensitivity (62.5%) and specificity (71.4%).

The findings of the present study suggest that the use of tTGA IgA antibody as a suitable method for screening a population for celiac disease should be reconsidered^[42,47]. It was only by means of our follow-up examinations that we were able to identify subjects with celiac disease with false-negative antibody titers. Otherwise, the prevalence of celiac disease in our collective would have been too low. With the 50% response rate to our celiac disease questionnaire, it cannot be excluded that there may be other undetected false-negative antibody results. A definite conclusion regarding the reliability of this antibody test method is difficult: on the one hand, the number of patients in the different collectives is very small; also, there have been only very few studies to date in which all antibody-positive patients have been biopsied^[2,8,23].

Quantitative video capsule endoscopy has been described in the literature as a new method in diagnosing celiac disease^[48,49]. The findings of these studies show that quantitative image analysis corresponds to the degree of villous atrophy. These studies, however, show some limitations; hence, the value of this new method must be investigated in further studies.

A limiting factor in the present study certainly relates to the study design itself. The EMIL study was not originally conceived to determine the prevalence of celiac disease. As a result, all study participants had to be sent a questionnaire following completion of the initial EMIL study, the response rate to which stood at only 50%. A further disadvantage is the inclusion in our collective of patients who had already been diagnosed with celiac

Table 4 Clinical presentation, histology and human leukocyte antigen findings in subjects with celiac disease

No.	Age (yr), sex	Histology	HLA DQ2	Clinical presentation
Classical celiac disease				
2	58, F	IIIc	Pos	Diarrhea, abdominal cramps, angular cheilitis, osteoporosis, depression, brittle nails, hypothyroidism
4	20, F	IIIa-IIIc	Pos	Diarrhea, weight variability, circulatory collapse, cheilosis, depression, anxiety, insomnia, brittle nails, restless-leg syndrome
5	52, F	IIIb	Pos	Fatigue, 1 intestinal cramping, nausea, vomiting, weight loss, paresthesias in the hands, joint, bone and abdominal pains, anxiety, depression, Sicca syndrome, muscle cramps
17	56, M	IIIc	Pos	Weight loss, abdominal cramping, depressive phases, muscle cramps, anal eczema, meteorism, diarrhea following consumption of wheat beer
18	62, F	IIIc	Pos	Diarrhea, weight loss, fatigue, bone pain, alopecia, osteoporosis, cheilosis, bronchiectasia
Atypical celiac disease				
1	40, M	0, IIIc	Pos	Psoriatic lesion on the head
3	42, F	IIIc	Pos	No symptoms, suspected lactose intolerance
Transient celiac disease				
16	18, F	NoHisto, ED 1985	Neg	Diarrhea, weight loss, Sicca syndrome, lactose intolerance since 2004

HLA: Human leukocyte antigen; No.: Subject number; Age: In years; NoHisto: Histological findings unavailable; F: Female; M: Male; Histology: Histological findings classified according to Marsh stage; NA: Not available; Pos: Positive; Neg: Negative.

disease. Also problematic is the impact on the standardization of examination conditions of the retroactive refocusing of the study on determining the prevalence of celiac disease. Patients were referred for endoscopy to several gastroenterologists in private practice in Leutkirch. Biopsies returned between one and seven tissue samples. The histological assessment of the biopsy material was performed by pathologists in different centers.

In conclusion, the findings of the present study show a prevalence of 0.37% for celiac disease, which is comparable to that reported in other European studies. The use of a combination of several antibody test methods for screening examinations appears useful.

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COMMENTS

Background

Celiac disease may present with typical clinical signs or patients may be oligo-symptomatic or completely asymptomatic. It is associated with specific, often serious complications. As all forms of celiac disease yield the same risk for complications and especially T cell lymphoma, it is paramount to detect not only the classical form but also patients with atypical disease manifestation.

Research frontiers

Prevalence rates for celiac disease vary for different populations. This may also be due to the scarcity of data from representative samples. Serological antibody

tests constitute the most important screening tools. In the literature the sensitivity and specificity of transglutaminase immunoglobulin-A, endomysium antibody and antigliadin antibody tests differ.

Innovations and breakthroughs

This is the first study to prospectively assess the prevalence of celiac disease in a representative population sample of adults in Germany. In comparison to results of the small bowel biopsy the sensitivity and specificity of serological antibody tests for celiac disease were lower than previously described in the literature.

Applications

Their results add to an accurate estimate of the prevalence of celiac disease in the general population. The use of a single antibody test for screening purposes must be called into question.

Peer review

Data over recent years have generally noted increased recognition and diagnosis of celiac disease (CD), with rates approaching 2% in some settings. Rates however, appear to vary between geographical regions. This study based in one German city examined several serological tests and gastroenterology symptoms in a large population of adults. The determined prevalence of less than 0.5% is consistent with some previous data, but substantially less than rates in other countries. Interestingly, only 2 of these 2157 adults had previously been thought to have CD, emphasising that CD is often not recognised in routine clinical practice.

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CYP24A1 inhibition facilitates the anti-tumor effect of vitamin D3 on colorectal cancer cells

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Abstract

AIM: The effects of vitamin D3 have been investigated on various tumors, including colorectal cancer (CRC). 25-hydroxyvitamin-D3-24-hydroxylase (CYP24A1), the enzyme that inactivates the active vitamin D3 metabolite 1,25-dihydroxyvitamin D3 (1,25-D3), is considered to be the main enzyme determining the biological half-life of 1,25-D3. During colorectal carcinogenesis, the expression and concentration of CYP24A1 increases significantly, suggesting that this phenomenon could be responsible for the proposed efficacy of 1,25-D3 in the treatment of CRC. The aim of this study was to investigate the anti-tumor effects of vitamin D3 on the human

CRC cell line Caco-2 after inhibition of the cytochrome P450 component of CYP24A1 activity.

METHODS: We examined the expression of CYP24A1 mRNA and the effects of 1,25-D3 on the cell line Caco-2 after inhibition of CYP24A1. Cell viability and proliferation were determined by means of sulforhodamine-B staining and bromodeoxyuridine incorporation, respectively, while cytotoxicity was estimated via the lactate dehydrogenase content of the cell culture supernatant. CYP24A1 expression was measured by real-time reverse transcription polymerase chain reaction. A number of tetralone compounds were synthesized to investigate their CYP24A1 inhibitory activity.

RESULTS: In response to 1,25-D3, CYP24A1 mRNA expression was enhanced significantly, in a time- and dose-dependent manner. Caco-2 cell viability and proliferation were not influenced by the administration of 1,25-D3 alone, but were markedly reduced by co-administration of 1,25-D3 and KD-35, a CYP24A1-inhibiting tetralone. Our data suggest that the mechanism of action of co-administered KD-35 and 1,25-D3 does not involve a direct cytotoxic effect, but rather the inhibition of cell proliferation.

CONCLUSION: These findings demonstrate that the selective inhibition of CYP24A1 by compounds such as KD-35 may be a new approach for enhancement of the anti-tumor effect of 1,25-D3 on CRC.

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Key words: Colorectal cancer; CYP24A1 inhibition; Vitamin D3; Tetralone derivatives; Caco-2 cell culture

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INTRODUCTION

Epidemiologic studies have suggested that maintenance of an adequate level of vitamin D may reduce the incidence and development of several types of tumors, including breast, prostate and colorectal cancers (CRC)^[1-4]. The role of vitamin D deficiency in the development of CRC, and the potential use of vitamin D in the treatment of CRC have been the focus of a number of studies, as CRC is one of the most common cancers^[5].

There is a vast array of evidence suggesting a protective effect of vitamin D against CRC^[6-8]. There is an inverse association between the serum level of 25-hydroxy vitamin D3 (25-D3) and the risk of CRC^[1,9]. In ulcerative colitis, low expression of the vitamin D receptor (VDR) is associated with an elevated risk of the development of CRC^[10]. An inadequate dietary intake of vitamin D and a vitamin D deficiency promote the development and growth of CRC in mice^[3,11]. In elderly women, higher plasma levels of 25-D3 are accompanied by a lower risk of CRC^[12]. Further studies have shown that vitamin D may have a preventive role not only in CRC, but also in other cancers of the alimentary tract^[13]. Nevertheless, the exact cellular pathway for the putative anti-tumor effect of vitamin D remains unclear. The action of 1,25-dihydroxyvitamin-D3 (1,25-D3) through the nuclear VDRs is delayed, but the immediate responses triggered from the cell by cytosolic VDRs acting through Ca²⁺ influx might also play an important role in this process^[14]. However, the application of 1,25-D3 in tumor treatment is restricted due to its tendency to cause hypercalcemia^[15].

The anti-tumor efficacy of vitamin D in tumor cell cultures is somewhat contradictory^[16-19]. Some cancer cell lines are more susceptible to vitamin D treatment than others^[19,20], and the vitamin D-sensitive cell cultures have been shown to resemble early-stage tumors^[20]. During the progression of the cancer, this susceptibility is gradually lost, but the underlying pathophysiological process of this loss is not clear. Though numerous clinical studies have been conducted with vitamin D or its analogs, the anti-tumor results were largely disappointing^[21]. The current evidence suggests that a relationship does exist between vitamin D and cancer, but the strength of this relationship appears to weaken on progression from the preclinical to the clinical situation^[22]. Thus, further examinations are needed to identify factors influencing the anti-tumor effect of vitamin D on tumor cells.

The mitochondrial enzyme cytochrome P450 component of 25-hydroxyvitamin-D3-24-hydroxylase (CYP24A1), which is the major 1,25-D3-inactivating enzyme, is considered to be an essential factor determining the biological half-life of 1,25-D3. Previous immunohisto-

chemical studies have shown that the level of CYP24A1 rises significantly as the course of colorectal carcinogenesis progresses^[20,23]. This fact might explain why 1,25-D3 cannot exert its anti-tumor effect in many pathological situations. It has also been demonstrated that the higher the level of CYP24A1, the more malignant the CRC^[24]. A concomitantly increased expression of the proliferation marker Ki-67 in human CRC samples suggests that the overexpression of CYP24A1 reduces the local availability of 1,25-D3, and hence its antiproliferative effect^[24]. Other mechanisms to may be involved in the development of 1,25-D3 insensitivity such as the downregulation of the VDRs^[25].

In the present study, we set out to investigate the effects of 1,25-D3 on CRC cells after the inhibition of CYP24A1 activity.

MATERIALS AND METHODS

CYP24A1 inhibitors

The ability of tetralones to inhibit CYP24A1 is less than that of theirazole counterparts, but a greater degree of selectivity can be achieved with tetralones through the mechanism of their binding to the active site. Instead of binding to the heme iron, they interact with the active site if the enzyme through hydrogen bonds and van der Waals forces^[26]. Thirteen new 2-substituted-benzyl-6-methoxy-1-tetralones synthesized in the Department of Organic Chemistry in Szeged were utilized in the present study.

The method employed for the preparation of the tetralones^[27] involved the condensation of commercially available 6-methoxy-1-tetralone with benzaldehyde or a substituted benzaldehyde (Figure 1). 6-methoxy-1-tetralone (1) was dissolved in 4% ethanolic KOH solution, the appropriate benzaldehyde (2a-m) was added, and the reaction mixture was stirred at room temperature for 1-8 h until the starting material had disappeared (thin layer chromatography monitoring), and then allowed to stand overnight. The precipitate that formed was filtered off, washed with water, purified by flash chromatography on silica gel, and recrystallized from ethanol. The synthesis of the hydroxy derivative necessitated initial protection of the hydroxy group in the 4-hydroxybenzaldehyde with a tetrahydropyranyl group, which was stable under the basic ethanolic KOH condensation conditions. The protecting group was removed by heating with aqueous hydrochloric acid in a mixture of ethyl acetate and ethyl methyl ketone. In the next step, the 2-substituted-benzylidene-6-methoxy-1-tetralones (3a-m) were dissolved in ethyl acetate, and hydrogenated at 1 atm in the presence of Pd/C as catalyst for 1 h at room temperature. The catalyst was subsequently removed by filtration through a bed of silica gel, the solvent was evaporated *in vacuo*, and purification by flash chromatography on silica gel furnished the 2-substituted-benzyl-6-methoxy-1-tetralones (4a-m).

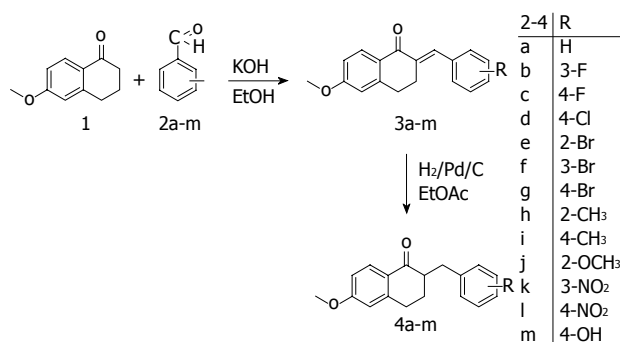


Figure 1 Outline of the procedure for the synthesis of the tetralones.

The resulting tetralones were dissolved individually in dimethyl sulfoxide at a concentration of 10 mmol/L and stored at 4 °C until use. In cell culture experiments, compounds (4a-m) were dissolved in sterile culture medium (GIBCO's OPTI-MEM, Life Technologies-Invitrogen, Carlsbad, CA, United States) to the desired concentration. 1,25-D3 at 1 and 10 nmol/L and an untreated control were also applied in these experiments.

Cell culturing

The human epithelial colorectal adenocarcinoma cell line Caco-2 obtained from ECACC was maintained in Dulbecco's Modified Eagle Medium (D-MEM, Sigma, St. Louis, MO, United States) supplemented with 10% fetal calf serum (FCS, Sigma) and 1% antibiotic, antimycotic solution (Sigma) at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were cultured in 6-, 24- and 96-well plates, and all measurements were carried out in triplicate. The cell line was genotyped and identified as Caco-2 in 2011 on the basis of the results of STR analysis (DSMZ Profile Database, www.dsmz.de). Twenty-four hours before treatment, the medium was changed to GIBCO's OPTI-MEM (Life Technologies-Invitrogen, Carlsbad, CA, United States). All experiments were carried out with cells from passages 5-25.

Cell viability assay

The protein dye sulforhodamine-B (SRB), was used to test various tetralone derivatives in various concentrations for various incubation times in 96-well plates to determine the effects of the compounds alone and in the presence of 1,25-D3 on the Caco-2 cell number. After removal of the culture medium, 100 µL of trichloroacetic acid was used to fix the cells during an incubation period of 30 min. The plates were then rinsed 5 times with distilled water. The cells were stained with a 0.4% solution of SRB (Sigma) in acetic acid for 30 min. After removal of the excess dye solution the plates were rinsed 4 times with 1% acetic acid solution and allowed to dry at room temperature. The bound SRB was dissolved in unbuffered Tris-HCl and the plates were shaken for 5 min. The plates were measured in an Infinite M200 reader (Tecan AG, Männedorf, Switzerland) at 520 nm.

Cytotoxicity measurement

Levels of cytotoxicity were quantified after treatment through measurement of the lactate dehydrogenase (LDH) levels in the wells by using the Cytotoxicity Detection Kit^{PLUS} (Roche, Indianapolis, IN, United States). The greater the number of cells that die due to the cytotoxic effect, the higher the amount of LDH in the medium. The experiments were carried out in accordance with the kit manufacturer's instructions.

Cell proliferation assays

Cell proliferation was quantified by measurement of the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into the cellular DNA by means of Cell Proliferation enzyme-linked immunosorbent assay, BrdU (colorimetric) (Roche). The experiments were carried out in accordance with the manufacturer's instructions.

RNA isolation and Taqman probe-based real-time RT-PCR

RNA was isolated through use of the High Pure RNA Isolation Kit (Roche) as prescribed in the manufacturer's instructions. The isolated RNA was translated by using Moloney murine leukemia virus reverse transcriptase in accordance with the manufacturer's instructions (Promega, Madison, WI, United States). Predesigned and validated gene-specific TaqMan Gene Expression Assays from Life Technologies (Life Technologies, Foster City, CA, United States) were used in triplicate for quantitative real-time polymerase chain reaction (PCR) according to the manufacturer's protocol. Each set contained gene-specific forward and reverse primers and fluorescence-labeled probes. The probes span an exon junction and do not detect genomic DNA [ABI Taqman assay No's are hs00167999_m1 and hs99999905_m1, for CYP24A1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively]. The PCR assays were carried out with the following protocol: denaturation for 10 min at 95 °C, and 45 cycles of denaturation for 15 s at 95 °C, annealing and extension for 1 min at 60 °C. The PCR reaction volume of 20 µL contained 2 µL cDNA, 10 µL of TaqMan 2x Universal PCR Master Mix NoAmpErase UNG (Life Technologies), 1 µL of gene-specific TaqMan Gene Expression Assay Mix and 7 µL of water. GAPDH was used as a housekeeping gene to normalize for RNA loading. Samples were analyzed using the ABI Prism 7500 real-time PCR system (Life Technologies). Relative quantification (RQ) studies were carried out on collected data (threshold cycle numbers, referred to as Ct) with the 7500 System SDS software 1.3 (Life Technologies).

Statistical analysis

Data were analyzed by using SPSS for Windows, release 18 (IBM, Armonk, NY, United States). Final data are presented as the means ± SD of at least three independent measurements. Statistical analysis was performed with the unpaired Student *t*-test; results with *P* ≤ 0.05 were con-

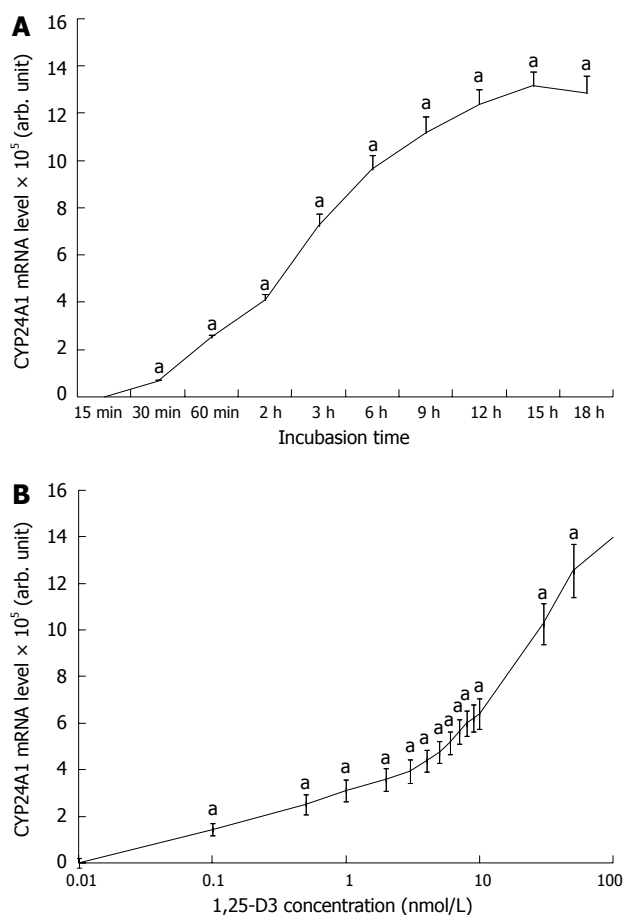


Figure 2 Time and dose dependent-changes in CYP24A1 mRNA expression in response to 1,25-D3 administration. A: Time course of changes in the cytochrome P450 component of the 25-hydroxyvitamin D3-24-hydroxylase (CYP24A1) mRNA expression in Caco-2 cells after the addition of 100 nmol/L active vitamin D3 metabolite 1,25-dihydroxyvitamin D3 (1,25-D3) to the cell culture supernatant. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-normalized CYP24A1 expression levels are shown as a percentage of the CYP24A1 level of the untreated control cells. Points indicate means ± standard deviation (SD) (^a*P* < 0.05 vs untreated control); B: Dose-dependent changes in CYP24A1 mRNA levels in Caco-2 cells after the addition of different amounts of 1,25-D3. GAPDH-normalized CYP24A1 expression levels are shown as a percentage of the CYP24A1 level of the untreated control cells. Points indicate means ± SD (^a*P* < 0.05 vs untreated control).

sidered statistically significant.

RESULTS

Time and concentration-dependent changes in CYP24A1 mRNA expression after vitamin D3 treatment

An increase in CYP24A1 mRNA level of six orders of magnitude was observed after a brief period of 1,25-D3 treatment. The increase in CYP24A1 mRNA expression was very rapid and it could be observed after 30 min of 1,25-D3 administration, and reached a maximum after 12-16 h of incubation (Figure 2A). After 4 h of incubation in the presence of 1 and 10 nmol/L 1,25-D3, the level of CYP24 mRNA was elevated to 311405-fold and 612801-fold, respectively, relative to the untreated controls (Figure 2B).

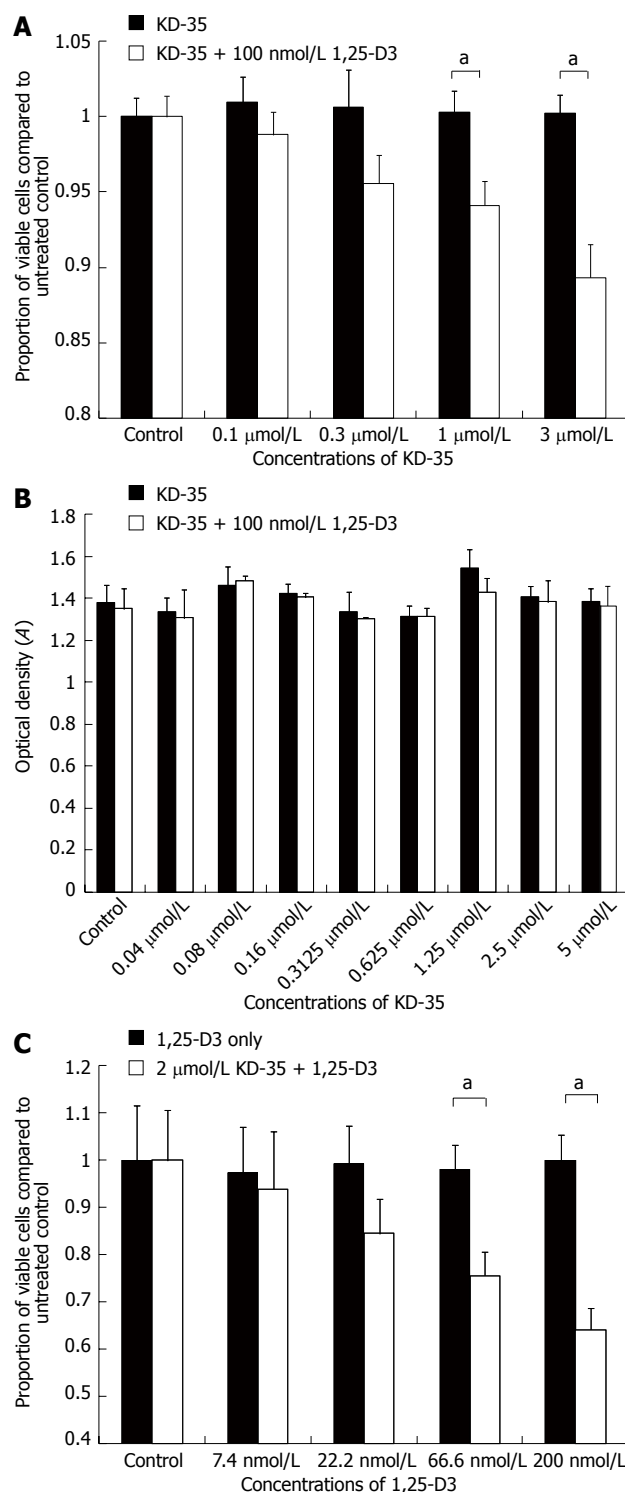


Figure 3 Cell proliferation, lactate dehydrogenase activity and proliferation studies in the presence of KD-35 and 1,25-D3. A: Changes in the number of viable Caco-2 cells (sulforhodamine-B staining) in the presence of different concentrations of KD-35. Selected wells were treated with 100 nmol/L active 1,25-D3. Data are means ± SD (^a*P* < 0.05 between KD-35 and KD-35 + 1,25-D3 treated cells); B: Changes in the lactate dehydrogenase (LDH) activity of the cell culture supernatant in response to KD-35 with or without 1,25-D3. Data are means ± SD. No significant changes in LDH activity were seen after treatment; C: Changes in the proliferation of Caco-2 cells (5-bromo-2'-deoxyuridine incorporation) in response to different concentrations of 1,25-D3. White bars indicate combined treatment with the given 1,25-D3 concentration + 2 μmol/L KD-35. Data are means ± SD. Significance levels were calculated between each sample and the untreated control sample (^a*P* < 0.05 between 1,25-D3 and 1,25-D3 + KD-35 treated cells).

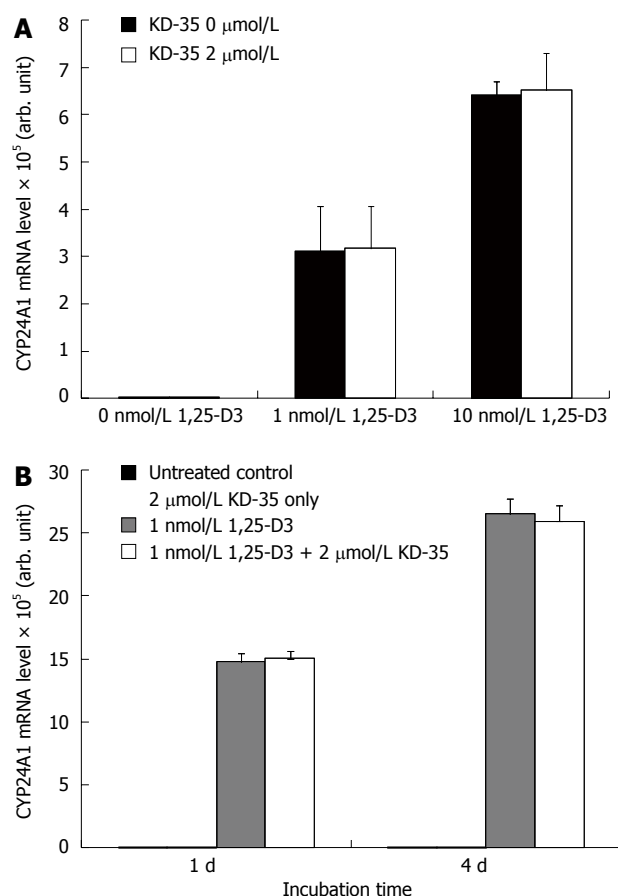


Figure 4 KD-35 has no effect on CYP24A1 mRNA expression. A: Changes in CYP24A1 mRNA levels in Caco-2 cells incubated with different concentrations of 1,25-D3 for 4 h with or without KD-35. Data are means \pm SD. No significant changes in mRNA levels were seen with or without KD-35; B: Effects of KD-35 and KD-35 + 1,25-D3 on CYP24A1 mRNA expression in Caco-2 cells. Data are means \pm SD. No significant change in mRNA levels was seen with or without KD-35 at any time point.

Effects of tetralone derivatives on Caco-2 cell line

Certain of the tetralones were found to decrease the Caco-2 cell viability but only after 2-4 d of incubation with 1,25-D3. These compounds were tested at various concentrations for various periods to optimize the effect of 1,25-D3 in reducing the total Caco-2 cell count. Finally, compound KD-35 was selected for further and detailed investigations.

Effects of KD-35 on Caco-2 cell line

When Caco-2 cells were incubated for 4 d in the presence of 100 nmol/L 1,25-D3 with 0.1, 0.3, 1 or 3 μ mol/L KD-35, the cell number was reduced by 2.17%, 5.07%, 6.18% and 10.93%, respectively, relative to the controls treated with only 100 nmol/L 1,25-D3 or 3 μ mol/L KD-35 (Figure 3).

Results of the cytotoxicity test

To determine the cause of the decrease in viable cell number in the presence of KD-35 and 1,25-D3, we measured LDH concentration in the cell suspension. The concentration of KD-35 ranged between 0.04 μ mol/L

and 5 μ mol/L. Half of the wells were treated with KD-35 and 100 nmol/L 1,25-D3, the other half were treated with KD-35 only. All experiments were carried out in triplicate. Incubation lasted for 4 d. In all of the experimental setups, the LDH concentrations did not differ significantly in the presence of KD-35 alone or in combination with 1,25-D3 (Figure 3).

Results of the cell proliferation assay

In the presence of 2 μ mol/L KD-35, the following concentrations of 1,25-D3 were used: 7.4, 22.2, 66.6 and 200 nmol/L. Half of the wells were treated only with 2 μ mol/L KD-35. Incubation lasted for 4 d. After incubation, the 5-BrdU label was added for an additional 2 h. The reduction in cell number relative to the control was 3.43%, 14.81%, 22.49% and 35.81%, respectively, compared to the wells with 1,25-D3 only (Figure 3).

Changes in CYP24A1 mRNA expression

The amount of CYP24A1 mRNA expressed in the presence of various concentrations of KD-35 did not differ from that of the untreated controls. The CYP24A1 mRNA expression did not depend significantly on the duration of incubation with KD-35 (Figure 4).

DISCUSSION

We have identified a new tetralone compound, KD-35, that effectively and markedly stimulates the anti-proliferative effect of 1,25-D3 in the CRC cell line Caco-2.

CYP24A1, a member of the cytochrome P450 (CYP450) enzyme superfamily is the key enzyme in the metabolism of vitamin D neutralizing the active metabolite 1,25-D3, and thereby controlling its concentration in the tissues. The CYP450 enzymes all display an iron-containing heme domain at the active site. There are two types of enzyme blockers: azoles and non-azoles^[28]. The *N*-heterocyclic ring of azoles is linked directly to the iron in the heme domain and, although this inhibition is very potent, it is not selective. Since the other enzymes involved in vitamin D metabolism (CYP27A1 and CYP27B1) are also members of the CYP450 superfamily, this type of nonselective inhibition is not specific for CYP24A1.

The enzyme inhibitory effect of non-azoles is mediated through hydrogen bonds and hydrophobic interactions with the active site of the enzyme. This is a more flexible mechanism which may permit significant selectivity though the inhibitory effect may be less than that of azoles^[26]. We investigated 13 tetralones (non-azoles) in a search for a compound that is effective locally in the colon and is not strongly absorbed, so that the risk of adverse systemic effects is minimized.

Most of the 13 tetralones were either toxic or ineffective, even in the presence of 1,25-D3. Only in the presence of KD-35 did 1,25-D3 markedly inhibit Caco-2 cell proliferation without pronounced cytotoxicity of the tetralone alone. Such inhibition was not observed in the absence of KD-35. Unfortunately, two of the three

most effective tetralones exhibited much higher cytotoxicity at higher concentrations than KD-35. The question arises as to whether KD-35 exerts its effect *via* CYP24A1 inhibition. We did not measure CYP24A1 enzyme activity directly since this is technically extremely difficult. It is also complicated to measure the intermediates of the CYP24A1 reaction. Moreover, a simple enzyme kinetic measurement would not reveal whether the compound enters the cell. We therefore chose an indirect approach: to prove the biological efficacy of the compound. KD-35 was found to exert an effect that allowed 1,25-D3 to reduce Caco-2 cell proliferation effectively, as reflected by an altered BrdU incorporation. Direct cytotoxicity was excluded by the LDH measurements, and no change in CYP24A1 mRNA expression was detected in response to KD-35, which ruled out alterations in protein synthesis. Obviously, no direct evidence was obtained to support direct enzyme inhibition, but an alternative mechanism is highly unlikely with this non-azole.

Two major pathways are mediated through the VDRs: the Wnt-beta-catenin pathway, which is responsible for the loss of adherent cell type, and the E-cadherin pathway, which is responsible for cell-to-cell adhesion and cell differentiation^[29,30]. The administration of 1,25-D3 suppresses the Wnt-beta-catenin pathway and induces the expression of E-cadherin. The Wnt-beta-catenin pathway is constitutionally overregulated in most CRCs, due to the mutation of several members of the pathway (APC, AXIN2, *etc.*)^[29]. There are other participants in colorectal carcinogenesis, such as estrogen receptors, which elevate the number of VDRs in the mucosal cells of the alimentary tract, or SNAIL, which inhibits the E-cadherin pathway and expression of VDRs^[30-35]. Another important factor in the mucosal cell transition toward adenocarcinoma is an elevated level of CYP24A1, the intracellular concentration of which correlates with the dignity of the tumor^[24].

Our results corroborate the earlier finding^[36] that the presence of 1,25-D3 dramatically stimulates the expression of CYP24A1 in CRC cells^[24,37]. Two vitamin D-responsive elements are present in the promoter region of CYP24A1^[38]. Through this pathway, 1,25-D3 stimulates its own destruction through metabolism into inactive forms by enhancing the expression of CYP24A1^[39].

Besides the genomic effects, there have also been reports of immediate nongenomic mechanisms. A possible mode of action is activation of the RhoA-ROCK-p38MAPK-MSK signaling pathway. This pathway mediates the induction of CST5, which is possibly responsible for tumor suppression and the level of CYP24A1; as a negative feedback mechanism, this eliminates 1,25-D3 from the cell^[40]. VDRs found in other tumor cell membranes may bind 1,25-D3, and the complex could induce a rapid influx of Ca²⁺ into the cell^[40], which activates RhoA-ROCK and then the p38MAPK-MSK-1 pathway. Besides the nongenomic activation of this pathway, a vitamin D-responsive element can also be identified in the -1k promoter region of the *RhoA* gene (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>).

RhoA plays an important role in the induction of CDH1/E-cadherin, which is crucial for the acquisition of the polarity and adhesive phenotype of cancer cells^[29].

In view of these data, the elevation of CYP24A1 expression might be a self-defense mechanism of tumor cells. By inhibiting the inactivating enzyme, the amount of active vitamin D or its analogs required to elicit their marked anti-tumor effect could be reduced *in vivo*, thereby preventing elevation of the serum Ca²⁺ level and avoiding hypercalcemia^[36]. The inhibition of CYP24A1 may allow 1,25-D3 to exert its anti-tumor effect, in this way leading to a new approach in the treatment of CRC in the future.

COMMENTS

Background

The effects of vitamin D3 have been investigated on various tumors, including colorectal cancer (CRC). The cytochrome P450 component of 25-hydroxyvitamin D3-24-hydroxylase (CYP24A1), the enzyme that inactivates the active vitamin D3 metabolite 1,25-dihydroxyvitamin-D3 (1,25-D3) is considered to be the main enzyme determining the biological half-life of 1,25-D3. During colorectal carcinogenesis, the expression and concentration of CYP24A1 increases significantly, suggesting that this phenomenon could be responsible for the controversial efficacy of 1,25-D3 in the treatment of CRC. In the present study, authors set out to investigate the effects of 1,25-D3 on CRC cells after the inhibition of CYP24A1 activity.

Research frontiers

The anti-tumor effect of vitamin D3 has been a focus of interest during the last 10-15 years. However, vitamin D3 cannot exert this important effect in a number of tumors. The reasons for this have been investigated intensively. One possible explanation for the reduced anti-tumor efficacy of vitamin D3 is the accelerated neutralization of the active vitamin D3 compound in certain cases, *e.g.*, CRC, liver and papillary thyroid cancers.

Innovations and breakthroughs

The authors synthesized a number of compounds potentially able to inhibit the action of CYP24A1, the enzyme neutralizing the effects of vitamin D3. One of these compounds, KD-35, had inhibitory potential without an apparent toxic effect. In the presence of KD-35, vitamin D3 markedly inhibited the growth of CRC cells.

Applications

Selective inhibition of the CYP24A1 by compounds such as KD-35 may permit a new approach to enhancement of the anti-tumor effect of 1,25-D3 on CRC.

Peer review

The authors tackled an interesting topic for investigation. The manuscript is investigating the association between CYP24A1 inhibition and anti-tumor effect of 1a, 25-dihydroxyvitamin-D3 in Caco-2 CRC line. A careful assessment was considered using appropriate cell assays. A major finding of the study was that Caco-2 cell viability and proliferation were markedly reduced in response to 1,25-D3 when the CYP24A1 was inhibited (by KD-35, one of the tetralone compounds).

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Establishment of mouse intestinal myofibroblast cell lines

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Abstract

AIM: To establish novel intestinal myofibroblast (IMF) cell lines from mouse colonic mucosa and investigate their biological characters.

METHODS: Primary IMFs were isolated from mucosal tissues of mouse colon that was denuded of epithelial cells and smooth muscle layer. For immortalization, primary IMFs were transfected with simian virus 40 large T antigen (designated as LmcMF). We also isolated some primary IMFs that spontaneously became immortalized without transfection (designated as SmcMF). To check immortality and normality of these cells, we examined their proliferative ability and contact inhibition. Moreover, the expression levels of proteins characterizing IMFs [including α -smooth muscle actin (α -SMA), vimentin, desmin, and type I collagen] and proteins associated with the immune response [such as toll-like

receptor 4 (TLR-4), CD14, MD2, $\text{I}\kappa\text{B}\alpha$, and p-p38] were determined by Western blotting. The localization of several myofibroblast protein markers was also detected by immunofluorescence staining.

RESULTS: The cell growth assay results show that both LmcMF and SmcMF cells proliferated logarithmically at least up to passage 20. In addition, the contact inhibition assays show that LmcMF and SmcMF stopped growing after the cells reached confluence. These data suggest that these 2 types of cells were immortalized without losing contact inhibition of growth. Moreover, both LmcMF and SmcMF, like primary IMFs, showed spindle-shaped appearance. The expression levels of key myofibroblast protein markers, including α -SMA, vimentin, and desmin, were also examined by the Western blotting and immunofluorescence analyses. Our results show that these cells were positive for α -SMA and vimentin, but not desmin, as well as that both LmcMF and SmcMF expressed type I collagen at a lower level than primary IMFs. Finally, we investigated the expression level of lipopolysaccharide (LPS) receptor-related proteins, as well as the response of the cells to LPS treatment. We found that the TLR4, CD14, and MD-2 proteins were present in LmcMF and SmcMF, as well as in primary IMFs, and that all these cells responded to LPS.

CONCLUSION: We established 2 novel IMF cell lines from mouse colonic mucosa, namely, LmcMF and SmcMF, both of which were able to respond to LPS.

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Key words: Cell line; Colon; Lipopolysaccharide; Mouse; Myofibroblast

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INTRODUCTION

The gastrointestinal mucosa is in contact with the extracorporeal environment and is exposed to various antigens and molecules that are mainly derived from ingested food and commensal bacteria^[1]. Such antigens do not induce inflammation in healthy individuals, because intestinal epithelial cells form a physical barrier protecting against luminal bacteria and toxic substances. To achieve this protective function, differentiation and proliferation of epithelial cells need to be properly regulated. Accumulating evidence has recently revealed that subepithelial intestinal myofibroblasts (IMFs), which are located subjacent to the epithelium, play crucial roles in regulating epithelial cells^[2-4].

IMFs belong to the myofibroblast family, which includes several functionally related cells, such as lung contractile interstitial cells, pancreatic stellate cells, and orbital and synovial fibroblasts^[5]. IMFs are spindle-shaped or stellate cells that exhibit phenotypic characteristics of both fibroblasts and smooth muscle cells^[6,7]. In addition, it has been shown that IMFs, like other mesenchymal cells, synthesize collagen^[6,8,9]. Notably, IMFs orchestrate diverse events in gastrointestinal health and diseases, including epithelial differentiation and development, mucosal repair, carcinogenesis, and inflammatory responses^[3,4,10]. IMFs express toll-like receptor 4 (TLR-4), which is activated by lipopolysaccharide (LPS), a component of gram-negative bacteria, subsequently leads to secretion of proinflammatory mediators^[10-13]. Moreover, IMFs, as nonprofessional antigen-presenting cells in the intestinal mucosa, induce proliferation of both resting CD4⁺ T cells and regulatory T cells in a major histocompatibility complex (MHC) class II-dependent manner to maintain intestinal mucosal tolerance^[14,15]. Furthermore, enterotoxins are known to engage MHC class II molecules on IMFs, which in turn leads to secretion of inflammatory mediators^[16].

Despite the crucial role of IMFs in gastrointestinal health and diseases, the understanding of their underlying regulatory mechanism is limited. One of the major issues that hinder the advance of IMF research is the lack of IMF cell lines that show myofibroblastic phenotypes without stimulation. Typically, primary IMFs isolated from intestine are used within passages 2-6, because their phenotypes change and the cells stop growing after repeated passages. In addition, CCD-18Co cells, which have been used as a human colon myofibroblast cell line, still need to be treated with transforming growth factor- β (TGF- β) for the expression of α -smooth muscle actin (α -SMA)^[17]. In this present study, to address these issues, we separated mouse intestinal myofibroblast cell lines (namely, LmcMF and SmcMF) and examined their properties.

MATERIALS AND METHODS

Culture of mouse intestinal myofibroblasts and mouse embryonic fibroblasts

C57BL/6J mice purchased from Charles River Japan

(Yokohama, Japan) were maintained in compliance with the guidelines of the Animal Care and Use Committee of Yamaguchi University. Mouse IMFs were isolated as previously described, with slight modifications^[18]. Briefly, a segment of the proximal colon was detached from the mesenterium, and mucosal layers were completely denuded of epithelial cells by repeated ($\times 3$) 30-min incubation in 1 mmol/L EDTA-Hanks' balanced salt solution at 37 °C. Subsequently, smooth muscle layers were detached from mucosal layers with tweezers. De-epithelialized mucosal samples were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Tokyo, Japan) containing 100 mL/L fetal bovine serum (FBS) at 37 °C in a 50 mL/L CO₂ atmosphere. Consequently, myofibroblast cells that migrated from mucosal tissues formed colonies. In this study, primary IMFs at passages 2-6 were used. Mouse embryonic fibroblasts (MEFs) isolated from embryos of C57BL/6J mice at embryonic day 11.5 were also grown under the same condition described above.

Antibodies and cell lines

The following antibodies were used in this study: anti- α -SMA and anti-vimentin antibodies from Sigma (Tokyo, Japan); anti-desmin and anti-tubulin antibodies from Thermo Scientific (Yokohama, Japan); anti-TLR4, anti-CD14, and anti-actin antibodies from Santa Cruz (CA, United States); anti-MD2 antibody from AbD Serotec (Kidlington, United Kingdom); anti-I κ B α and anti-p-p38 antibodies from Cell Signaling (MA, United States); anti-Collagen type I antibody from Merck (Tokyo, Japan); anti-valosin-containing protein (VCP) antibody from Gene Tex (CA, United States); horseradish peroxidase (HRP)-conjugated mouse anti-rabbit Ig Light Chain from ECM biosciences (KY, United States); and HRP-conjugated donkey anti-mouse IgG from R and D Systems (MN, United States). A mouse mammary gland epithelial cell line, Eph4, and a mouse macrophage-like cell line, RAW264.7, were grown in DMEM supplemented with 100 mL/L FBS at 37 °C in a 50 mL/L CO₂ atmosphere.

Immortalization of IMFs

EGIP-EF1a-Large T-IRES-Puro, a lentiviral plasmid expressing simian virus 40 (SV40) large T antigen (LT) but not small T antigen (ST), was obtained from Addgene (ID No. 18922; MA, United States)^[19]. Using Lipofectamine LTX (Invitrogen), lentivirus was produced by transfecting Lenti-X 293T cells in 60-mm dishes with 3 μ g of EGIP-EF1a-Large T-IRES-Puro, 2.3 μ g of a packaging plasmid (psPAX2), and 1.3 μ g of a coat protein plasmid expressing vesicular stomatitis virus G protein (pDM2.G). Viral supernatants were collected after 48 h, and filtered (0.22 μ m). Primary IMFs were infected with virus for 8 h. With puromycin treatment for 6 d, immortalized LT-positive cells were selected and designated as LmcMF.

To obtain spontaneously immortalized IMFs, pri-

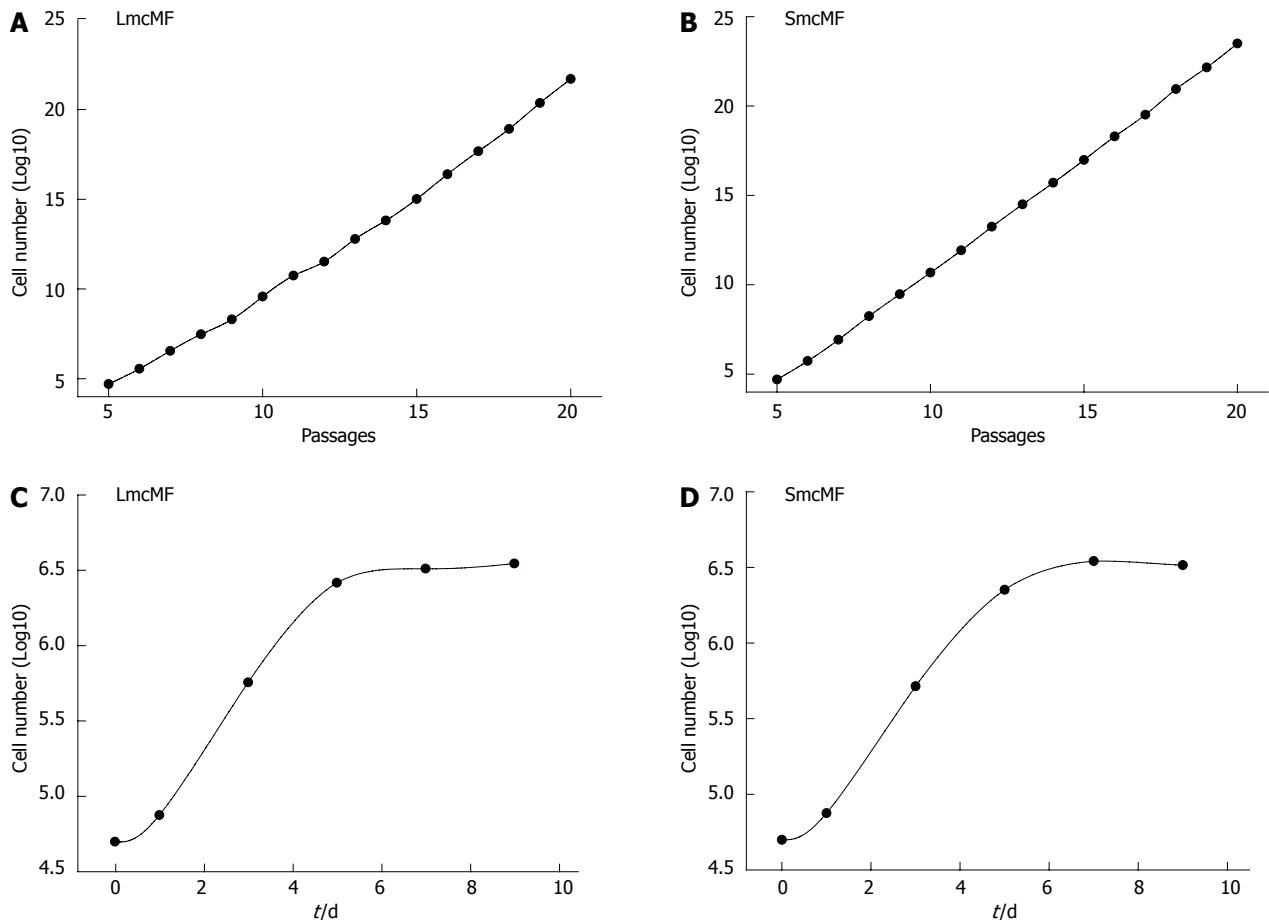


Figure 1 Proliferation and contact inhibition of LmcMF and SmcMF. A, B: The cells were subcultured every 3 d, and the growth rate of LmcMF (A) and SmcMF (B) were assessed as described; C, D: Contact inhibition of LmcMF (C) and SmcMF (D).

mary IMFs were repeatedly passaged until cells that kept proliferating emerged. These spontaneously immortalized cells were designated as SmcMF.

Cell growth and contact inhibition assays

To determine the proliferative ability of IMF cells, 5×10^4 cells were plated in 35-mm dishes and passaged every 3 d, and the number of cells was subsequently counted using a hemocytometer. For the contact inhibition assay, 5×10^4 cells were plated in 35-mm dishes, and the number of cells was counted on the next day and then every other day.

Immunofluorescence staining

Primary IMFs, as well as LmcMF and SmcMF, were grown on glass coverslips and subsequently fixed with 4% formaldehyde for 20 min at room temperature. Cells were permeabilized with 0.2% Triton X-100 in PBS-T (PBS containing 0.05% Tween 20) for 60 s and then blocked with 3% skim milk in PBS-T. After incubation with the first antibodies overnight at 4 °C, Alexa594-conjugated secondary antibodies (Alexa Fluor® 594 goat anti-mouse IgG or Alexa Fluor® 594 donkey anti-goat IgG; Invitrogen) were added and incubated for 1 h at room temperature. Nuclei were counterstained with SYTOX Green (Life Technologies, CA, United States).

Finally, fluorescence images were captured by a confocal laser-scanning microscope (LSM510; Zeiss, Tokyo, Japan).

Western blotting

The western blot analysis was performed as previously described^[18]. Briefly, cells were lysed in a buffer consisting of 50 mmol/L Tris-HCl (pH 8.0), 5 mmol/L EDTA (pH 8.0), 5 mmol/L EGTA (pH 8.0), 1% Triton X-100, 1 mmol/L Na_3VO_4 , 20 mmol/L sodium pyrophosphate, and Roche Complete Protease Inhibitor Cocktail (Roche, Tokyo, Japan). Amersham Hybond ECL Nitrocellulose Membranes (GE Healthcare, Buckinghamshire, United Kingdom) were blocked with 0.5% skim milk and treated with specific antibodies. Protein bands were detected using the ECL Western blotting Detection Reagents (GE Healthcare) or the Western Lightning ECL Pro (PerkinElmer, MA, United States) and visualized using a LAS-3000 mini luminescence imager (Fujifilm, Tokyo, Japan).

RESULTS

Immortalization of IMFs without losing contact inhibition of growth

First, we investigated the immortalization of 2 differ-

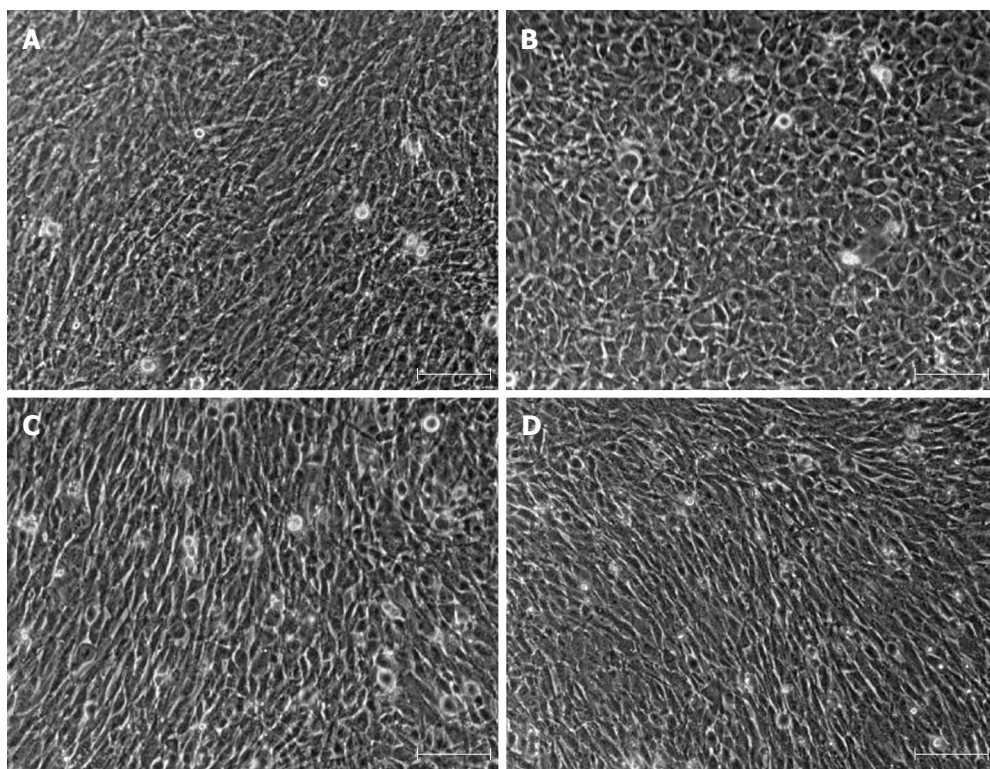


Figure 2 Phase-contrast microscopic images of primary intestinal myofibroblasts, mouse embryonic fibroblasts, LmcMF, and SmcMF. A: Primary intestinal myofibroblasts; B: Mouse embryonic fibroblasts; C: LmcMF; D: SmcMF were cultured to confluence, and the phase-contrast microscopic images were taken. Representative images are shown. Scale bars indicate 200 μ m.

ent IMF cell lines, LmcMF and SmcMF. The cells (5×10^4 cells) were seeded in 35-mm dishes and subcultured every 3 d during the examination of the cell growth rate. As shown in Figure 1A and B, both LmcMF and SmcMF cells proliferated logarithmically at least up to passage 20, and their doubling times were 19.1 and 17.2 h, respectively. Primary IMFs, on the other hand, immediately stopped growing under this experimental condition, since they require a high cell density for proliferation.

For the contact inhibition assay, the cells (5×10^4 cells) were seeded in 35-mm dishes and cultured up to 9 d (Figure 1C and D). The cells became confluent around day 5 and reached the plateau phase by day 6 or 7. These data indicate that LmcMF and SmcMF were immortalized without losing contact inhibition of growth.

LmcMF and SmcMF show myofibroblastic phenotypes

IMFs have been defined as spindle-shaped or stellate cells that are positive for α -SMA and vimentin, and very weakly positive or negative for desmin^[7]. As shown in Figure 2A and B, primary IMFs exhibited spindle-shaped appearance, whereas MEFs were round-shaped. Both LmcMF and SmcMF, like primary IMFs, showed spindle-shaped appearance (Figure 2C and D).

The expression levels of key myofibroblast protein markers, including α -SMA, vimentin, and desmin, were subsequently examined by the Western blotting and immunofluorescence analyses. We observed that primary IMFs and LmcMF expressed an equivalent amount of

α -SMA, whereas the α -SMA protein level in SmcMF was relatively low (Figure 3A and B). As expected, α -SMA was not detectable in the mouse mammary gland epithelial cell line, Eph4, which was used as a negative control. We also found that vimentin was present in both LmcMF and SmcMF, almost at the same level as that in primary IMFs, but was not detectable in the negative control RAW 264.7 cells (Figure 3C and D). Moreover, desmin, though not present in LmcMF, was detected at very low levels in SmcMF, compared to its expression level in intestinal smooth muscle tissues (positive control) (Figure 3E and F). Furthermore, both LmcMF and SmcMF expressed type I collagen, however, at a lower level than that in primary IMFs (Figure 3G). As the negative control, RAW 264.7 cells did not express type I collagen. Overall, these results suggest that LmcMF and SmcMF share almost the same characteristics with intestinal myofibroblasts.

To confirm the expression pattern of α -SMA and vimentin in IMFs, we next performed the immunofluorescence analysis. In a cell, actin transits between its globular (G-) and filamentous (F-actin) states, and G-actin can be visualized by immunofluorescence microscopy in a spreading pattern, because they are monomeric actin^[20]. As shown in Figure 4A, we observed strong filamentous α -SMA expression in primary IMFs. While LmcMF also showed filamentous α -SMA fibers, the amount of globular α -SMA was higher than that in primary IMFs. Most of the α -SMA protein in SmcMF existed in a

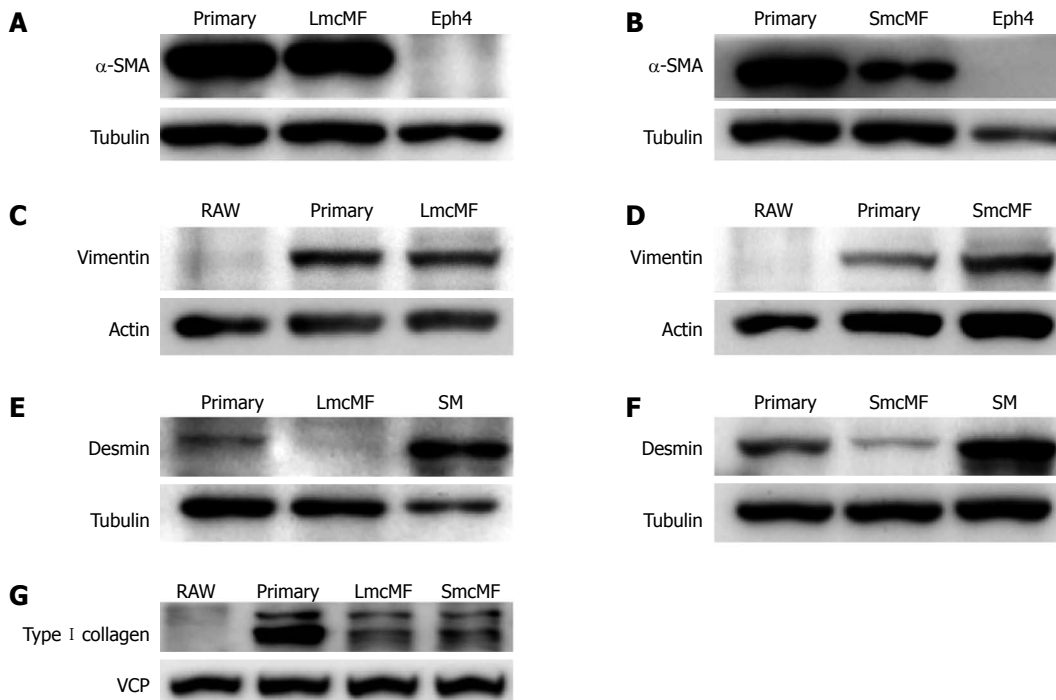


Figure 3 Expression levels of characteristic markers in LmcMF and SmcMF. A, B: The expression levels of α -smooth muscle actin (α -SMA); C, D: Vimentin; E, F: Desmin; G: Type I collagen in primary intestinal myofibroblast (primary); LmcMF (A, C, and E), and SmcMF (B, D, and F) were investigated by Western blotting. Representative blots from 2 independent experiments are shown. Tubulin, actin, and valosin-containing protein (VCP) were used as loading controls.

globular α -SMA form. On the other hand, vimentin was predominantly localized around the nucleus of primary IMFs and LmcMF but diffused throughout SmcMF cells (Figure 4B).

Responses of LmcMF and SmcMF to LPS

It is well known that the LPS receptor complex is composed of TLR4, CD14, and MD-2 and that both CD14 and MD-2 are required for the activation of TLR4^[21]. A study has previously shown that primary IMFs expressed all these 3 components and that the exposure of cells to LPS activated the NF- κ B and p38 MAPK pathways^[10]; therefore, we assessed whether the same holds true for LmcMF and SmcMF. Our results show that although the expression level of TLR4 was low in primary IMFs, the levels in LmcMF and SmcMF were much higher (Figure 5A). As for CD14, the expression level was lower in LmcMF, compared to primary IMFs and SmcMF, and the expression level of MD-2 was relatively higher in primary IMFs and lower in SmcMF, compared to LmcMF.

Finally, we investigated the reactivity of primary IMFs, LmcMF, and SmcMF to LPS. We found that LPS treatment (20 ng/mL) induced the degradation of I κ B α , as well as the phosphorylation of p38 MAPK (Figure 5B-D). While the maximal levels of I κ B α degradation and p38 MAPK phosphorylation were detected at 30 min after LPS treatment, their expression was restored to basal levels within 2 h. Consistent with the previous study (performed in primary IMFs)^[10], our results demonstrate that both LmcMF and SmcMF preserve myofibroblastic phenotypes.

DISCUSSION

IMFs play an important role in intestinal injury, inflammation, fibrosis, and tissue repair; however, their exact function and involvement are still not completely understood^[6,8,10,22]. One issue that hinders the development of IMF research is the lack of IMF cell lines. In this present study, we established 2 IMF cell lines, namely, LmcMF and SmcMF, both of which were immortalized without losing contact inhibition of growth. LmcMF was established by transfecting primary IMFs with plasmid DNA encoding the LT of replication origin-defective SV40. It is known that 2 oncoproteins, LT and ST, encoded by the SV40 early region play an essential role in inducing transformation of mammalian cells^[23]. In addition, previous studies have also shown that LT can immortalize cells but cannot induce neoplastic transformation without ST^[23]. Moreover, it has been shown that immortalization of mouse cells can be achieved by transfecting only with LT^[24]. Based on these findings, we used a plasmid that expresses LT, but not ST, for our immortalization experiments of IMFs. On the other hand, SmcMF was derived from cells that spontaneously immortalized. It has been reported that murine cells tend to immortalize spontaneously in culture, because mouse somatic cells have the telomerase activity^[25].

IMFs can be distinguished from other types of mesenchymal cells, such as smooth muscle cells and fibroblasts, by the expression of 3 protein markers: α -SMA, vimentin, and desmin. It was reported that IMFs are positive for α -SMA and vimentin, but negative or very

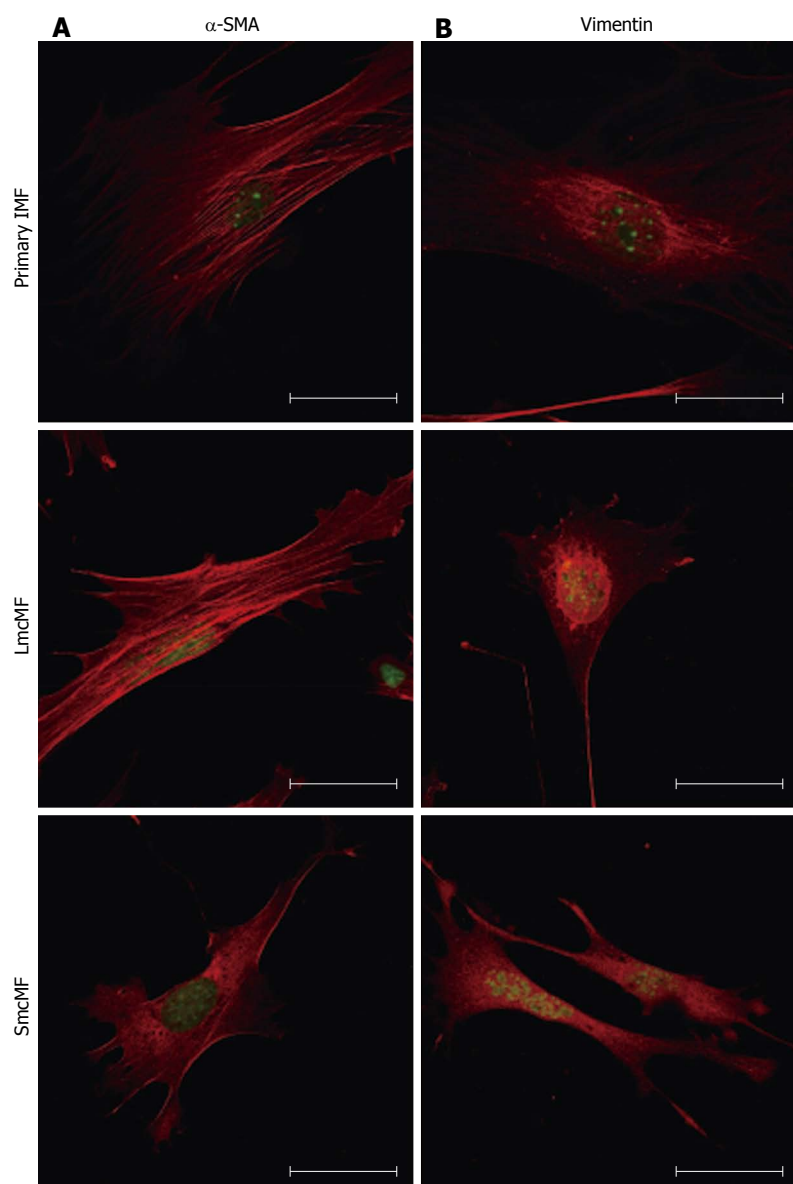


Figure 4 Immunofluorescence staining for α -smooth muscle actin and vimentin in primary intestinal myofibroblasts, LmcMF, and SmcMF. Primary intestinal myofibroblasts (IMFs), LmcMF, and SmcMF were immunostained with α -smooth muscle actin (α -SMA) (A) and vimentin (B) (red). SYTOX Green was used for nuclear labeling (green). Representative images are shown. Scale bars indicate 40 μ m.

weakly positive for desmin^[7]. In contrast, smooth muscle cells are α -SMA-positive, vimentin-negative, and strongly positive for desmin, whereas typical fibroblasts, such as dermal fibroblasts, are negative for α -SMA and desmin, but positive for vimentin^[7,8,26]. In the present study, we observed that all 3 cell lines (primary IMFs, LmcMF, and SmcMF) expressed both α -SMA and vimentin, suggesting that these cells share the same characteristics and that LmcMF and SmcMF can be used as intestinal myofibroblast cell lines. It is also noted that the doubling time of these cells is within a day, whereas that of human primary IMFs is about 5 d^[27]. Therefore, the use of LmcMF and SmcMF will be helpful for improving the efficiency of IMF research.

There are, however, some differences among these cells. For instance, the α -SMA expression level was

slightly lower in SmcMF, and α -SMA was organized into stress fibers in primary IMFs and LmcMF, but not in SmcMF. The vimentin expression level was slightly higher in SmcMF, whereas the type I collagen levels in LmcMF and SmcMF were relatively lower than that in primary IMFs. We propose that these differences may depend on the differentiation stage of the cells. In the present study, very low levels of desmin were detected in primary IMFs and SmcMF. It has been reported that primary human colonic myofibroblasts and CCD-18Co cells do not express desmin^[28], whereas some lesion myofibroblasts do^[7]. These differences in the desmin expression may occur by cell differentiation or by the differences between animal species tested (for example, mice *vs* humans); however, to clarify this, additional experiments are necessary.

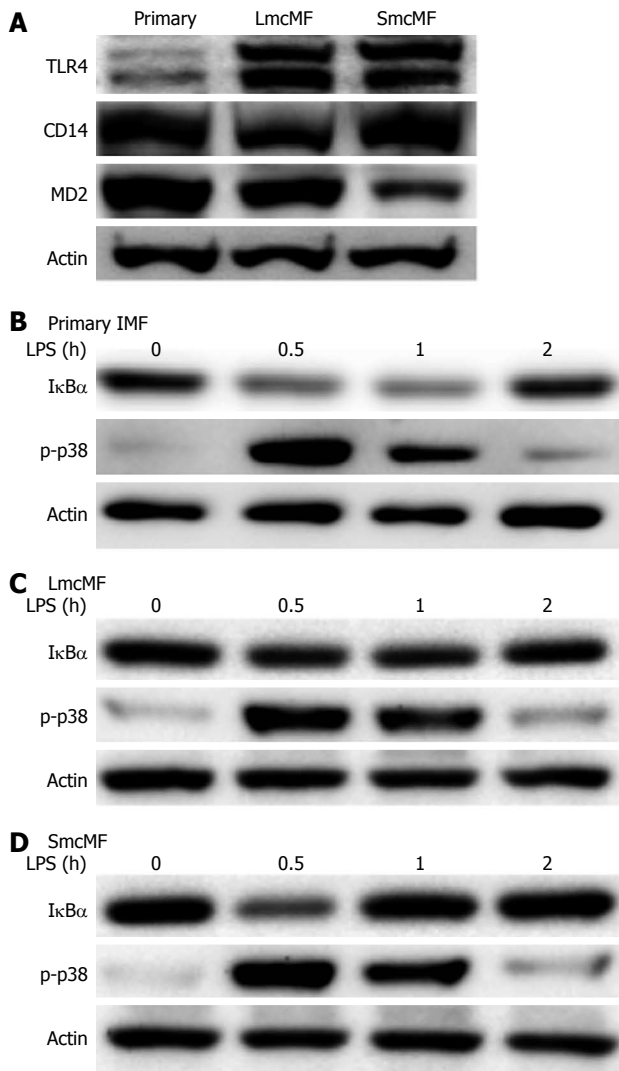


Figure 5 Expression of lipopolysaccharide-related proteins and responses of primary intestinal myofibroblasts, LmcMF, and SmcMF to lipopolysaccharide. A: The expression levels of indicated proteins in primary intestinal myofibroblasts (IMFs) (primary), LmcMF, and SmcMF were investigated by Western blotting; B-D: Primary IMFs (B), LmcMF (C), and SmcMF (D) were stimulated with lipopolysaccharide (LPS) (20 ng/mL) for indicated periods. The IκBα degradation and p38 MAPK phosphorylation were determined by Western blotting. Representative blots from 3-4 independent experiments are shown. Actin was used as a loading control.

The differentiation of fibroblasts into myofibroblasts is an important step in tissue repair and fibrosis^[29]; however, it is a complex process and has not been completely elucidated. It was reported that CCD-18Co cells exhibit fibroblastic and myofibroblastic phenotypes and that TGF-β stimulates their differentiation into myofibroblasts^[17]. CCD-18Co cells have also been used to investigate the mechanism of differentiation into myofibroblasts during wound healing processes. However, CCD-18Co cells poorly differentiate into myofibroblasts^[30]; therefore, LmcMF and SmcMF may be useful to help reveal the role of differentiated myofibroblasts in tissue repair.

Accumulating evidence has shown that IMFs play a crucial role in local immune regulation in the intestine^[10,14,15]; therefore, it is important that the developed cell lines show immune responses to external factors. Consistent with previous report^[10], all three types of IMF expressed TLR4, CD14, and MD-2. We also demonstrate that LmcMF and SmcMF, similar to primary IMFs, were able to respond to LPS stimulation.

In the present study, we observed that the TLR4 expression levels in LmcMF and SmcMF were higher than that in primary IMFs. It is possible that TLR4 was downregulated in primary IMFs and then recovered in LmcMF and SmcMF, since during the cell isolation procedure the intestinal tissues and cells were exposed to a large amount of LPS that existed in the intestinal lumen. Interestingly, although the TLR4 expression level in primary IMFs was low, LPS induced almost the same responses, including IκBα degradation and p38 MAPK phosphorylation, in all 3 types of IMFs. Together, our results indicate that the amount of TLR4 protein in primary IMFs was sufficient to respond to LPS.

In conclusion, we have successfully established 2 novel types of myofibroblast cell lines from mouse colon, namely, LmcMF and SmcMF, both of which expressed the key myofibroblast markers. Moreover, these cell lines were immortalized (rather than transformed to neoplastic cells) and were able to respond to LPS, like primary IMFs. Furthermore, these cell lines proliferate much faster than primary IMFs, and therefore they can be used efficiently. Nevertheless, for the development of IMF research using LmcMF and SmcMF, more experiments are necessary to determine their properties.

COMMENTS

Background

With respect to the gastrointestinal mucosa, accumulating evidence has shown that subepithelial intestinal myofibroblasts (IMFs), which are located subjacent to the epithelium, play crucial roles in regulating epithelial cells. IMFs orchestrate diverse events in gastrointestinal health and diseases, including epithelial differentiation and development, mucosal repair, carcinogenesis, and inflammatory responses. Therefore, it is important to establish IMF cell lines, which can be used for clarifying the role of IMFs.

Research frontiers

Despite the crucial role played by IMFs in gastrointestinal health and diseases, the understanding of their underlying regulatory mechanism is limited. A major issue that hinders the advance of IMF research is the lack of IMF cell lines that exhibit myofibroblastic phenotypes. In this study, authors established 2 IMF cell lines, namely, LmcMF and SmcMF, both of which were immortalized without losing contact inhibition of growth, and were able to respond to lipopolysaccharide, like primary IMFs.

Innovations and breakthroughs

For the IMF research, the 2 following cell types are available: freshly isolated IMFs (primary IMFs) and IMF-like cell lines (such as CCD-18Co cells). However, primary IMFs typically change phenotypes within passage 6 and stop growing after repeated passages. In addition, CCD-18Co cells need to be treated with TGF-β for the expression of key myofibroblast protein markers. To the best of our knowledge, this report is the first to establish novel types of myofibroblast cell lines from the mouse colon. Authors developed 2 cell lines, namely, LmcMF and SmcMF, both of which expressed the key myofibroblast markers without

stimulation.

Applications

Because IMFs play crucial roles in regulating epithelial cells, it is important to develop IMF cell lines. The establishment of 2 cell lines is helpful for improving the efficiency of IMF research.

Peer review

This study characterizes 2 IMF cell lines isolated from the mouse colon. This is a straightforward descriptive report and is methodological in nature; essentially, the authors have generated a potentially useful cell line resource that may be used to further myofibroblast research into tissue repair and fibrosis, immune regulation within the intestine, and other aspects of myofibroblast function. Because only a few good myofibroblast cell lines are currently available, it is important to create new myofibroblast cell lines for *in vitro* studies, and the authors have generated 2 IMF cell lines by using 2 different approaches.

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Expression of interleukin-22/STAT3 signaling pathway in ulcerative colitis and related carcinogenesis

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by Western blotting.

RESULTS: Patients with active UC had significantly more IL-22, IL-23, IL-22R1 and p-STAT3-positive cells than the patients with inactive UC and normal controls. Furthermore, IL-22 and related proteins were closely related to the severity of the colitis. The expression of IL-22 and IL-22R1 in the tissue of initial UC was stronger than in that of chronic UC, whereas the expression of p-STAT3 was significantly increased in chronic UC tissues. In dysplasia tissues, the expression level of IL-22 and related proteins was higher compared with controls. Mouse colitis model showed that expression of IL-22, IL-22R1 and IL-23 was increased with time, p-STAT3 and the downstream gene were also remarkably upregulated.

CONCLUSION: IL-22/STAT3 signaling pathway may be related to UC and UC-induced carcinogenesis and IL-22 can be used as a biomarker in judging the severity of UC.

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Abstract

AIM: To investigate the expression of interleukin (IL)-22 and its related proteins in biopsy specimens from patients with ulcerative colitis (UC) and UC-related carcinogenesis.

METHODS: Biopsy specimens were obtained from patients with inactive ($n = 10$), mild-to-moderately active ($n = 30$), severely active ($n = 34$), initial ($n = 30$), and chronic UC ($n = 44$), as well as UC patients with dysplasia ($n = 10$). Specimens from patients without colonic abnormalities ($n = 20$) served as controls. Chronic colitis in experimental mice was induced by 2.5% dextran sodium sulfate. The expression levels of IL-22, IL-23, IL-22R1 and phosphorylated STAT3 (p-STAT3) were determined by immunohistochemistry. Bcl-2, cyclin D1 and survivin expression was detected

Key words: Ulcerative colitis; Ulcerative colitis-related carcinogenesis; Interleukin-22; Interleukin-22R1; STAT3

Core tip: This study investigates the expression of interleukin (IL)-22, IL-22R1, IL-23, and STAT3 in ulcerative colitis (UC) and UC-related carcinogenesis (UC-CRC) tissues from human and mouse. The results showed that IL-22 and related proteins were closely related to the severity of colitis, and the expression level of IL-22 and related proteins was higher in dysplasia tissues. IL-22/STAT3 signaling pathway was related to UC and UC-CRC. IL-22 can be used as a biomarker for determining the severity of UC and as an interesting therapeutic target in active UC and UC-CRC.

Yu LZ, Wang HY, Yang SP, Yuan ZP, Xu FY, Sun C, Shi RH. Expression of interleukin-22/STAT3 signaling pathway in ulcer-

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INTRODUCTION

Ulcerative colitis (UC) is a subtype of chronic inflammatory bowel disease (IBD) of the large intestine. The disease is characterized by a dysregulated mucosal immune response. This aberrant immune response leads to the secretion of harmful cytokines that destroy the gastrointestinal tract epithelium, thereby causing further inflammation.

Several inflammatory cytokines have been associated with IBD, including the interleukins IL-1 and IL-6, tumor necrosis factor (TNF)- α , and interferon (IFN)- γ . Among the inflammatory cytokines implicated in IBD pathogenesis, much interest has been focused on the recently-identified cytokine IL-22. IL-22 is a member of the IL-10 subfamily; its production is highly dependent on IL-23 in T-helper 17 (Th17)^[1-3], Th1^[4,5], NK-22^[6,7], and CD11c⁺^[8] cells. IL-22 is expressed by the novel Th22 cell lineages^[9] and innate lymphoid cells (ILCs)^[10]. IL-22-producing ILCs in humans are responsive to IL-23 signaling, as potentially important mediators of IBDs^[10]. Th22 and Th17 cells may be implicated in the pathogenesis of several chronic inflammatory and autoimmune diseases such as IBD, psoriasis, ankylosing spondylitis, and rheumatoid arthritis^[11-16].

IL-22 signaling is established when the cytokine binds to a heterodimeric receptor complex of IL-22R1 and IL-10R2. Given that IL-10R2 is a ubiquitous protein, cellular IL-22 responsiveness is mainly determined by IL-22R1 expression. IL-22R1 is specifically expressed in nonleukocytic cells such as those of the pancreas, skin, kidney, liver, and colon. IL-22R1 expression is detectable in epithelial cells of these organs, but not in their immune cells^[17-20]. Therefore, IL-22 is unique among the cytokines because it cannot mediate autocrine or paracrine functions among leukocytes. Instead, IL-22 transmits information between leukocytes and the nonleukocytic cell compartment.

The STAT3 pathway for transcription activation appears to be a major mode of IL-22 signal transduction. Activated STAT3 is translocated from the cytoplasm to the nucleus, where it regulates genes involved in cell apoptosis, proliferation, migration, and survival. STAT3 has important functions in several autoimmune diseases. IL-22 mediates IL-23-induced acanthosis and dermal inflammation in psoriasis and IBD through the activation of STAT3^[21]. When activated by IL-22, STAT3 can aggravate colitis by promoting the expression of inflammatory factors such as IL-8, IFN- γ , and the matrix metalloproteinases (MMPs)^[22,23]. Previous studies investigated the role of IL-22 in UC and UC-related carcinogenesis (UC-CRC)^[24-26]. These studies revealed that the IL-22/

STAT3 pathway is involved in UC pathophysiology and carcinogenesis through the activity of inducible nitric oxide synthase (iNOS), DMBT1, and REG α ^[24-26]. However, the IL-22-induced phosphorylated STAT3 (p-STAT3) was considered a defense mechanism because it enhanced mucus production and goblet cell replacement in mouse models for acute colitis and wound healing^[27-29]. Thus, the role of the IL-22/STAT3 signaling pathway in UC remains unclear. The current study investigates the significance of IL-22 and the IL-22/STAT3 signaling pathway in UC and UC-CRC, as well as its value as a therapeutic target for both diseases.

MATERIALS AND METHODS

Patients and tissue samples

Colon biopsy *via* endoscopy was performed on 74 patients with UC, including 31 females and 43 males (median age, 45.9 years; range, 17-87 years). The controls included 6 females and 14 males (mean age, 45.6 years; range, 33-55 years). All samples were obtained at the First Affiliated Hospital of Nanjing Medical University from 2009 to 2011.

This study was approved by the Research Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. Written informed consent was obtained from each patient. The diagnosis of UC was based on clinical, endoscopic, and histological findings. The patient characteristics and histological data are summarized in Table 1.

Experimental mouse models of UC

The mouse experiments were conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the United States National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University. Male ICR mice were purchased and maintained at the Centre of Animal Facility, Nanjing Medical University. The mice were sacrificed by cervical dislocation. All efforts were made to minimize suffering. Chronic colitis was induced by administering 2.5% dextran sodium sulfate (DSS; ICN Biomedicals Inc., Irvine, CA, United States) to the drinking water in three seven-day cycles, which had a five-day recovery period after each cycle. During the recovery period, mice drank only normal water. The age-matched control mice received only normal drinking water throughout the entire study.

Immunohistochemistry

All the tissues were fixed overnight in 4% paraformaldehyde at 4 °C, processed, and cut into 5 μ m-thick sections. The sections were then deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked by 3% H₂O₂ for 10 min at room temperature. Antigen retrieval was performed by 15 min of boiling in the pre-heated buffer (10 mmol/L of sodium citrate, pH = 6.0)

Table 1 Clinical characteristics of ulcerative colitis patients

	Normal control <i>n</i> = 20	Histological activity of UC			Types of UC		UC with dysplasia <i>n</i> = 10
		Inactive <i>n</i> = 10	Mild-moderate <i>n</i> = 30	Severe <i>n</i> = 34	Initial <i>n</i> = 30	Chronic <i>n</i> = 44	
Gender (<i>n</i>)							
Male	14	5	16	22	16	27	6
Female	6	5	14	12	14	17	4
Mean age ³ , yr	40.6 ± 12.5	41.2 ± 13.3	44.7 ± 13.2	43.2 ± 17.1	42.8 ± 17.3	46.5 ± 13.6	44.7 ± 16.6
Mean duration of disease ³ , yr	-	6.14 ± 4.2	4.3 ± 2.3	3.7 ± 3.1	0.8 ± 0.6	5.1 ± 0.6 ¹	10.5 ± 1.4 ²
Extent of disease (<i>n</i>)							
Extensive colitis	-	0	8	19	8	19	3
Left-side colitis	-	4	13	9	13	13	4
Proctitis	-	6	9	6	9	12	3
Treatment (<i>n</i>)							
Aminosalicylates	-	8	25	20	16	35	5
Corticosteroids	-	4	5	18	4	15	3
Immunosuppressive agent	-	0	0	1	0	1	0
Biological agent	-	0	0	0	0	0	0
None	-	3	2	0	10	5	2

¹The mean duration of disease in the chronic group (5.1 ± 0.6 years) was significantly longer than in the initial group (0.8 ± 0.6 years; $P < 0.001$); ²The mean duration of disease in the ulcerative colitis (UC) with dysplasia group (10.5 ± 1.4 years) was significantly longer than in other groups ($P < 0.05$); ³Data are expressed as mean ± SD.

at 200 W in a microwave. The slides were transferred to a humidifier and blocked by incubating in 5% normal goat serum at room temperature for 1 h. The polyclonal rabbit antibodies used in this study included anti-p-STAT3 tyrosine 727, anti-IL-22, anti-IL-22R1, and anti-IL-23 (ab30647, ab18499, ab5984 and ab115759, respectively); these antibodies were diluted in 5% normal goat serum at ratios of 1:200, 1:200, 1:200 and 1:400, respectively. The sections were incubated in the respective antibodies overnight at 4 °C. The slides were subsequently incubated in the secondary antibody goat anti-rabbit IgG-biotin (B8895; Sigma-Aldrich, St. Louis, MO, United States) at room temperature for 40 min. The sections were incubated in the ABC-peroxidase solution (Ultrasensitive™ S-P kit, kit 9719; Maixin-Bio, China) for 30 min at room temperature, followed by counterstaining with hematoxylin. The immunohistochemistry (IHC) results were analyzed using Image Pro-Plus.

Western blotting analysis

Proteins were extracted from the mouse tissues and quantified using a commercial protein assay (Bio-Rad Laboratories, CA, United States). The protein samples (30 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Immunoblot analysis was conducted using antibodies against total STAT3, p-STAT3 (S727), Bcl-2, cyclin D1, and survivin (Abcam Inc, MA, United States). The results were visualized using the chemiluminescent Pierce ECL Substrate Western blotting detection system (Thermo Scientific, IL, United States) and exposure to autoradiography film (Kodak XAR film).

Statistical analysis

The results were expressed as the mean ± SD. The two

groups were compared using the Student's *t* test or the Mann-Whitney *U* test, as appropriate. All statistical analyses were performed using the SPSS statistical software (version 13.0). $P < 0.05$ was considered to be statistically significant.

RESULTS

Patient characteristics

As shown in Table 1, we collected biopsy specimens from 20 healthy controls, 10 patients with inactive UC, 64 patients with active UC (including 30 patients with mild-to-moderate active UC and 34 patients with severely active UC), 30 patients with initial UC attacks, 44 patients with chronic UC, and 10 UC patients with dysplasia. The duration of disease was significantly longer in the groups with chronic UC and UC with dysplasia than in the other groups ($P < 0.05$). No other significant differences were observed among the other groups, regardless of the treatment used.

IL-22 expression in patients with different degrees of inflammation

Immunohistochemical analysis showed that the IL-22 protein was mainly expressed in inflammatory cells of the colonic lamina propria, but not in the normal controls (Figure 1A). Tissues with mild-moderate and severe UC had significantly higher expression levels than those with inactive UC and normal colon tissues (mild-moderate *vs* normal, $P < 0.001$; mild-moderate *vs* inactive, $P = 0.02$, $P < 0.05$; severe *vs* normal, $P < 0.001$; severe *vs* inactive, $P < 0.001$; Figure 2A). Moreover, significantly more IL-22-positive cells were present in tissues of severe UC than in those of mild-moderate UC (severe *vs* mild-moderate, $P < 0.001$; Figure 2A). The results indicate that

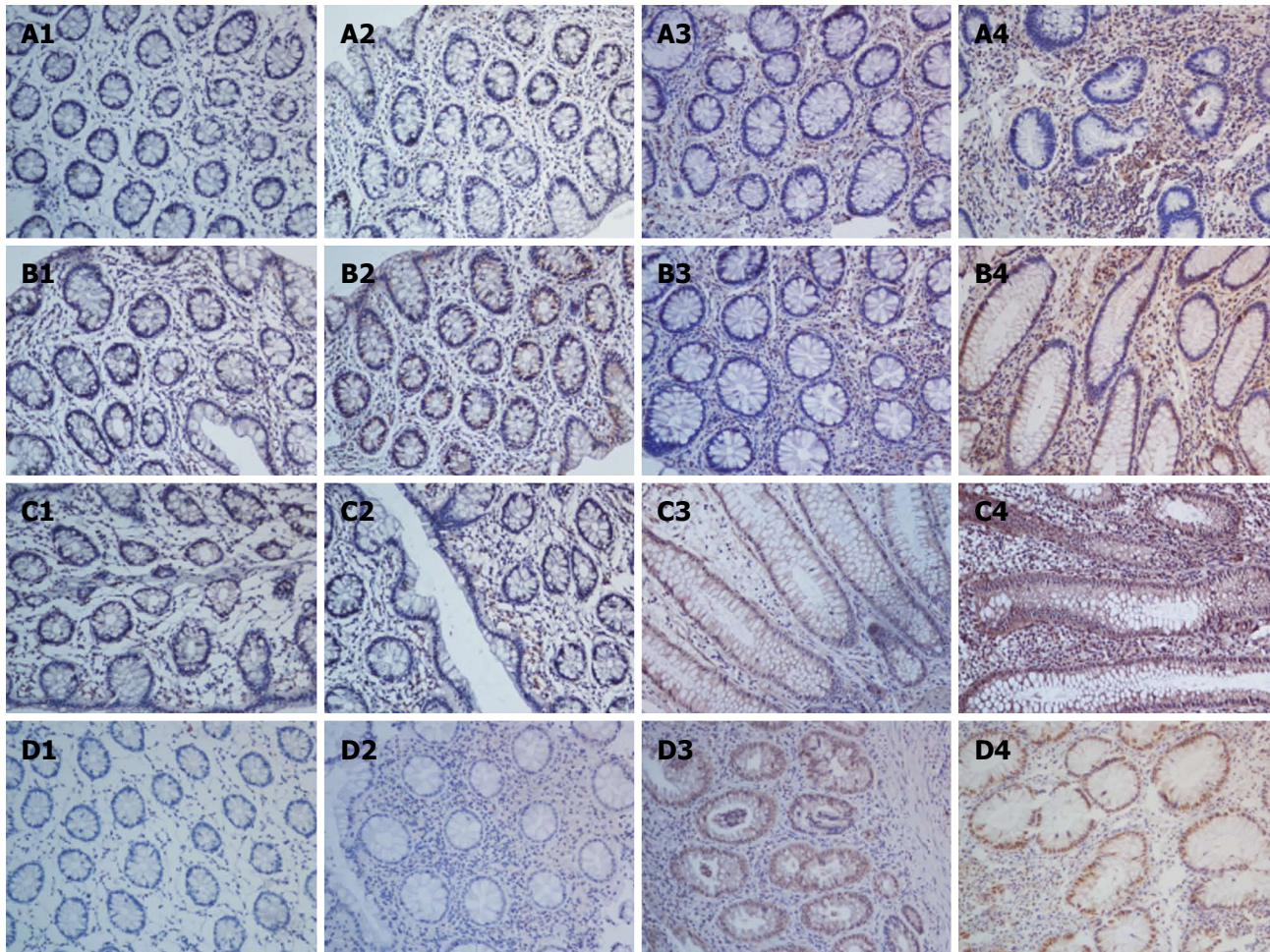


Figure 1 Expression and distribution of interleukin-22 and its related proteins in colonic biopsy specimens as analyzed by immunohistochemistry ($\times 200$). A1-A4: Expression and distribution of interleukin (IL)-22 in colonic biopsy specimens from the control, inactive ulcerative colitis (UC), mild-moderate UC, and severe UC tissues, respectively; B1-B4: The expression and distribution of IL-23 in colonic biopsy specimens from the control, inactive UC, mild-moderate UC, and severe UC tissues; C1-C4: The expression and distribution of IL-22R1 in colonic biopsy specimens from the control, inactive UC, mild-moderate UC, and severe UC tissues; D1-D4: The expression and distribution of p-STAT3 (S727) in colonic biopsy specimens from the control, inactive UC, mild-moderate UC, and severe UC tissues.

the expression of IL-22 was related to UC severity.

Expression of IL-22-related proteins in patients with different degrees of inflammation

Previous studies have demonstrated that IL-22 production is highly dependent on IL-23 in Th17, NK-22, and lymphoid tissue-inducer cells^[1-3,6,7]. Thus, we analyzed IL-23 expression in UC tissues using IHC. The results indicated that IL-23 was significantly highly upregulated in active UC, as compared with inactive UC and the controls (mild-moderate *vs* normal, $P < 0.001$; mild-moderate *vs* inactive, $P < 0.001$; severe *vs* normal, $P < 0.001$; severe *vs* inactive, $P < 0.001$; Figure 2B). Similarly, the IL-23 expression was stronger with severe UC than with mild-moderate UC (severe *vs* mild-moderate, $P = 0.01$, $P < 0.05$; Figure 2B). The positive region of IL-23 in UC was mostly confined to the intestinal epithelial cells (IECs) and inflammatory cells of the colonic lamina propria (Figure 1B).

We identified IL-22R1, another key molecule that is necessary for signal transmission. IL-22R1 was mainly

localized in the IECs (Figure 1C). IL-22R1 was overexpressed in active UC (mild-moderate *vs* normal, $P < 0.001$; mild-moderate *vs* inactive, $P = 0.0002$, $P < 0.001$; severe *vs* normal, $P < 0.001$; severe *vs* inactive, $P = 0.0002$, $P < 0.001$; severe *vs* mild-moderate, $P = 0.007$, $P < 0.01$; Figure 2C).

Based on the downstream effects of IL-22, the activation of STAT3 was determined by staining with p-STAT3 at the S727 residue. IHC analysis showed that in the colon tissues resected from patients with UC, the p-STAT3 (S727) protein was mainly expressed in the nucleus of epithelial cells (Figure 1D). Its expression was significantly upregulated in the active UC tissues, particularly in severe UC (severe *vs* normal, $P < 0.001$; severe *vs* inactive, $P < 0.001$; severe *vs* mild-moderate, $P = 0.0045$, $P < 0.01$; mild-moderate *vs* normal, $P < 0.001$; mild-moderate *vs* inactive, $P = 0.0017$, $P < 0.01$; Figure 2D).

IL-22 expression in patients with different types of UC

According to the clinical diagnostic standards, we classified UC into two types: initial and chronic UC. IHC

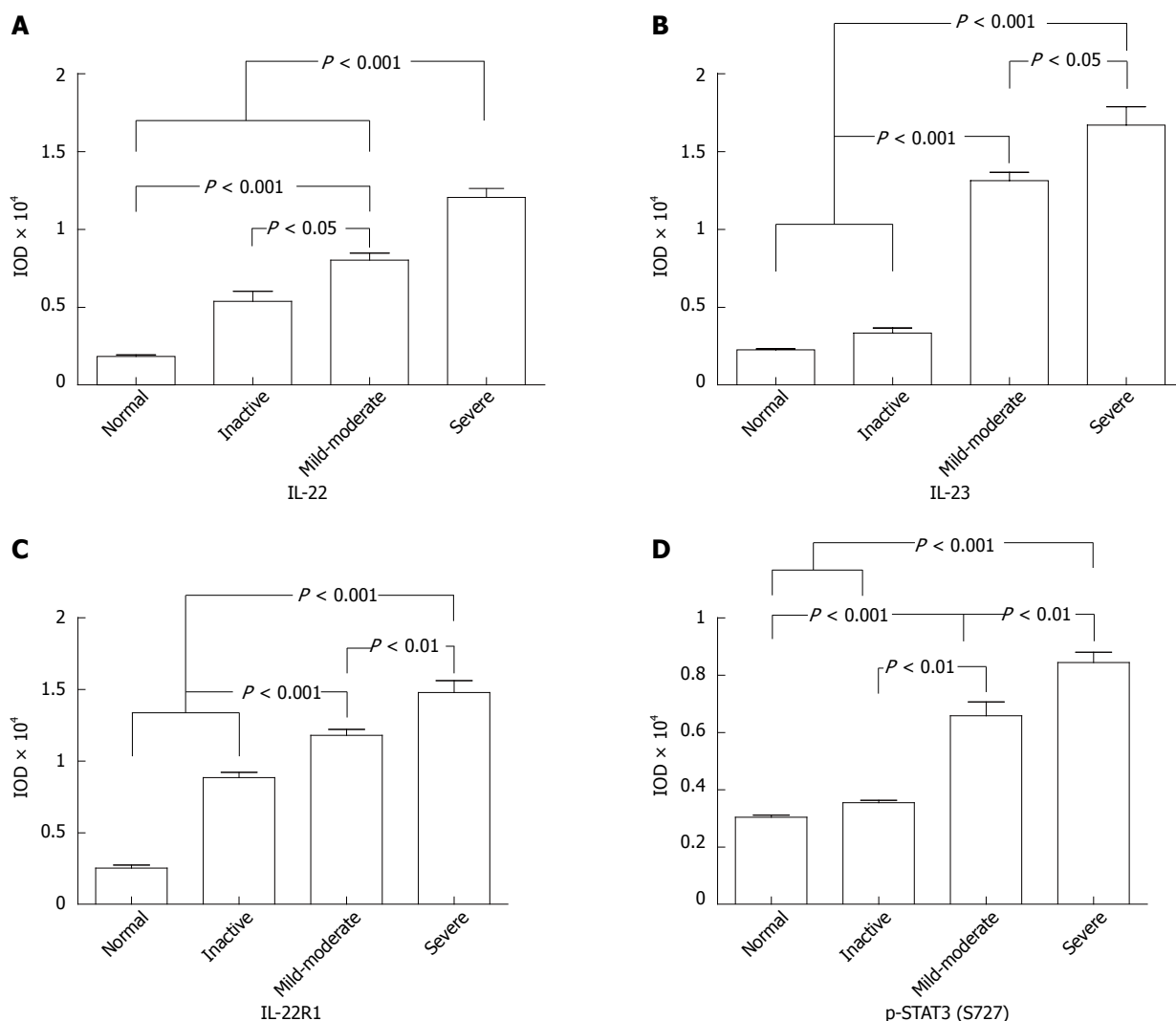


Figure 2 Statistical evaluation of interleukin-22 and its related proteins in human colonic biopsy specimens from the controls and patients with inactive, mild-moderate, and severe ulcerative colitis. The average integrated optical density (IOD) was obtained by analyzing five random fields for each slide, which were evaluated using Image-Pro Plus software (v. 5.0), for the immunohistochemistry staining of interleukin (IL)-22 (A), IL-23 (B), IL-22R1 (C), and p-STAT3 (S727) (D) in human colonic biopsy specimens. The expression levels of IL-22 (A), IL-23 (B), IL-22R1 (C), and p-STAT3 (D) were positively correlated with colitis severity.

indicated that IL-22 was significantly upregulated in the initial UC tissues than in the chronic UC tissues (initial *vs* chronic, $P = 0.0077$, $P < 0.01$; Figures 3A and 4A).

IL-22-related protein expression in patients with different types of UC

Similar to IL-22, IL-22R1 was more strongly expressed in the tissues of initial UC than in those of chronic UC (initial *vs* chronic, $P < 0.001$; Figures 3C and 4C). By contrast, the number of p-STAT3-positive cells was significantly higher in chronic UC tissues than in initial UC (chronic *vs* initial, $P = 0.03$, $P < 0.05$; Figures 3D and 4D). However, no significant differences were detected in terms of the IL-23 expression in these two groups (Figures 3B and 4B).

IL-22 expression in patients with UC-CRC (dysplasia)

A positive correlation exists between the IL-22-positive cells and the severity of colitis in patients with UC. We investigated IL-22 expression in biopsy specimens from

patients with UC-CRC (dysplasia) and analyzed the IL-22 levels in UC with dysplasia (Figure 5A). Significantly more IL-22-positive cells were observed in the dysplasia group than in the inflammatory group ($P = 0.02$; Figure 6A).

Expression of IL-22-related proteins in patients with UC-CRC (dysplasia)

Given that IL-22 was highly upregulated in UC tissue with dysplasia, we studied the expression of the receptor IL-22R1, its upstream IL-23, and its downstream p-STAT3 (S727) in UC tissues with dysplasia, as compared with active and inactive UC tissues. The expression levels of IL-22R1, IL-23, and p-STAT3 were significantly higher in UC tissues with dysplasia than in the control group (IL-22R1: dysplasia *vs* active, $P = 0.02$; IL-23: dysplasia *vs* active, $P = 0.01$; p-STAT3: dysplasia *vs* active, $P = 0.02$; Figure 6B-D). The increased expression was strictly found at the dysplastic tissues of the patients (Figure 5B-D).

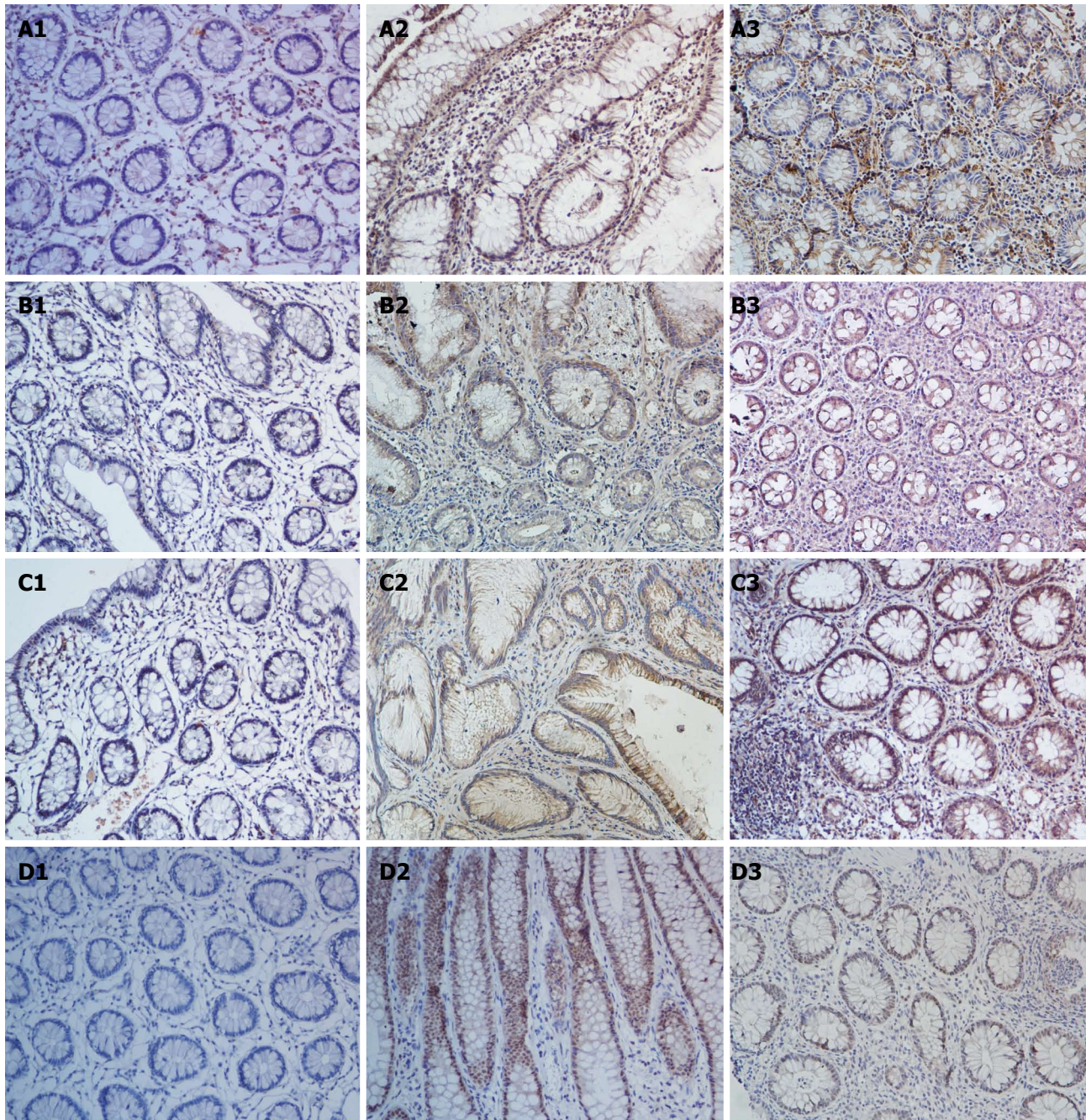


Figure 3 Expression and distribution of interleukin-22 and related proteins in human colonic tissues from controls and patients with chronic and initial ulcerative colitis as detected by immunohistochemistry ($\times 200$). A1-A3: Expression and distribution of interleukin (IL)-22 in ulcerative colitis (UC) tissues from the control, chronic UC, and initial UC groups; B1-B3: Expression and distribution of IL-23 in UC tissues from the control, chronic, and initial UC groups; C1-C3: Expression and distribution of IL-22R1 in UC tissues from the control, chronic, and initial UC groups; D1-D3: Expression and distribution of p-STAT3 in UC tissues from the chronic and initial UC groups.

Expression of IL-22 and its related proteins in DSS-induced mouse models of chronic colitis

We induced experimental colitis by treating mice with DSS to study the role of IL-22 and its related proteins in the disease. Dynamic IL-22, IL-23, and IL-22R1 expression levels were investigated by IHC. p-STAT3 activity and its downstream gene expression were confirmed by Western blotting analysis at different time points (at days 40, 80, and 120). The expression levels of IL-22, IL-22R1, and IL-23 were increased with time (Figure 7A).

STAT3 activation and the activity of its downstream cell proliferation-related genes, such as Bcl-2, cyclin D1, and survivin, were also investigated. All these genes had sustained expression over time (Figure 7B).

DISCUSSION

IBDs such as Crohn's disease and ulcerative colitis are chronic inflammatory disorders of the gastrointestinal tract. Although their etiology is not completely under-

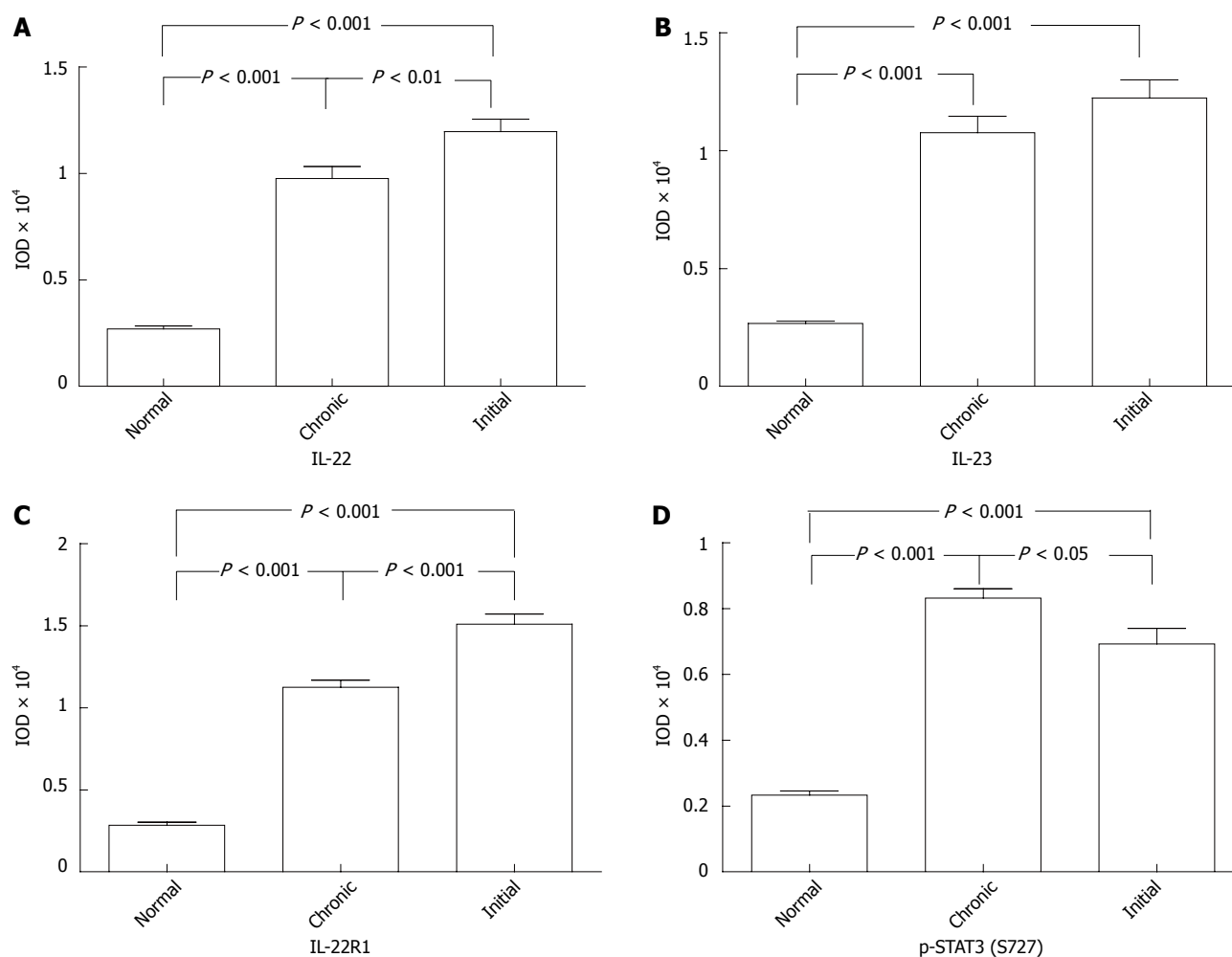


Figure 4 Statistical evaluation of interleukin-22 and its related proteins in human colonic biopsy specimens from controls and patients with chronic and initial ulcerative colitis. The average integrated optical density (IOD) for the immunohistochemistry staining of interleukin (IL)-22 (A), IL-23 (B), IL-22R1 (C), and p-STAT3 (S727) (D) in colonic tissues with initial and chronic ulcerative colitis (UC). Significantly higher expression levels of IL-22 and IL-22R1 were observed in the initial UC group as compared with the chronic UC group. p-STAT3 was highly expressed in the chronic UC group.

stood, initiation and aggravation of the inflammatory process seem to be related to a massive local mucosal immune response. IL-22 belongs to the IL-10 family of cytokines; it has recently been shown to be preferentially expressed by Th17 and Th22 cells. These cells have been identified in the pathogenesis of certain chronic inflammatory diseases, including colitis, psoriasis, and rheumatoid arthritis. IL-22 targets innate immune pathways because of the restricted expression of IL-22 receptors on nonleukocytic cells, such as epithelial cells, keratinocytes, and hepatocytes; however, it does not recognize T- or B-cells^[17-20]. Studies using genetically-engineered mice have demonstrated that epithelial STAT3 activation in DSS-induced colitis is dependent on IL-22, rather than on IL-6. Both IL-22 and STAT3 activation in epithelial cells is important for wound-healing, as demonstrated by *in vivo* experiments^[27]. Sugimoto *et al.*^[28] found that the IL-22/STAT3 pathway contributes to the rapid amelioration of local intestinal inflammation by enhancing the production of membrane-bound mucins (Muc1, -3, -10, and -13) in a mouse model of acute colitis. However, IL-22 is also considered an inflammatory driver in IBD

by acting on human colonic subepithelial myofibroblasts to stimulate secretion of proinflammatory cytokines such as MMPs, IL-1, IL-8, and INF- γ ^[30]. Highly elevated serum levels of IL-22 were correlated with disease severity in patients with Crohn's disease (CD)^[17]. Colitis mouse models have indicated that highly elevated IL-22 expression may directly or indirectly induce inflammation^[31]. Moreover, the IL-22/STAT3 signaling pathway is important in inflammation and carcinogenesis during UC *via* its upregulation of iNOS and MMP production, respectively^[23,24]. Here, we demonstrate that IL-22 contributes to the inflammatory severity of UC and UC-CRC by activating STAT3 in IECs.

The UC microenvironment is composed of IECs, macrophages, immunocytes, and so on. The interactions among these cells involve their secreted cytokines and consist of a positive feedback loop with persistent activation of the STAT3-enabling progression of UC. Similar to IL-23 and IL-22R1, an IL-22 positive feedback loop in the UC microenvironment was demonstrated in this study. Our results indicated the IL-22 overexpression in the inflammatory cells of the colonic lamina propria in

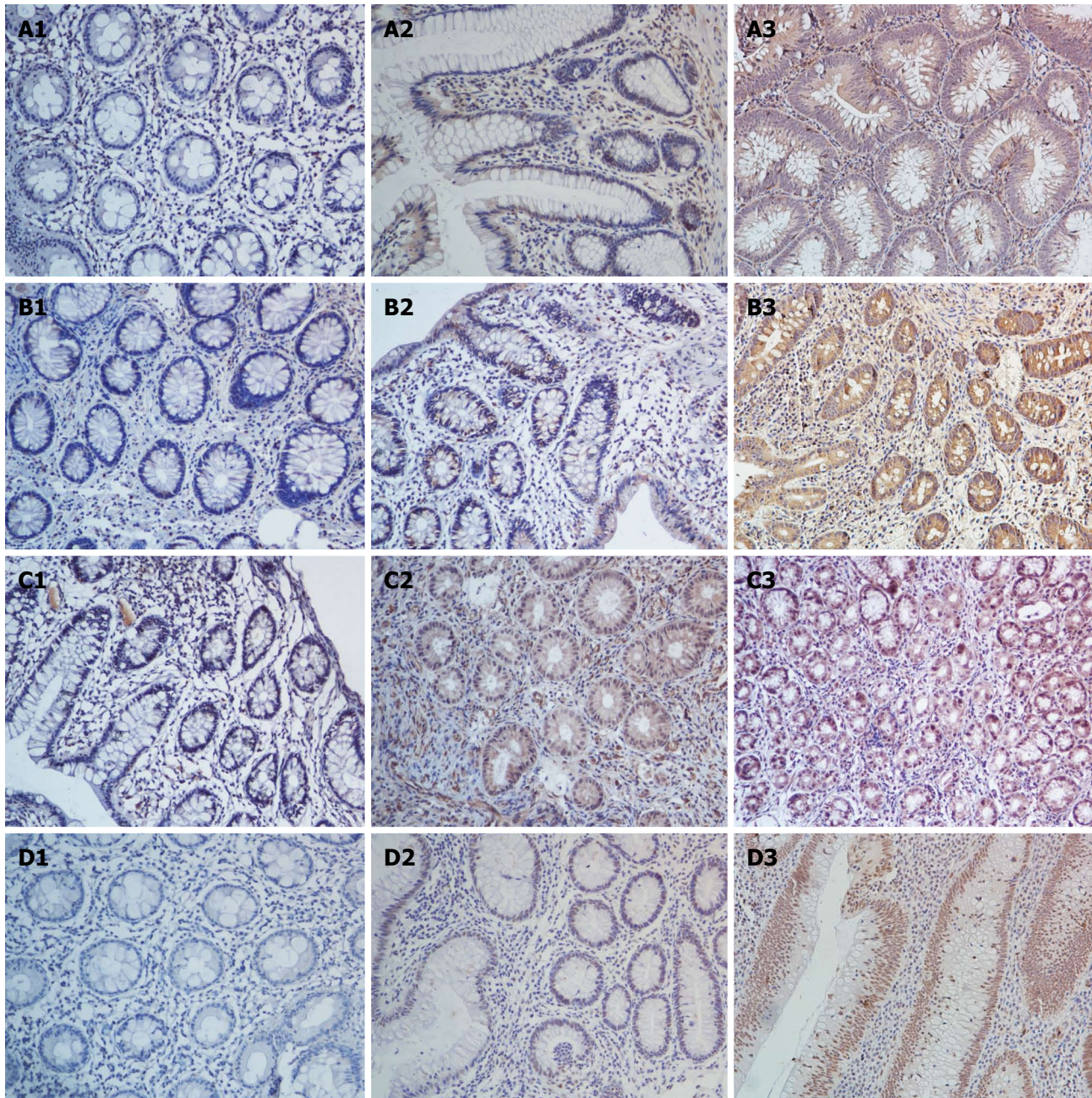


Figure 5 Expression and distribution of interleukin-22 and its related proteins in human colonic tissues from patients with inactive ulcerative colitis, active ulcerative colitis, and ulcerative colitis with dysplasia, as detected by immunohistochemistry ($\times 200$). A1-A3: Expression and distribution of interleukin (IL)-22 in inactive ulcerative colitis (UC), active UC, and UC with dysplasia tissues; B1-B3: Expression and distribution of IL-23 in inactive UC, active UC, and UC with dysplasia tissues; C1-C3: Expression and distribution of IL-22R1 in inactive ulcerative colitis (UC), active UC, and UC with dysplasia tissues; D1-D3: Expression and distribution of p-STAT3 (S727) in inactive UC, active UC, and UC with dysplasia tissues.

UC tissues. Moreover, the sustained activation of STAT3 signaling in IECs was verified. Simultaneously, IL-22R1 expression was enhanced in IECs, thereby ensuring the transmission of the IL-22 signal.

The STAT3-regulated proinflammatory cytokine IL-23^[32] was likewise overexpressed in human UC. IL-23 activates innate immune cells to secrete pro-inflammatory cytokines such as IL-1 β , TNF- α , and IL-6, as well as maintains the expansion of Th17 cells that express IL-22^[33].

IL-22 is increased in active Crohn's disease and pro-

motes proinflammatory gene expression^[17]. We demonstrated that IL-22 is more highly expressed in active UC than in inactive UC and the normal control. Thus, the increased IL-22 signaling in active IBD supports the potential of an IL-22 signaling blockade as a therapeutic strategy for IBD. Furthermore, IL-23, IL-22, STAT3, and IL-22R1 are closely related to the colitis severity. Thus, positive feedback loops can further exacerbate inflammation. If left unchecked, these pathways may lead to the chronic immune pathology that is characteristic to IBD. Therefore, IL-22 could be used as a biomarker for deter-

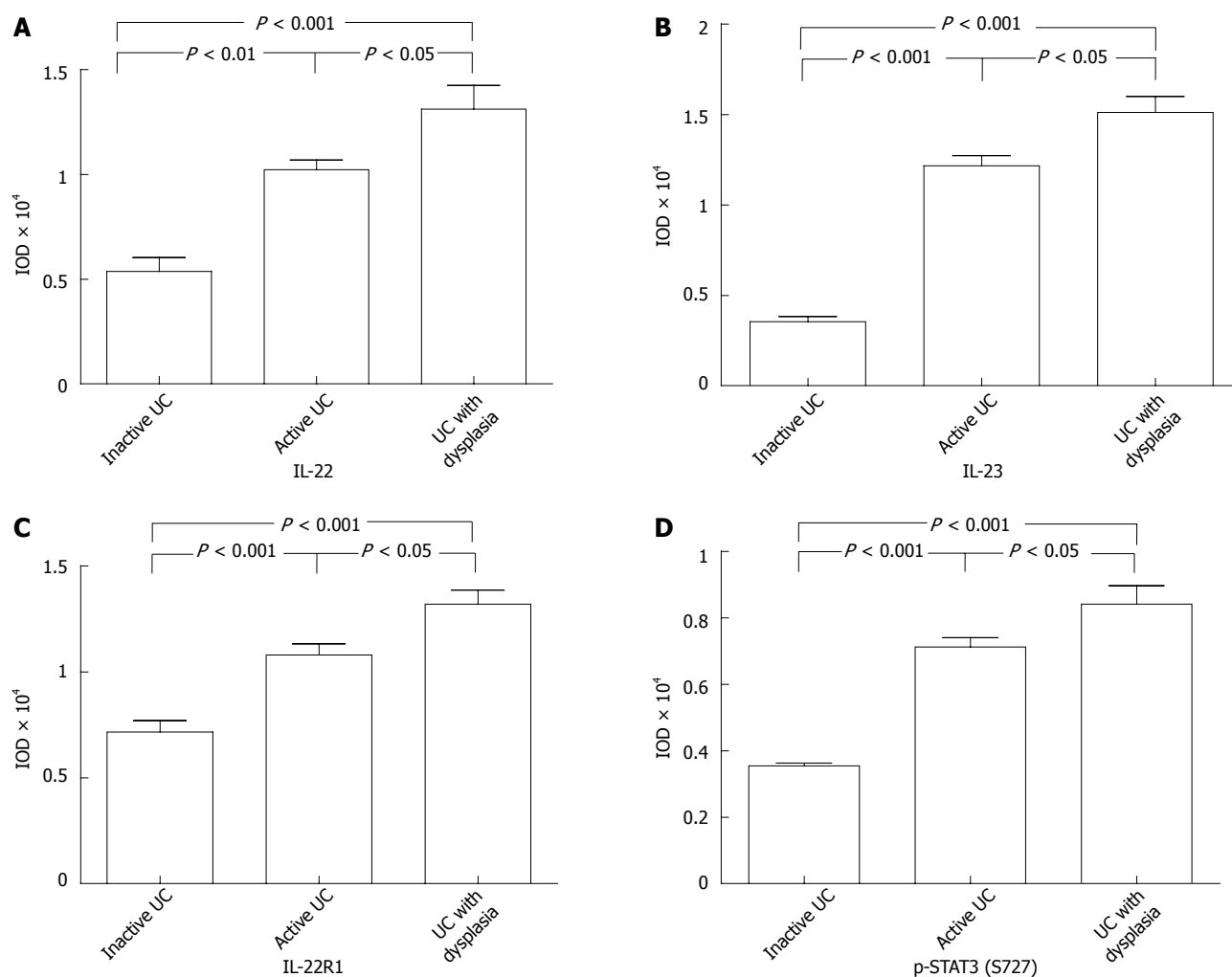


Figure 6 Statistical evaluation of interleukin-22 and its related proteins in human colonic biopsy specimens from patients with inactive ulcerative colitis, active ulcerative colitis, and ulcerative colitis with dysplasia. The average integrated optical density (IOD) for immunohistochemistry staining of interleukin (IL)-22 (A), IL-23 (B), IL-22R1 (C), and p-STAT3 (S727) (D) in colonic tissues with ulcerative colitis (UC) and UC-related carcinogenesis (UC-CRC). Significantly higher expression levels of IL-22, IL-23, IL-22R1, and p-STAT3 were observed in the dysplasia group, as compared with the inactive and active UC groups.

mining UC severity.

STAT3 is a transcription factor that is activated by the binding of several cytokines, hormones, and growth factors to their respective receptors, including IL-22, IL-6, IL-23, and IL-1 β ^[34]. We propose that STAT3 signaling is disrupted in chronic inflammation and carcinogenesis. The expression levels of total STAT3 and p-STAT3 in patients with UC were persistently elevated, with a positive correlation to the degree of inflammation^[22]. Moreover, STAT3 is constitutively activated in a variety of human cancers, including colorectal cancer. This transcription factor is crucial in cancer cells because it regulates the transcription of genes involved in cell survival, apoptosis, and other cellular processes. Morikawa *et al.*^[34] found that p-STAT3 is significantly associated with poor prognosis in a data set of 724 colorectal cancers. Furthermore, STAT3 signaling has been reported to induce cancer-promoting inflammation and to inhibit antitumor immunity^[35,36]. IL-6, a main activator of STAT3, has been proven to be important for promoting UC and UC-CRC^[37]. IL-22, another inflammatory factor that

predominantly activates STAT3, has been verified in the chronic hepatitis and hepatocellular carcinoma (HCC) microenvironment; it induces tumor growth, inhibits apoptosis, and promotes metastasis *via* STAT3 activation^[38]. Consistent with other studies on chronic hepatitis and HCC, our results demonstrated that the expression levels of IL-23, IL-22, IL-22R1, and STAT3 are consistently highly expressed in human UC tissues. IL-22 and p-STAT3, in particular, are more constitutively upregulated in the chronic colitis than in the controls. During the chronic phase of the DSS-induced mice colitis model, IL-22, IL-22R1, and IL-23 were highly expressed over time. Likewise, p-STAT3 and its downstream Bcl-2, cyclin D1, and survivin genes were remarkably upregulated. Furthermore, the expression of IL-22, p-STAT3, IL-23, and IL-22R1 were significantly elevated in human UC tissues with dysplasia, as compared with inactive and active UC tissues. Our results showed that the expression levels of IL-22 and IL-22R1 were more highly elevated in the acute colitis phase, which is in accordance with earlier studies^[27,28]. We propose that IL-22 may ameliorate

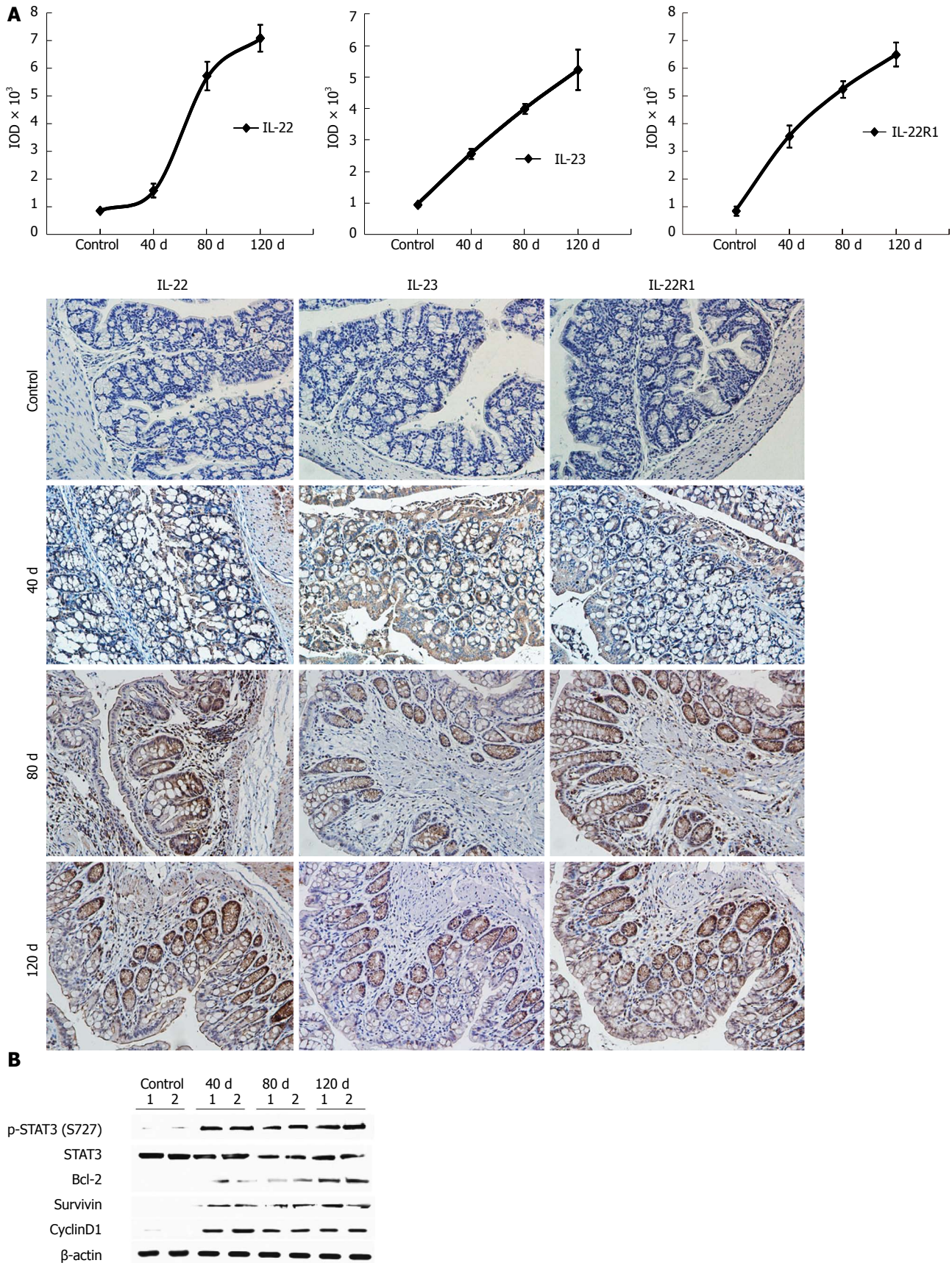


Figure 7 Expression of interleukin-22 and its related proteins in the mouse chronic colitis model. A: The average integrated optical density (IOD) for the immunohistochemistry staining of interleukin (IL)-22, IL-23, and IL-22R1 in mouse ulcerative colitis and normal tissues at different time points: days 40, 80, and 120. The expression of IL-22, IL-22R1, and IL-23 gradually increased with time; B: Western blotting detection of p-STAT3 (S727), total STAT3, Bcl-2, survivin, and cyclin D1 after DSS administration. Expression levels were all normalized to β -actin. All genes showed sustained expression over time.

intestinal inflammation by enhancing mucus production and goblet cell replacement in the early phase of inflammatory response. However, the persistent expression of IL-22 during the chronic phase of UC can strongly activate STAT3 phosphorylation in IECs, which is associated with the progression of human UC and UC-CRC^[37] by upregulating genes for cell proliferation, anti-apoptosis, and survival.

In conclusion, our study provides clinical evidence that the IL-22/STAT3 signaling pathway is related to UC and UC-CRC. Moreover, IL-22 can be used as a biomarker for determining the severity of UC and as an interesting therapeutic target in active UC and UC-CRC.

COMMENTS

Background

It has been previously reported that interleukin (IL)-22, one of the cytokines secreted by Th17 cells, promotes a protective and inflammatory effect in inflammatory bowel disease (IBD) through STAT3 signaling activation.

Research frontiers

The IL-22/STAT3 signaling pathway plays an important role in several autoimmune diseases, such as psoriasis, IBD, and so on. When activated by IL-22, STAT3 can aggravate colitis by promoting the expression of inflammatory factors such as IL-8, interferon- γ , and matrix metalloproteinases. However, some studies have found that the IL-22 induced phosphorylation of STAT3 is a defense mechanism that enhances mucus production and goblet cell replacement in mouse models of acute colitis and wound-healing. Thus, the role of the IL-22/STAT3 signaling pathway in ulcerative colitis (UC) remains unclear.

Innovations and breakthroughs

IL-22 may ameliorate intestinal inflammation by enhancing mucus production and goblet cell replacement during the early phase of the inflammatory response. However, the persistent expression of IL-22 in chronic phase of UC can strongly activate the phosphorylation of STAT3 in intestinal epithelial cells. p-STAT3 is associated with the progression of human UC and UC-related carcinogenesis (UC-CRC) because it upregulates the genes for cell proliferation, anti-apoptosis, and survival.

Peer review

The authors report about the expression of IL-22, IL-22R1, IL-23, and STAT3 in biopsies of human UC and UC-related carcinogenesis. The study is well performed; it gives an overview on the expression of the before-mentioned factors in active and chronic UC and correlates IL-22 expression with disease severity.

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Prognostic and survival analysis of 837 Chinese colorectal cancer patients

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Abstract

AIM: To develop a prognostic model to predict survival of patients with colorectal cancer (CRC).

METHODS: Survival data of 837 CRC patients undergoing surgery between 1996 and 2006 were collected and analyzed by univariate analysis and Cox proportional hazard regression model to reveal the prognostic factors for CRC. All data were recorded using a standard data form and analyzed using SPSS version 18.0 (SPSS, Chicago, IL, United States). Survival curves were calculated by the Kaplan-Meier method. The log rank test was used to assess differences in survival. Univariate hazard ratios and significant and independent predictors of disease-specific survival and were identified

by Cox proportional hazard analysis. The stepwise procedure was set to a threshold of 0.05. Statistical significance was defined as $P < 0.05$.

RESULTS: The survival rate was 74% at 3 years and 68% at 5 years. The results of univariate analysis suggested age, preoperative obstruction, serum carcinoembryonic antigen level at diagnosis, status of resection, tumor size, histological grade, pathological type, lymphovascular invasion, invasion of adjacent organs, and tumor node metastasis (TNM) staging were positive prognostic factors ($P < 0.05$). Lymph node ratio (LNR) was also a strong prognostic factor in stage III CRC ($P < 0.0001$). We divided 341 stage III patients into three groups according to LNR values (LNR1, $LNR \leq 0.33$, $n = 211$; LNR2, $LNR 0.34-0.66$, $n = 76$; and LNR3, $LNR \geq 0.67$, $n = 54$). Univariate analysis showed a significant statistical difference in 3-year survival among these groups: LNR1, 73%; LNR2, 55%; and LNR3, 42% ($P < 0.0001$). The multivariate analysis results showed that histological grade, depth of bowel wall invasion, and number of metastatic lymph nodes were the most important prognostic factors for CRC if we did not consider the interaction of the TNM staging system ($P < 0.05$). When the TNM staging was taken into account, histological grade lost its statistical significance, while the specific TNM staging system showed a statistically significant difference ($P < 0.0001$).

CONCLUSION: The overall survival of CRC patients has improved between 1996 and 2006. LNR is a powerful factor for estimating the survival of stage III CRC patients.

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Key words: Colorectal cancer; Prognostic factors; Cox proportional hazard regression; Lymph node ratio

Core tip: Recent reports and reviews have highlighted the importance of metastatic lymph node and Lymph

node ratio (LNR) in predicting prognosis of colorectal cancer (CRC). We found that the histological grade, depth of bowel wall invasion, and number of metastatic lymph nodes were the most important prognostic factor for CRC without consideration of the interaction of the tumor node metastasis staging system. LNR was a powerful factor for estimating the survival of stage III CRC. This paper presents new results on the 5-year overall survival and prognostic factors in Chinese CRC patients.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies and one of the most common causes of cancer-related death worldwide^[1]. An estimated 143460 new cases of CRC will be diagnosed this year, and 51690 patients will succumb to their disease in the United States alone^[2]. Meanwhile, with the continuous aging of the population and an increased tendency to adopt a western lifestyle, the incidence of CRC and its related mortality is gradually increasing and it has become the fifth most common of all cancers in China^[3,4]. Thus, the importance of CRC as a public health problem is increasing in China.

Over the past two decades, the 5-year overall survival of CRC patients has improved. Some advanced CRC patients have received clear survival benefits due to the practice of resecting liver metastases and advances in surgical techniques^[5]. For those patients who have missed the opportunity for surgery, chemotherapy is still the main treatment. Although the overall survival of advanced CRC patients is still poorer than for early stage patients, it is encouraging that the combination of chemotherapy and targeted drugs may have the potential to improve survival.

In clinical practice, clinicians need an accurate outcome prediction of CRC patients to devise an appropriate therapeutic strategy. However, many variables may influence the prognosis, including both patient and tumor characteristics^[6]. Therefore, we conducted the present study to explore the relevant factors affecting the prognosis of CRC patients using existing data in the Second Affiliated Hospital of Zhejiang University College of Medicine, China.

MATERIALS AND METHODS

Patients and clinical data

A total of 837 patients with CRC that underwent surgery

at the Department of Surgical Oncology at the Second Affiliated Hospital of Zhejiang University College of Medicine from January 1996 to December 2006 were enrolled from our database. All clinical cases and their follow-up data were recorded. The data included sex, age at diagnosis, clinical symptoms, severe complications, location of the primary tumor, histological type, tumor differentiation, lymphovascular invasion, depth of invasion, numbers of retrieved lymph nodes and metastatic lymph nodes, date of surgery, date of recurrence (if applicable), cause of recurrence (if applicable), date of death (if applicable), cause of death (if applicable), postoperative treatment, and date of follow-up. This study consisted of stages I-IV CRC patients. No local or systemic treatment had been conducted preoperatively. Patients' blood samples were collected before their operation and their carcinoembryonic antigen (CEA) levels were analyzed. Specimens were fixed in formalin and stained with hematoxylin-eosin (HE) and used for histopathological evaluation. The 6th and the 7th editions of the Union for International Cancer Control (UICC) classification were used to categorize colorectal carcinomas. Rectal cancer was defined as carcinomas with a distal margin of 15 cm from the anal verge measured with a rigid endoscope.

Follow-up duration

All patients were followed up at 3-mo intervals for the first 2 years, and 6-mo intervals for 3-5 years. Follow-up was completed for the entire study population by March 2011, and the median follow-up period was 45 mo. The baseline of the study cases are shown in Table 1 (six cases of double primary CRC were excluded from Table 1).

Statistical analysis

All data were recorded using a standard data form and analyzed using SPSS version 18.0 (SPSS, Chicago, IL, United States). Survival curves were calculated by the Kaplan-Meier method. The log rank test was used to assess differences in survival. Univariate hazard ratios and significant and independent predictors of disease-specific survival and were identified by Cox proportional hazard analysis. The stepwise procedure was set to a threshold of 0.05. Statistical significance was defined as $P < 0.05$.

RESULTS

A total of 837 patients with CRC were enrolled. The 3-year and 5-year survival for all 837 patients was 74% and 68%, respectively. Table 2 summarizes the univariate analysis results of different clinical and pathological features.

Most patients ($n = 808$) were diagnosed in middle age (median age: 60 years, range: 19-91 years) and 29 were diagnosed at ≤ 35 years of age. Patients were divided into four groups according to age at diagnosis: age1 ≤ 35 years, age2 36-59 years, age3 60-74 years, and age4 ≥ 75 years (Figure 1A). A significant difference in 5-year

Table 1 Basic data for patients with colorectal cancer *n* (%)

Basic data	Colon cancer (<i>n</i> = 437)	Rectal cancer (<i>n</i> = 394)
Sex		
Male	245 (56.1)	245 (62.2)
Female	192 (43.9)	149 (37.8)
Age at operation ¹ (yr)	60.9 ± 13.1	58.3 ± 12.7
Dukes' staging		
A	38 (8.7)	81 (20.6)
B	181 (41.4)	117 (29.7)
C	166 (38.0)	172 (43.7)
D	49 (11.2)	23 (5.8)
Status of resection		
Curative	356 (81.5)	349 (88.6)
Palliative	62 (14.2)	33 (8.4)
Undefined	19 (4.3)	12 (3.0)
Tumor size		
≥ 5 cm	154 (35.2)	64 (16.2)
< 5 cm	247 (56.5)	263 (66.8)
Undefined	36 (8.2)	67 (17.1)
Histological differentiation grade		
Well	93 (21.3)	118 (29.9)
Moderate	184 (42.1)	180 (45.7)
Poor	108 (24.7)	61 (15.5)
Undefined	52 (11.9)	35 (8.9)
Lymphovascular invasion		
Positive	14 (3.2)	10 (2.5)
Negative	423 (96.8)	384 (97.5)
Perineural invasion		
Positive	11 (2.5)	3 (0.8)
Negative	423 (96.8)	391 (99.2)
Invasion of adjacent organs		
Positive	37 (8.5)	15 (3.8)
Negative	396 (90.6)	379 (96.2)
Undefined	4 (0.9)	0 (0.0)

¹Data are expressed as mean ± SD. Six cases of double primary colon and rectal cancer were not included.

survival was found between these four groups: age1 65%, age2 66%, age3 74%, and age4 53% ($P = 0.002$).

Among the 837 patients, 495 were male and 342 were female. There was no sex difference in survival ($P = 0.834$). Clinical features of 437 colon cancer patients and 394 rectal cancer patients were recorded. We also found six cases of double primary colon cancer and rectal cancer. In spite of a higher incidence of colon cancer, there were no significant differences in survival between patients with colon cancer and rectal cancer.

There were 25 patients who had a family history of CRC. It seemed that they had a trend toward better survival than the other 812 patients without a CRC-related family history. The difference was not statistically significant; 3-year survival was 91% *vs* 73% and 5-year survival was 82% *vs* 68% ($P = 0.391$).

According to the results of univariate analysis, patients with obvious clinical symptoms, such as tumor-related obstruction, perforation, diarrhea, constipation, and change of bowel habits had a shorter survival (Table 2). However, only the difference in tumor-related obstruction was statistically significant. The 3-year and 5-year

Table 2 Univariate analysis of the prognostic factors for patients with colorectal cancer

	<i>n</i>	3-YSR	5-YSR	<i>P</i> value ¹
Age group (yr)				0.002
Age1 (≤ 35)	29	65%	65%	
Age2 (36–59)	370	73%	66%	
Age3 (60–74)	334	78%	74%	
Age4 (≥ 75)	104	61%	53%	
Sex				0.834
Male	495	73%	67%	
Female	342	74%	69%	
Family history of CRC				0.391
Negative	812	73%	68%	
Positive	25	91%	82%	
Obstruction				0.000
Negative	790	76%	70%	
Positive	45	39%	35%	
Perforation				0.629
Negative	824	74%	68%	
Positive	11	68%	68%	
Bleeding				0.116
Negative	289	69%	66%	
Positive	546	76%	69%	
Diarrhea				0.421
Negative	750	75%	68%	
Positive	85	65%	63%	
Constipation				0.415
Negative	776	74%	68%	
Positive	59	72%	66%	
Habits changes				0.547
Negative	531	74%	69%	
Positive	304	73%	66%	
Serum CEA level				0.042
≤ 5 ng/mL	661	74%	69%	
> 5 ng/mL	172	71%	62%	
Status of resection				0.000
Curative	711	80%	74%	
Palliative	95	29%	22%	
Tumor location				0.705
Colon cancer	437	73%	69%	
Rectal cancer	394	74%	66%	
Double primary of colon and rectal cancer	6	75%	75%	
Tumor size				0.004
< 5 cm	516	77%	71%	
≥ 5 cm	218	67%	62%	
Histological differentiation grade				0.001
Well	212	78%	71%	
Moderate	366	73%	65%	
Poor	170	62%	60%	
Pathological types				0.036
Non-mucous cell carcinoma	663	76%	70%	
Mucous cell carcinoma	141	63%	59%	
Lymphovascular invasion				0.000
Negative	813	75%	69%	
Positive	24	44%	36%	
Perineural invasion				0.057
Negative	820	74%	68%	
Positive	14	42%	42%	
Invasion of adjacent organs				0.000
Negative	781	75%	70%	
Positive	52	43%	33%	

¹*P* values were made by log-rank test. 3-YSR: 3-year accumulative survival rate; 5-YSR: 5-year accumulative survival rate; CEA: Carcino-embryonic antigen; CRC: Colorectal cancer.

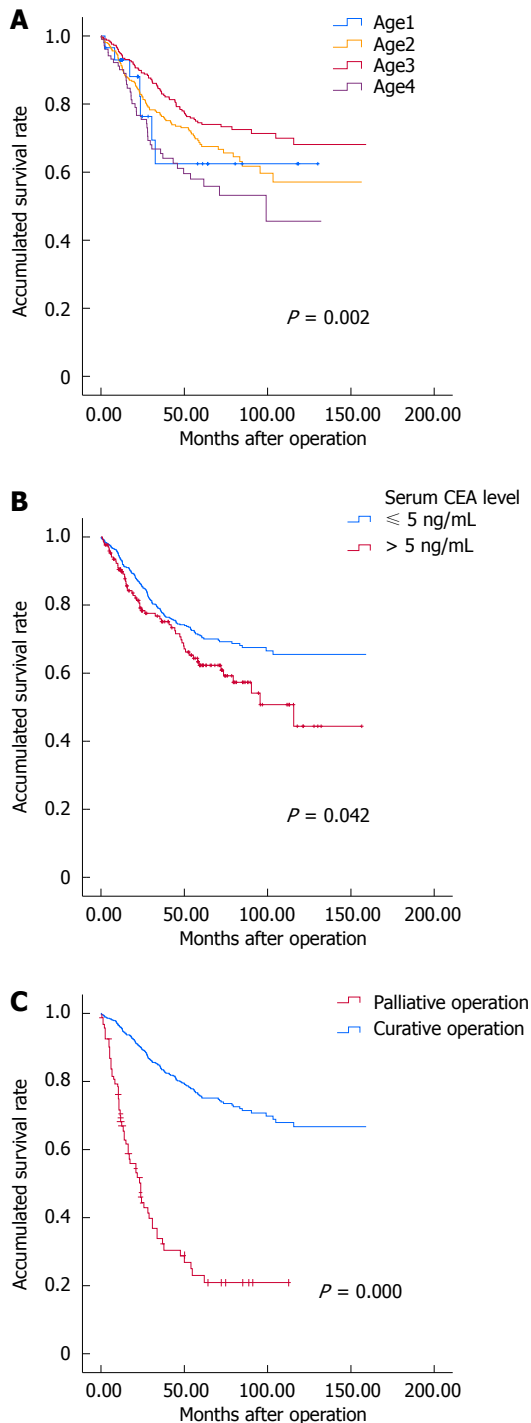


Figure 1 Survival curves of colorectal cancer patients. A: In different age groups; B: With different carcino-embryonic antigen levels; C: With different operation status. CEA: Carcinoembryonic antigen.

survival of 45 patients with preoperative bowel obstruction was 39% and 35% respectively *vs* 76% and 70% in patients without symptoms ($P < 0.0001$). In addition to the clinical symptoms, serum carcino-embryonic antigen (CEA) level is commonly used as a screening and predictive factor for CRC patients (Figure 1B). In our study, the prognosis for patients with high CEA levels of > 5 ng/mL at diagnosis was worse than those who with low CEA levels; 3-year survival was 71% *vs* 74% and 5-year

survival 62% *vs* 69% ($P = 0.042$).

Surgery plays an important role in the treatment of CRC, and radical resection of tumors also has a major influence on prognosis. In our study, 711/837 CRC patients underwent curative surgery, while 95 had palliative surgery due to serious complications or for other reasons (Figure 1C). Compared with patients who had curative surgery, there was a significant decrease in postoperative survival in patients who had palliative surgery; 3-year survival of 80% *vs* 29% and 5-year survival of 74% *vs* 22% ($P < 0.0001$). This confirms that curative surgery is one of the crucial factors affecting prognosis of CRC patients. In addition, the maximum length of the primary lesion, tumor differentiation, histological type, depth of bowel wall invasion, lymphovascular invasion, and invasion of adjacent organs may affect the prognosis of CRC patients ($P < 0.05$, Table 2).

Currently, the TNM staging system is widely accepted for tumor staging globally, and also represents the main staging system in our country. The 6th revision is regarded as being a significant improvement in CRC staging and the 7th revision is considered to be a major turning point in the evolution of cancer staging^[7]. Regardless of the edition used for staging, survival of CRC patients gradually declined with increase in depth of infiltration of the primary tumor, the number of positive lymph nodes estimated, and status of distant metastases (Table 3, Figure 2). We also found that survival of stage IIIA patients was better than of stage IIB patients regardless of which edition was used to classify postoperative staging: with the 6th edition, 5-year survival of stage IIB and IIIA was 75% and 87% ($P < 0.0001$), and for the 7th edition, 5-year survival of stages IIB and IIIA was 75% and 91% ($P < 0.0001$).

LNR is defined as the ratio of positive lymph nodes divided by the total number of retrieved lymph nodes, and does not depend on the number of lymph nodes harvested^[8]. It is considered to be an independent factor that reflects survival of CRC patients, especially those with stage III disease. We calculated the LNR values of 341 stage III cases. The mean LNR was 0.34 (median: 0.25, range: 0-1). Patients were divided into the following three LNR subgroups: LNR1, LNR ≤ 0.33 , $n = 211$; LNR2, LNR 0.34-0.66, $n = 76$; and LNR3, LNR ≥ 0.67 , $n = 54$ (Figure 3). Survival among these three groups was significantly different ($P < 0.0001$).

After we calculated the positive factors by univariate analysis, we used multivariate analysis (Cox proportional hazard model) to find the most significant prognostic factors (Table 4). First, we analyzed the interaction of the positive clinicopathological factors from univariate analysis, and multivariate analysis showed that histological grade, depth of bowel wall invasion, and number of metastatic lymph nodes affected the prognosis of CRC patients ($P < 0.05$). We performed another two separate multivariate analyses with the 6th and 7th TNM staging systems. We found that histological grade was no longer a positive item when considering the interaction of the

Table 3 Univariate analysis of tumor node metastasis staging system for patients with colorectal cancer

6 th edition of TNM staging system					7 th edition of TNM staging system				
	<i>n</i>	3-YSR	5-YSR	<i>P</i> value		<i>n</i>	3-YSR	5-YSR	<i>P</i> value
pT				0.000	pT				0.000
T1	35	100%	100%		T1	35	100%	100%	
T2	128	87%	86%		T2	128	87%	86%	
T3	324	73%	66%		T3	324	73%	66%	
T4	345	66%	59%		T4a	303	69%	62%	
					T4b	42	45%	33%	
Undefined	5	78%	78%		Undefined	5	78%	78%	
pN				0.000	pN				0.000
N0	445	86%	80%		N0	444	86%	80%	
N1	224	68%	61%		N1a	103	71%	63%	
N2	168	48%	43%		N1b	120	66%	61%	
					N1c	2	50%	/	
					N2a	82	54%	43%	
					N2b	86	42%	42%	
pM				0.000	pM				0.000
M0	765	78%	73%		M0	765	78%	73%	
M1	72	28%	18%		M1a	49	29%	19%	
					M1b	23	26%	17%	
Stage				0.000	Stage				0.000
I	121	93%	93%		I	121	93%	93%	
II A	173	88%	81%		II A	173	88%	81%	
II B	125	85%	75%		II B	121	85%	75%	
III A	33	87%	87%		II C	5	100%	100%	
III B	168	68%	61%		III A	33	91%	91%	
III C	141	53%	48%		III B	199	69%	61%	
IV	72	28%	18%		III C	109	47%	44%	
					IV A	49	29%	19%	
					IV B	23	26%	17%	
Undefined	4	100%	100%		Undefined	4	100%	100%	

3-YSR: 3-year accumulative survival rate; 5-YSR: 5-year accumulative survival rate; TNM: Tumor node metastasis.

TNM staging system (Table 4). Results for the 6th and 7th TNM staging systems in multivariate analysis showed significant differences (Table 4, *P* < 0.0001). Another two factors, the depth of bowel wall invasion and the number of metastatic lymph nodes, showed a positive statistical significance, regardless of which TNM staging system was used (Table 4, *P* < 0.05). Besides, with the increase in the number of metastatic lymph nodes with each level, the relative risk of death of CRC patients will increase 1.093 times without consideration of an exact clinical staging. However, this risk decreased to 1.037 times using the 6th TNM staging system and 1.047 times using the 7th system.

DISCUSSION

CRC is the fifth most common cancer in China^[3]. The morbidity and mortality of CRC have shown a clear upward trend in both urban and rural areas over the past 30 years. Although there has been an improvement in surgical techniques and treatment, the 5-year overall survival of CRC is still hovering around 60%. Park *et al*^[9] have reported a 5-year survival rate of 67.2% in 2230 cases of CRC. In China, Lv *et al*^[10] has reported 5-year survival rates of 58.4% and 64.5% 383 cases in colon and rectal cancer patients, respectively. In our study, the 3-year and 5-year survival of CRC patients was 74% and 68%, respectively.

The postoperative 5-year survival increased to 74% in our hospital, compared with 66% during 1980-1999^[11,12].

From 1980 to the 1990s, rectal cancer accounted for the main part of the incidence of CRC in China^[4,11]. However, data from Table 2 showed a higher proportion of colon cancer than rectal cancer in our hospital from 1996 to 2006; with 437 cases *vs* 394 cases. Other researchers have reported similar results, which suggests that the proportion of rectal cancer cases is gradually declining^[13-15]. Although the reason for the change is unclear, some experts have suggested that the higher incidence of colon cancer might be a complex result of changes in dietary habits, the higher rate of diagnosis of colon cancer, etiological changes, and the increased incidence of right colon cancer^[16-20].

In addition to the change in location of disease, the age at onset has also changed. Previously, CRC had a higher incidence in elderly people^[21]. However, recent results at home and abroad have found that detection of CRC in the younger population is increasing^[22]. CRC in young patients is generally considered a more aggressive disease, which presents at a later stage and has poorer pathological features^[23,24]. Zhong *et al*^[25] have reported only a 27.51% 5-year survival rate in young Chinese patients with CRC. In our study, the 5-year survival in the low-age group (age1) was 65%, which was slightly lower than the overall rate (68%), although it had improved

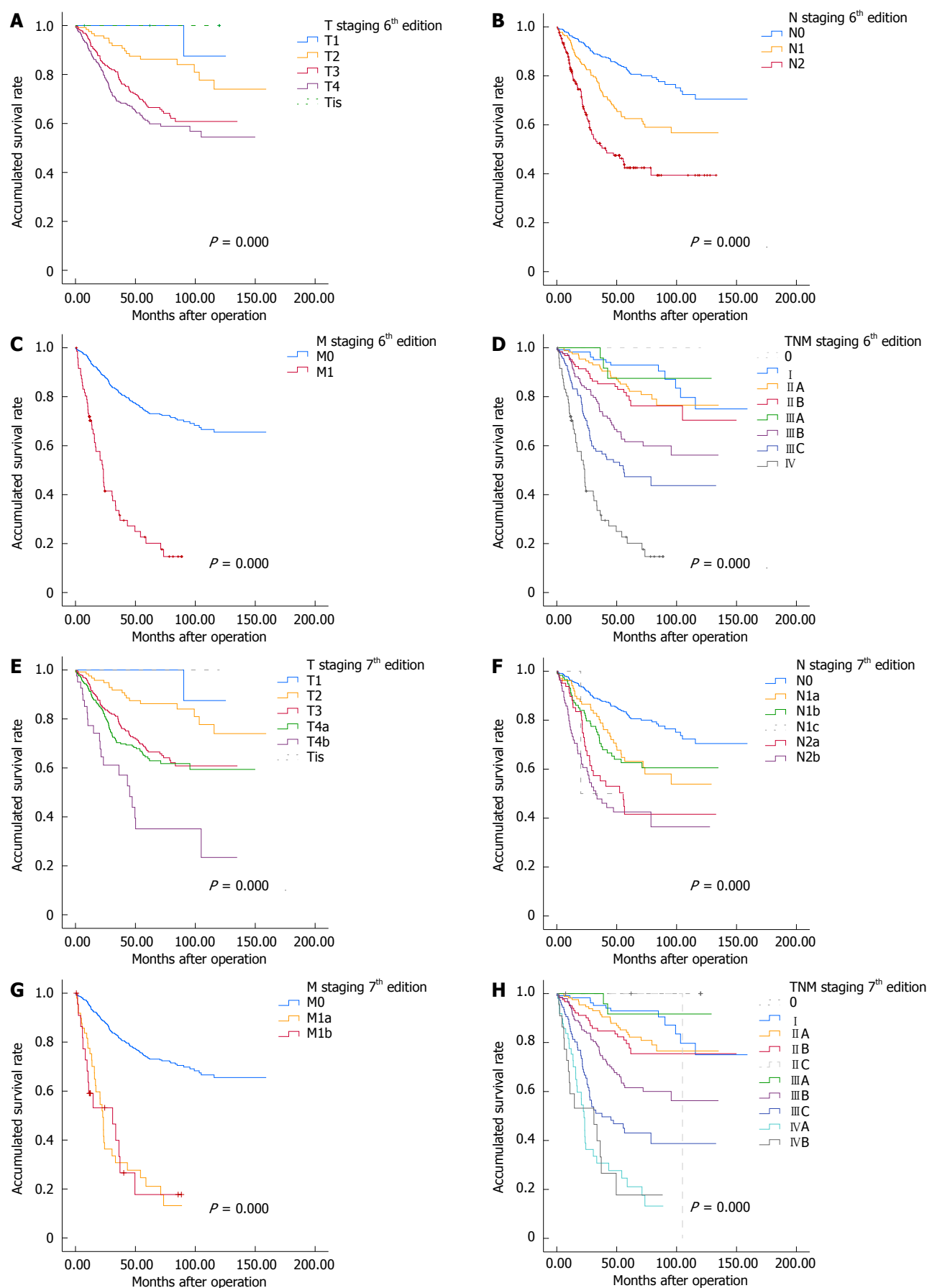


Figure 2 Survival curves of colorectal cancer patients. A-D: According to the 6th edition of the tumor node metastasis classification; E-H: According to the 7th edition of the tumor node metastasis classification.

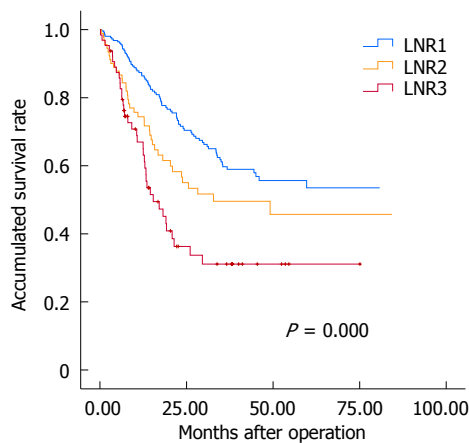


Figure 3 Survival curves of colorectal cancer patients in different lymph node ratio groups. LNR: Lymph node ratio.

from the 5-year survival rate of 53% in patients aged ≤ 40 years at our hospital between 1980 and 1999^[26]. It should be noted that there is no international standard definition of young or old, and the definition of low age in our study is different from that used by Cai *et al.*^[26]. The overall survival between different age groups showed a significant difference in univariate analysis (Figure 1A), but failed to show a significant difference in the multivariate analysis.

Some reports have suggested that several clinicopathological features contribute to the unfavorable prognosis of CRC in young patients^[27-29]. A review of the literature has suggested that younger patients with CRC, without relevant predisposing risk factors, have more advanced stages of disease, more aggressive histopathological characteristics, and a poorer prognosis compared with older patients^[24]. However, there is also some evidence to show that cancer-related survival in young CRC patients seems no less favorable compared with older patients^[30-32].

The current international standard for CRC staging is the TNM system. The 7th edition of TNM staging, developed by the UICC and American Joint Committee on Cancer, has undergone some significant changes from the 6th edition. We tested which of the two versions could predict survival more accurately. Results of univariate analysis showed values in both staging systems were statistically significant prognostic factors ($P < 0.05$). Figure 2D and H demonstrate the differences from stage I to stage IV disease. Similarly, both the 6th and 7th TNM staging systems were effective for judging the clinical survival and prognosis of CRC based on the results of multivariate analysis. The results also suggest a higher relative risk of death in CRC patients with more metastatic lymph nodes with an unclear clinical staging. It is worth noting that the patients with stage IIIA disease had a better survival than patients with stage IIB disease, as determined from the follow-up data. It might be explained by stage IIIA patients routinely receiving chemotherapy after their operation as part of current clinical practice, while stage IIB patients do not. Some authors also hold the view that lower survival of stage II CRC patients might be

Table 4 Multivariate analysis (Cox proportional hazard model) of prognostic factors

	P value	RR	95%CI
Without interplay tumor node metastasis staging system			
Age group	0.060	1.193	0.993-1.434
Obstruction	0.241	1.011	0.993-1.030
Tumor size	0.257	1.002	0.998-1.006
Serum CEA level	0.690	0.996	0.978-1.015
Status of resection	0.082	1.005	0.999-1.012
Histological grade	0.007	0.991	0.984-0.998
Pathological types	0.817	0.999	0.992-1.006
Depth of bowel wall invasion	0.000	1.047	1.028-1.067
Lymphovascular invasion	0.695	0.974	0.854-1.111
Invasion of adjacent organs	0.942	0.998	0.949-1.050
Number of metastatic lymph nodes	0.000	1.093	1.073-1.114
With interplay 6 th tumor node metastasis staging system			
Age group	0.054	1.194	0.997-1.430
Obstruction	0.386	1.008	0.990-1.028
Tumor size	0.259	1.002	0.998-1.006
Serum CEA level	0.789	0.997	0.979-1.017
Status of resection	0.136	1.005	0.999-1.011
Histological grade	0.114	0.995	0.988-1.001
Pathological types	0.290	0.996	0.989-1.003
Depth of bowel wall invasion	0.014	1.028	1.006-1.050
Lymphovascular invasion	0.758	0.981	0.869-1.108
Invasion of adjacent organs	0.840	0.994	0.935-1.056
Number of metastatic lymph nodes	0.006	1.037	1.010-1.065
6 th TNM staging	0.000	1.471	1.344-1.610
With interplay of 7 th tumor node metastasis staging system			
Age group	0.094	1.168	0.974-1.400
Obstruction	0.434	1.008	0.989-1.027
Tumor size	0.289	1.002	0.998-1.006
Serum CEA level	0.768	0.997	0.978-1.016
Status of resection	0.184	1.004	0.998-1.010
Histological grade	0.109	0.995	0.988-1.001
Pathological types	0.283	0.996	0.989-1.003
Depth of bowel wall invasion	0.023	1.025	1.003-1.048
Lymphovascular invasion	0.779	0.983	0.873-1.107
Invasion of adjacent organs	0.802	0.992	0.930-1.058
Number of metastatic lymph nodes	0.002	1.041	1.015-1.069
7 th TNM staging	0.000	1.354	1.261-1.454

RR: Relative risk; CEA: Carcino-embryonic antigen; TNM: Tumor node metastasis.

related to the particular biological behavior of stage II tumors^[33-35].

Lymph node metastasis is a significant component of TNM staging of CRC. Tumor stage and the number of lymph nodes retrieved at resection influence the accuracy of determining nodal status in CRC. They also influence the postoperative treatment strategy of CRC patients. In our study, we took the T, N and M stage as factors in univariate analysis and obtained positive results (Table 3, Figure 2). In addition, multivariate analysis demonstrated a strong relationship between the number of metastatic lymph nodes and survival of CRC patients (Table 4). The relative risk of death is increased with the number of metastatic lymph nodes. The number of lymph nodes found after surgical resection was positively associated with survival of patients with stage II and III colon cancer^[36,37]. An underestimation of the nodal stage may lead to a high risk of local recurrence and influence decisions regarding adjuvant therapy, as well as influenc-

ing the overall prognosis^[38-41]. According to the result of the INT-0089 trial, National Comprehensive Cancer Network Colon Cancer Clinical Practice Guidelines recommend that retrieval and examination of ≥ 12 lymph nodes can be regarded as adequate lymphadenectomy for accurate staging^[42].

There is a difference between the number of metastatic lymph nodes reported during surgery and the actual number of metastatic lymph nodes. The difference may result from many factors, including the extent of surgical dissection and the thoroughness of the pathologists. Cases with insufficient retrieval and undetected lymph nodes are not unusual in clinical practice, although the concept of taking a sufficient number of lymph nodes during surgery to ensure exact postoperative staging is currently agreed. Evaluating lymph node metastasis has become a prognostic factor for CRC, and LNR is an important component of staging. LNR has also been identified as being of significant prognostic value in breast and gastric cancer^[43,44]. Berger *et al*^[45] were the first to suggest LNR as an important prognostic factor after curative resection for CRC. It was then established as a powerful independent index of CRC that reflected the probability of positive lymph nodes based on the number of retrieved lymph nodes^[8,46-48]. In our study, we found a dramatic decrease in survival with an increase in LNR in stage III CRC patients ($P < 0.0001$, Figure 3).

Although the LNR has been emphasized as an important prognostic factor, quantification should be followed for clinical validity. Song *et al*^[49] have compared three prognostic factors of CRC and have concluded that LNR classification is a more reliable N classification than the nodal staging in the TNM system and LODDS:

$$\text{defined as } \log \frac{\text{pnod} + 0.5}{\text{tnod} - \text{nnod} + 0.5},$$

pnod is the number of positive lymph nodes, tnod is the total number of lymph nodes retrieved, and 0.5 is added to both numerator and denominator to avoid singularity^[49]. They believe that LNR is superior to the other two indexes for the following reasons: (1) LNR could contribute to accuracy in prognostic assessment; (2) when the retrieved lymph node numbers is insufficient, TNM nodal staging will be inappropriate for staging migration and will even underestimate prognosis; and (3) as a novel indicator for predicting the status of lymph nodes, evidence of LODDS in CRC is inadequate and is more difficult to calculate and inconvenient for clinical practice^[50]. When the number of examined lymph nodes is inadequate, LNR is a simple and powerful index to assess the prognosis of CRC patients.

In conclusion, based on the results from our study, we were delighted to find the overall survival in our hospital had improved between 1996 and 2006. Younger patients with CRC have attracted attention because of the increasing number of new cases, their adverse clinicopathological features, and poor prognosis. However, there is still a debate about the prognosis and clinicopathological

features of CRC in young compared to old patients. The pathogenesis and mechanism of disease are still unclear. The overall survival in patients with stage IIIA CRC was better than that in patients with stage IIB disease. This might be a combination of the special biological behavior of stage II CRC and the type of medical intervention for stage III CRC patients. The exact mechanisms of these problems and phenomena need further study.

By using multivariate analysis, we found that tumor histological grade, depth of bowel wall invasion, and metastatic lymph node numbers were independent prognostic factors for patients with CRC if we did not consider the exact clinical staging. We also found other important factors that could affect the prognosis of patients with CRC by univariate analysis, such as patient age, status of resection, and invasion of adjacent organs. The relative risk of death in CRC patients increases with the number of metastatic lymph nodes with an unclear clinical staging, which emphasizes the importance of correct clinical staging.

Surgeons know that a curative operation can greatly improve the overall survival of CRC patients, and resection of a sufficient number of lymph nodes is a necessity for proper postoperative staging. LNR is a powerful factor for assessment of prognosis in stage III CRC patients and is worthy of use in daily practice for evaluating a patient's risk of death. However, we should combine it with other complex factors that together can make a complete assessment so we can devise a proper plan for further treatment.

Besides appropriate treatment, a sensible follow-up plan should be given to CRC patients with full consideration of the factors mentioned above. Moreover, we should devise treatment strategies carefully based on the concept of individualized treatment according to each patient's clinical features, to improve survival and prognosis, especially for those patients with risk factors. In addition, early screening and surveillance by appropriate methods may improve the overall survival of CRC.

COMMENTS

Background

In recent years, the morbidity and mortality of colorectal cancer (CRC) has risen in the Chinese population. Although the 5-year overall survival of CRC patients has improved, the overall survival of advanced CRC patients is still poor. There are many impact factors that could influence the prognosis of CRC patients. Thus, a proper model for predicting the prognosis of CRC patients is necessary for both surgeons and physicians.

Research frontiers

Nowadays, the tumor, node, metastasis (TNM) staging system is approved and widely used for clinical staging of CRC patients. As the latest version of TNM staging system, the 7th edition of TNM staging system is considered to represent a major turning point in the evaluation of CRC staging. However, less information of the real assessment validity between the 6th and 7th versions is available in Chinese populations.

Innovations and breakthroughs

Recent reports and reviews have highlighted the importance of metastatic lymph node and lymph node ratio (LNR) in predicting prognosis of CRC patients. LNR is an easy but powerful index to evaluate prognosis in stage III CRC patients.

Applications

Using univariate analysis and Cox proportional hazard regression model, we found that histological grade, depth of bowel wall invasion, and number of metastatic lymph nodes were the most important prognostic factors for CRC without consideration of the interaction of the TNM staging system. LNR is a powerful factor for estimating the survival of stage III CRC patients.

Terminology

LNR is defined as the ratio of positive lymph nodes divided by the total number of retrieved lymph nodes, and does not depend on the number of lymph nodes harvested. It is considered an independent factor that reflects survival of CRC patients, especially those with stage III disease.

Peer review

This article is helpful and creative for clinical significance. The results of this article verified the predictive affection of tumor invasion, lymph node metastasis and lymph node ratio. Meanwhile, it concludes that the 6th and 7th National Comprehensive Cancer Network TNM staging systems are both effective to predict the survival of colorectal cancer patients.

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Short- and long-term efficacy of endoscopic balloon dilation in Crohn's disease strictures

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Abstract

AIM: To evaluate short- and long-term efficacy of endoscopic balloon dilation in a cohort of consecutive patients with symptomatic Crohn's disease (CD)-related strictures.

METHODS: Twenty-six CD patients (11 men; median age 36.8 year, range 11-65 years) with 27 symptomatic strictures underwent endoscopic balloon dilation (EBD).

Both naive and post-operative strictures, of any length and diameter, with or without associated fistula were included. After a clinical and radiological assessment, EBD was performed with a Microvasive Rigiflex through the scope balloon system. The procedure was considered successful if no symptom reoccurred in the following 6 mo. The long-term clinical outcome was to avoid surgery.

RESULTS: The mean follow-up time was 40.7 ± 5.7 mo (range 10-94 mo). In this period, forty-six EBD were performed with a technical success of 100%. No procedure-related complication was reported. Surgery was avoided in 92.6% of the patients during the entire follow-up. Two patients, both presenting ileocecal strictures associated with fistula, failed to respond to the treatment and underwent surgical strictures resection. Of the 24 patients who did not undergo surgery, 11 patients received 1 EBD, and 13 required further dilations over time for the treatment of relapsing strictures (7 patients underwent 2 dilations, 5 patients 3 dilations, and 1 patient 4 dilations). Overall, the EBD success rate after the first dilation was 81.5%. No difference was observed between the EBD success rate for naive ($n = 12$) and post-operative ($n = 15$) CD related strictures ($P > 0.05$).

CONCLUSION: EBD appears to be a safe and effective procedure in the therapeutic management of CD-related strictures of any origin and dimension in order to prevent surgery.

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Key words: Endoscopic balloon dilation; Crohn's disease; Strictures; Endoscopy; Gastrointestinal surgery

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INTRODUCTION

In the last two decades, the medical therapy for Crohn's disease (CD) has remarkably improved and the introduction of biological therapies has dramatically changed the therapeutic approach in both adults and children^[1,2]. However, CD still displays an unpredictable clinical course with high incidence of recurrence frequently leading to complications such as strictures, fistulas and abscesses^[3]. At the time of diagnosis, intestinal strictures may occur throughout the gastrointestinal tract in about 5% of patients, whereas up to one third of the patients develop an intestinal strictures within 10 years of disease activity, with majority of them occurring at the terminal ileum, ileo-colonic, and colonic level^[2,4,5].

CD-related strictures is defined as a constant luminal narrowing, which can remain clinically silent or manifest with prestenotic dilatation and obstructive symptoms, such as abdominal bloating, distention, and pain. As the result of the continuous healing response to the chronic inflammation within the intestinal walls, the intestinal stricture induces a progressive narrowing of the lumen and an increased pressure gradient around the stricture, which might ultimately result into the development of internal fistula proximally to the obstruction^[6]. Stricture-associated fistulas contribute to increase the disease severity and worsen the clinical management.

CD strictures generally show a poor response to medical therapies, and surgical bowel resection or surgical strictureplasty are often required^[5,7,8]. In patients with CD, intestinal surgery is needed for as many as 80% of CD patients, and a permanent stoma is required in more than 10% of CD patients^[3]. However, high rate of relapse (defined by recurrence of clinical symptoms) is also observed after surgical resection: 40% at 4 years after bowel resection^[9,10], and 50% at 10 to 15 years after ileocecal resection^[11,12]. Strictureplasty has also been associated with a risk of stricture relapse in 34% of the cases at 7.5 years^[13]. This implies that up to one third of CD patients will undergo more than one surgery in their life course^[14-16]. Patients with an early onset disease have an increased risk of surgical relapse and need of repeated resections, which in turn may result in a short bowel syndrome^[13,17-20].

Endoscopic balloon dilation (EBD) is a minimally invasive technique that can reduce or delay the need of surgery in patients with CD-related strictures^[21,22]. With the new generation of double or single endoscopic balloon enteroscopy, this procedure can be performed at almost any level of the gastrointestinal tract. Moreover, the EBD in CD strictures appears to be a safe technique with a low complication rate (0% to 10%)^[22-24]. It has been shown that the technical success rate of the endoscopic bal-

loon dilation is 95%^[25-27], with up to 47% of CD patients showing a long-term global benefit, *i.e.*, a surgery-free period at 3 year follow-up^[22,24].

In this prospective study, we aimed to assess the effectiveness and safety of the EBD in a cohort of consecutive CD patients with symptomatic intestinal strictures.

MATERIALS AND METHODS

Study cohort

Twenty-six consecutive CD patients (11 males, 15 females), presenting 27 symptomatic strictures (one patient was treated for two strictures), and complaining up to two intestinal symptomatic obstructive episodes as suspected by plain abdominal X-rays or contrast study in the preceding 6 mo, were prospectively enrolled into the trial between March 2004 and March 2011. Diagnosis of CD in both adult and children was based on widely agreed endoscopic and histological criteria^[28,29], and disease classification was done according to the Montreal criteria for adults and Paris criteria for children^[30,31]. A detailed personal and family history was obtained from each patient. The clinical assessment of the patients was performed through the pediatric Crohn's disease activity index (PCDAI) in children, and through the Crohn's disease activity index (CDAI) in adult^[32,33]. An PCDAI ≤ 10 and a CDAI ≤ 150 indicate inactive disease.

Prior to the endoscopic procedure, all patients underwent radiological assessment by abdominal ultrasound with eco-color doppler of mesenteric and the ileocecal region, and magnetic resonance imaging with contrast enhancement to confirm the suspected stricture and to investigate the presence of concomitant fistula or abscess. No exclusion criterion was applied in the selection of strictures to be treated with EBD. Naïve and post-surgical strictures, strictures associated with fistula or abscess, and strictures of any length and diameter were included in the study. In all patients surgery was considered for treatment.

EBD technique

Before the endoscopic procedure, all patients underwent the following tests and medications: standard laboratory blood tests including coagulation tests; mechanical intestinal bowel preparation (approximately 36 h before); and liquid diet (starting at least 12 h before).

The endoscopic dilations were performed under unconscious sedation, obtained by administering IV midazolam +/- meperidine or propofol, under constant monitoring of the vital parameters. All procedures were performed by the same endoscopist (de'Angelis GL), and lasted approximately 1 h. The EBD was carried out using Olympus PCF 140 (Olympus, Germany) and Olympus Ileoscopy single balloon SIF 180 (Olympus, Germany) (according to the stricture site).

The EBD was carried out with a hydrostatic Microvative Rigidflex through the scope balloon system (Microvative Endoscopic, Boston Scientific Corporation®, Natick,

Table 1 Clinical characteristics of the study cohort

Clinical characteristics of the study cohort (n = 26)	Data
Gender distribution (n)	
Male	11
Female	15
Pediatric/adult patients distribution (n)	
Pediatric	3
Adult	23
CD indexes of activity (mean ± SE)	
PCDAI (n = 3)	38 ± 7.2
CDAI (n = 23)	365 ± 75
Ongoing medical therapy (n)	
Azathioprine	20
Azathioprine + Infliximab	6
Mean age at the time of the CD diagnosis (yr) [mean ± SE (range)]	22.9 ± 2.8 (2-50)
Mean age at the time of the occurrence of the first stricture (yr) [mean ± SE (range)]	36.8 ± 3.6 (11-65)

CD: Crohn's disease; PCDAI: Pediatric Crohn's disease activity index; CDAI: Crohn's disease activity index.

Massachusetts, United States), with a diameter of 15-18 mm. The correct insertion and positioning of the balloon was checked by fluoroscopic control. After reaching the optimal placement through the stricture, the balloon was gradually inflated with water and gastrografin up to 15 mm of diameter, held for 90 s and then deflated. A second inflation up to 18 mm diameter for 90 s was always performed. In case of resilient strictures, the process of inflation was repeated up to 6 times in the same session, reaching progressively larger balloon diameters. Once the balloon dilation was accomplished, a combination of metilprednisolone (40 mg) diluted in 5 mL of normal saline solution were injected intra-lesionally with Olympus single use injector 0, 5 mm (Olympus, Japan). The ultimate step of the procedure consisted in examining the proximal bowel (30 cm above the stricture) in order to detect others possible lesions that were undetected by the pre-procedural assessing imaging.

In combination with underlying treatment, after EBD each patient was treated by administering prednisolone with a dosing scheme determined by body weight: 1.5 mg/kg daily (maximum allowed dose 60 mg daily) for 2 wk, followed by a 4 wk tapering course.

Clinical outcomes

The technical success of the procedure was defined as the passage of the endoscope through the stricture, reaching a diameter of approximately 15 mm. Procedure-related complications were defined as intestinal perforation, and active bleeding requiring surgery or blood transfusions. The long-term clinical success was defined as surgery was avoided all long the follow-up period by obtaining symptom relief with repeated EBD procedures. The short-term clinical success was defined as 6 mo symptom-free period after the EBD. The need of re-dilation was determined based on clinical and imaging criteria in association with persistence or reoccurrence of

Table 2 Clinical characteristics of Crohn's disease-related strictures

Stricture characteristics (n = 27)	Data
Nature of the stricture (n)	
Naive	12
Post-surgical	15
Location of the stricture (n)	
Upper gastrointestinal	1
Small intestine	2
Ileo-colonic	14
Colonic	10
Mean length (cm) [mean ± SE (range)]	4.6 ± 0.4 (2-12)
≤ 4 cm (n)	15
> 4 cm (n)	12
Mean diameter (mm) [mean ± SE (range)]	2.5 ± 0.2 (1-6)
≤ 5 mm (n)	26
> 5 mm (n)	1
Stricture associated fistula (n)	
Yes	5
No	22

obstructive symptoms. Surgery was reserved for strictures that did not respond to the medical and the endoscopic therapy.

Ethical considerations

The work carried out was in accordance with the principles laid down in the Declaration of Helsinki for biomedical research involving humans. All adult patients included in the study gave their consent for the use of their clinical data. For children, written consent was obtained from both parents and those older than 12 year signed a statement of assent.

Statistical analysis

Data were analyzed by using SPSS (IBM SPSS Statistics, Version 17.0.0 for Macintosh, Chicago, IL, United States). Kaplan-Meier analysis was performed for periods free of surgery and free of endoscopic re-dilation. Regression statistics were used to relate the clinical and demographic variables to the main outcome (*i.e.*, need of surgery). *P* value ≤ 0.05 was considered significant. Data are expressed as median and range, or mean ± SE, unless otherwise stated.

RESULTS

The patients' demographic and clinical characteristics, and the stricture characteristics are summarized in Tables 1 and 2 respectively. Forty-six EBD were performed for 27 symptomatic strictures occurred in the 26 CD patients. Of these, 15 patients had post-surgical strictures and 11 had naive strictures. The technical success of the endoscopic procedure was achieved in all patients without any endoscopic related complication (Figure 1).

The mean follow-up time of the cohort was 40.7 ± 5.7 mo (range 10-94 mo). All patients survived during the follow-up period. Of the 26 CD patients that were treated with EBD, only two failed to respond to the treat-

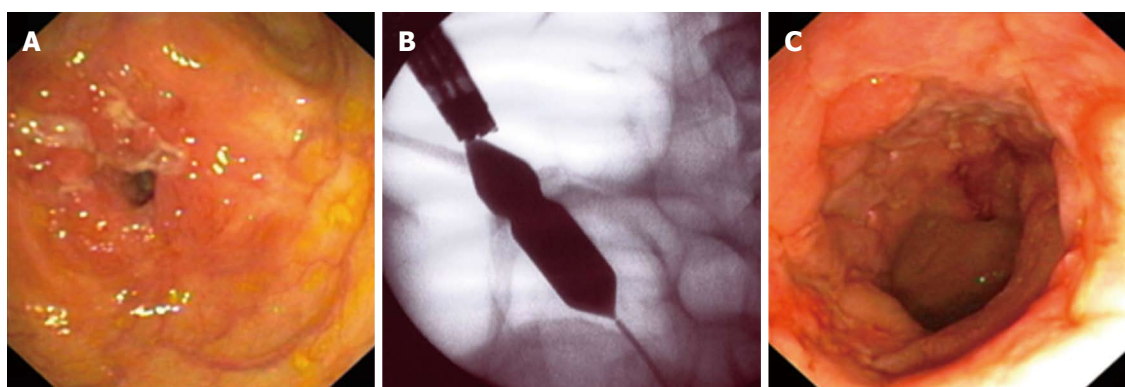


Figure 1 Images of Crohn's disease-related stricture in one patient. A: Direct visualization of the ileal stricture; B: Inflation of the endoscopic balloon under fluoroscopic control; C: Direct visualization of the bowel site after the endoscopic balloon dilation.

ment and underwent elective surgical laparoscopic stricture resection. Both patients requiring surgery presented ileocecal strictures associated with fistula. The overall long-term clinical success rate was 92.6% (24/26 patients remained free of surgery) (Figure 2A).

Of the 24 patients who did not undergo surgery all long the follow-up period, 11 patients received only 1 EBD, and 13 required further dilations over time for the treatment of relapsing strictures (7 patients underwent 2 dilations, 5 patients 3 dilations, and 1 patient 4 dilations). The mean time free of re-dilation between the first and the second EBD was 21.2 ± 5 mo. The cumulative percentages of patients free of re-dilation over the entire follow-up period are shown in Figure 2B.

Throughout the study population, the short-term clinical success rate was 81.5% (2 patients required surgery; 3 patients did not have symptom-free 6 mo) after the first EBD. After the second EBD, the clinical success rate was 92.3% (12/13 patients); after the third EBD, the clinical success rate was 83.3% (5/6 patients). Only one patient underwent a fourth EBD showing a clinical success. The subgroup analysis dividing the study population into two groups based on the nature of the strictures, *i.e.* naive vs post-operative, showed no statistical difference between groups in term of clinical success after repeated EBD. Indeed, after the first dilation, short-term clinical success was obtained in 93.3% of the post-operative strictures and 66.7% of the naive strictures [not significant (NS)]; after the second EBD, success was obtained in 100% of the post-operative strictures and in 80% of the naive ones (NS); after the third EBD, success was observed in 100% of the post-operative strictures and 66.7% of the naive strictures (NS). The cumulative percentages of patients free of re-dilation over the entire follow-up period for both naive and post-operative strictures are shown in Figure 2C.

Of the variables evaluated, the presence of stricture-associated fistula and the stricture location at the ileocecal level resulted significant predictive factors on the long-term negative clinical outcome, *i.e.*, the need of surgery (both $P = 0.002$). In fact, the 2 strictures that required surgery after the first EBD due to the failure of the

endoscopic procedure (*i.e.*, persistency of subocclusion symptoms) were sited at the ileocecal level and were associated with fistula. On the contrary, the sex and age of the patient, the nature of the strictures (naive *vs* post-surgical), the severity of CD activity, the dimension of the strictures (lengths and diameters), and the medical therapy did not influence the long-term clinical outcomes (Table 3).

DISCUSSION

The present study describes the clinical follow-up of a cohort of 26 CD patients presenting with symptomatic strictures and treated with EBD. The EBD appeared to be a safe technique that prevented the need of surgery in 92.6% of the patients during our follow-up period. The endoscopic treatment associated with the medical therapy influenced the natural history of the disease and thus it can be considered an effective strategy in the management of symptomatic strictures in CD patients.

EBD has become more and more used in the treatment of CD strictures since it demonstrated to be a safe and minimally invasive technique, while conserving the intestinal length. At the same time, the medical therapy is largely applied to manage the clinical course of this inflammatory disease and to control its clinical evolution. A combined medical and endoscopic therapy has shown to be effective in the treatment of CD-related strictures^[22,34]. However, standardized clinical guidelines and protocols are missing.

Our cohort study presents some points of strength and novelty. In fact, to describe the effectiveness of EBD and its influence of the natural history of the disease, we decided to recruit in the study consecutive CD patients without any strictures related exclusion criterion. Conversely to the other studies^[21,22,34,35], we considered strictures of any nature and lengths (up to 12 cm), with and without associated fistula. The objective was to analyze a population that is the most often seen in the everyday clinical practice compared to the highly selected cohorts of patients that are usually described in the literature. Our results demonstrated that the EBD can be

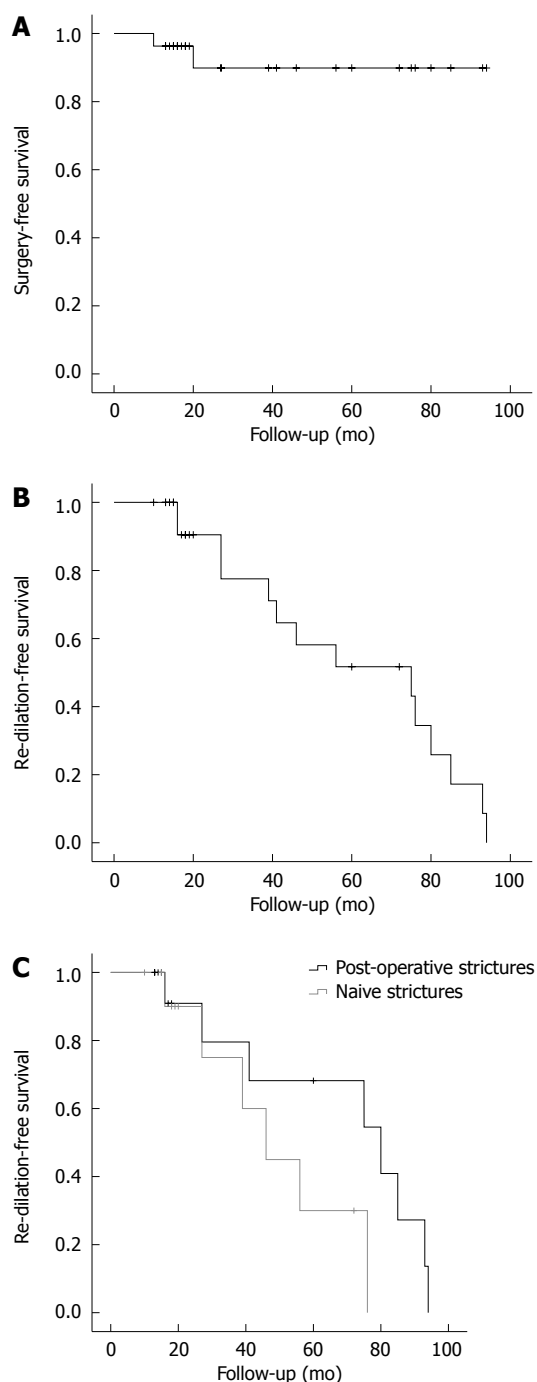


Figure 2 Kaplan-Meier curves for intervals free of surgery or endoscopic dilation. A: Kaplan-Meier curve for interval free of surgery after endoscopic balloon dilation or re-dilation; B: Kaplan-Meier curve for interval free of re-dilation during the follow-up period; C: Kaplan-Meier curve for the interval free of re-dilation over the follow-up period for patients with naive (in gray) and post-operative (in black) strictures.

effectively used also in CD related strictures longer than 4 cm in order to avoid or postpone surgery. The lengths of the strictures did not appear to be a contraindication for performing EBD as a first line treatment in contrast to previous studies^[24,34]. The EBD overall long-term success rate in our study is higher than the values of Hassan *et al.*^[34] in a recent systematic review, which reported a cumulative mean success rate of 67% (ranging from 41%

Table 3 Analyses of the influence of the clinical variables on the occurrence of surgery

Variables	No surgery (n)	Surgery (n)	P value
Male sex	10	1	NS
Adult patients	21	2	NS
Naive strictures	10	2	NS
Moderate disease activity	12	2	NS
Strictures length > 4 cm	11	1	NS
Strictures diameters ≤ 5 mm	24	2	NS
Strictures with fistula	3	2	0.002
Strictures at the ileocecal level	3	2	0.002
Pharmacological therapy (azathioprine)	18	2	NS

NS: Not significant.

to 100%) over an average follow-up time very close to one in the present study. In order to avoid surgery, EBD was repeated-up to 4 times during the follow with a high short-term clinical success rate after each procedure and a technical success rate of 100% with no procedure-associated complication. This result may be also related to the high-volume endoscopy center in which EBD were performed.

Based on these findings and in accordance with other studies^[22,24,34,35], EBD appeared to be a safe procedure with a very low complication rate when an endoscopist experienced in the management of bowel stricture performs it. In our study, we showed that EBD is safe and feasible even in a non-selected sample of CD patients. Moreover, the safety of the procedure may be related to the diameters of the endoscopic balloon used. In our study the 18 mm balloon was the largest applied, for not more than 90 s inflations and no more than 6 dilations per session. If prudence is respected in the selection of balloon dimensions, number of dilations, and progressive inflations, the intra-operative complications may be minimized^[22,23,36]. The procedural safety is essential in consideration of the need of frequent re-dilations over time in the same patient in order to obtain and maintain symptomatic relief.

EBD can be considered a valuable and safe alternative of surgical resection, but EBD and surgery must not be seen as mutually exclusive solutions for CD strictures. Rather EBD may be a complementary procedure that should be considered in both adult and pediatric patients in order to reach a symptom-free condition with a low risk associated.

As previously reported, we found that the stricture location at the ileocecal level appeared to be a significant negative predictor on the long-term clinical outcome, *i.e.*, the need of surgery^[24]. In our study, also the presence of fistula was associated with the occurrence of surgery. Notwithstanding, three patients with fistulizing phenotype (2 located at the colo-rectal anastomosis and 1 in the sigma), showed good clinical short and long-term outcomes after EBD, suggesting that the analysis results may represent a type II error and that larger groups could provide different results. Studies on EBD in CD

patients presenting strictures associated with fistula are scarce in the literature, since the majority of the previously published case series considered the presence of fistula as a patient's exclusion criterion^[21,27,35,37]. Although the paucity of data, in a similar clinical scenario the EBD should not be excluded a priori. Furthermore, it could be used, together with the medical therapy, as an option to bridge the patient to surgery in better performance conditions (*e.g.*, nutrition, inflammatory status), shifting from an emergency to an elective surgery. It is noteworthy that in patients with an adequate nutrition status in which the intestinal obstructive condition has been endoscopically managed (even if suboptimally), the response to surgery and the post-surgical complication rate (*e.g.*, anastomotic leakage) are generally improved^[38,39]. The other examined variables seem to not affect the clinical outcomes. However, we were not able to detect which variables are associated with clinical success after only one EBD, and which can predict the need of further dilations. These aspects should be studied in a larger sample of patients.

In our cohort, both naive and post-surgical strictures responded to the EBD treatment, without significant difference on the clinical outcomes as seen by other authors^[24,25]. Thus, EBD can be considered a valuable strategy in the management of both naive and post-surgical CD related strictures. In parallel, EBD resulted equally effective in adult and pediatric CD patients. It must be emphasized that the endoscopic management of CD strictures is cardinal in pediatrics because of the long life expectancy of these patients, who would more probably develop a short bowel syndrome if repeated surgical resections are performed. Moreover, many clinical concerns are related to the malnutrition and subsequent failure to thrive that will follow the obstructive condition in children if this is not immediately managed^[40].

Interestingly, all our patients were still medically both before and after the endoscopic treatment, mainly with azathioprine and/or biological therapy. Factors determining the development of strictures are not fully understood, but chronic and transmural inflammation probably plays a major role^[41-43]. Although the lack of data in the literature, azathioprine has been shown to reverse the inflammatory changes at the anastomotic site and to maintaining remission in CD patients^[44,45]. Conversely, because of reports of complete obstruction after infliximab in patients with or without initial stricture, its use has been contra-indicated in stenotic forms of CD^[46]. Theoretically, the rapid tissue healing induced by infliximab administration may result in marked architectural changes in the intestinal wall, which may lead to wall stricturing. However, strictures do not occur without inflammation, and chronic inflammation *per se* may lead to strictures. In fact, a long-term inflammatory process sustained by increased cytokine production leads to an excess of fibrotic response. On the other hand, substantial thickening of the mesenchymal layers is observed during mucosal repair. The control of chronic inflammation to prevent fibrosis and stenosis seems more important than the risk of fibrosis induced by treatment, thus justifying inflix-

imab infusions^[41]. However, the role of biological therapy in case of CD strictures remains controversial^[47,48]. In our cohort, we were not able to define the role and support of the two medical therapies on the clinical outcomes evaluated. With this objective, multicenter, randomized, controlled, blind clinical trials should be performed. The use of EBD is supported not only by the clinical risk/benefit ratio, but also by the costs associated to this procedure. In Italy, the entire EBD procedure (pre-endoscopy exams; hospitalization; medications; balloon dilation kit) is comprised between 1000 and 1200 Euros (reimbursed by the National Health System).

In conclusion, the EBD is not just an attractive treatment option in the management of CD-related strictures. The available literature provides quite enough evidence to support its use. However, clinical guidelines, especially on the combined medical and endoscopic therapy, are still lacking. Time has come to investigate EBD clinical benefits and success in large clinical studies in order to define and standardize the protocol of use.

COMMENTS

Background

Crohn's disease (CD)-related strictures are scarcely responsive to medical therapy and thus they are mainly treated surgically. Recently, endoscopic balloon dilation has been increasingly used in the treatment of CD-related strictures.

Innovations and breakthroughs

The present study evaluated the efficacy of endoscopic balloon dilation (EBD) in CD-related strictures of any length (up to 12 cm), both naive and post-operative, with or without associated fistula, and in both adult and pediatric patients. Strictures located in both upper and lower gastrointestinal were successfully treated using gastroscope, colonoscope or ileoscope according to the stricture site. Their results confirmed that over a mean follow-up period of 40.7 mo multiple endoscopic balloon dilations were safe and effective to manage CD-related strictures.

Applications

The present study supports EBD as a valuable option in the treatment of symptomatic CD patients. Moreover, EBD demonstrated as a safe technique, which can be repeated over time in order to avoid surgery, since it can be performed successfully also in relapsing patients who were previously endoscopically dilated. The associated complications are very rare (none in this study).

Terminology

EBD is an operative endoscopy procedure used to dilate intestinal strictures in unconscious patients. The dilation is carried out with a hydrostatic through the scope balloon system, which, once inserted and positioned through the stricture, is gradually inflated with water, held for 90 s and then deflated to obtain stricture dilation.

Peer review

de'Angelis *et al* report an interesting series of EBD of strictures in CD. The inclusion of both naive and postoperative strictures with or without associated fistula reflects the usual clinical scenario in CD strictures. Although the number of the cases included in the study is not so large, the conclusions are well presented and discussed. I agree with the authors that until clinical guidelines are available EBD would be a treatment option before surgery in this kind of patients.

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Narrow-band imaging with magnifying endoscopy is accurate for detecting gastric intestinal metaplasia

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Abstract

AIM: To investigate the predictive value of narrow-band imaging with magnifying endoscopy (NBI-ME) for identifying gastric intestinal metaplasia (GIM) in unselected patients.

METHODS: We prospectively evaluated consecutive patients undergoing upper endoscopy for various indications, such as epigastric discomfort/pain, anaemia, gastro-oesophageal reflux disease, suspicion of peptic ulcer disease, or chronic liver diseases. Patients underwent NBI-ME, which was performed by three blinded, experienced endoscopists. In addition, five biopsies (2 antrum, 1 angulus, and 2 corpus) were taken and examined by two pathologists unaware of the endoscopic

findings to determine the presence or absence of GIM. The correlation between light blue crest (LBC) appearance and histology was measured. Moreover, we quantified the degree of LBC appearance as less than 20% (+), 20%-80% (++) and more than 80% (+++) of an image field, and the semiquantitative evaluation of LBC appearance was correlated with IM percentage from the histological findings.

RESULTS: We enrolled 100 (58 F/42 M) patients who were mainly referred for gastro-esophageal reflux disease/dyspepsia (46%), cancer screening/anaemia (34%), chronic liver disease (9%), and suspected celiac disease (6%); the remaining patients were referred for other indications. The prevalence of *Helicobacter pylori* (*H. pylori*) infection detected from the biopsies was 31%, while 67% of the patients used proton pump inhibitors. LBCs were found in the antrum of 33 patients (33%); 20 of the cases were classified as LBC+, 9 as LBC++, and 4 as LBC+++. LBCs were found in the gastric body of 6 patients (6%), with 5 of them also having LBCs in the antrum. The correlation between the appearance of LBCs and histological GIM was good, with a sensitivity of 80% (95%CI: 67-92), a specificity of 96% (95%CI: 93-99), a positive predictive value of 84% (95%CI: 73-96), a negative predictive value of 95% (95%CI: 92-98), and an accuracy of 93% (95%CI: 90-97). The NBI-ME examination overlooked GIM in 8 cases, but the GIM was less than 5% in 7 of the cases. Moreover, in the 6 false positive cases, the histological examination showed the presence of reactive gastropathy (4 cases) or *H. pylori* active chronic gastritis (2 cases). The semiquantitative correlation between the rate of LBC appearance and the percentage of GIM was 79% ($P < 0.01$).

CONCLUSION: NBI-ME achieved good sensitivity and specificity in recognising GIM in an unselected population. In routine clinical practice, this technique can reliably target gastric biopsies.

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Key words: Narrow-band imaging; Magnification; Gastric intestinal metaplasia; Light blue crest; Gastric cancer; Endoscopy; Precancerous conditions; Gastric biopsy

Core tip: Gastric cancer is one of the most common neoplastic diseases in the Western world and has a poor prognosis and inconsistent signs and symptoms in the early phases. Narrow-band imaging with magnifying endoscopy was shown to be a valid method for intestinal metaplasia (IM) detection, this technique can reliably target which patients should be biopsied to evaluate IM and those who do not need biopsies. Moreover, a semi-quantitative evaluation of light blue crest appearance was feasible as there was a good correlation with the histological assessment of IM percentage.

Savarino E, Corbo M, Dulbecco P, Gemignani L, Giambruno E, Mastracci L, Grillo F, Savarino V. Narrow-band imaging with magnifying endoscopy is accurate for detecting gastric intestinal metaplasia. *World J Gastroenterol* 2013; 19(17): 2668-2675 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2668.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2668>

INTRODUCTION

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide^[1,2]. However, the mechanism of gastric carcinogenesis is unknown. The intestinal type tumour, according to the Lauren classification, is characterised by the presence of malignant cells forming glandular structures; in contrast to diffuse type carcinomas, these tumours are generally thought to be preceded by a sequence of precursor lesions. In this multistep model of gastric carcinogenesis, described several years ago by Correa *et al*^[3], *Helicobacter pylori* (*H. pylori*) causes chronic inflammation of the gastric mucosa, which slowly progresses through the premalignant stages of atrophic gastritis, intestinal metaplasia and dysplasia, intramucosal carcinoma and invasive neoplasia^[4].

Intestinal metaplasia (IM) is generally considered as the “field cancerization” in the gastric mucosa. At the cell level, intestinalized glands provide the cellular substrate that allows the gastric non-invasive neoplasia to develop. The reported progression rates of IM and dysplasia to cancer vary greatly from 0% to 10% per year for IM and from 0% to 73% per year for dysplasia^[1]. The prevalence of this condition in Europe varies by country^[5-9]; for instance, a Swedish group reported an IM prevalence of 23% in the general population^[10], while the prevalence has been estimated to be up to 25% in the Italian population, mainly occurring in the gastric antrum^[7,11-13].

In Western countries, premalignant gastric lesions and

early gastric cancer are generally diagnosed upon histologic examination of random biopsies, whereas in Asian countries, especially in Japan, the presence and extension of these lesions are frequently established during an endoscopy^[14-16]. These remarkable differences are explained by different training and attitudes towards the inspection of the stomach in countries with a high gastric cancer incidence.

The narrow-band imaging system (NBI) is based on the modification of optical filter spectral characteristics in the light source, which improves the visibility of mucosal structures. NBI may be combined with magnification endoscopy to obtain a clear visualisation of surface and vascular patterns^[1,2]. Earlier studies using the NBI system with magnification endoscopy (NBI-ME) in the gastric mucosa showed that the appearance of a light blue crest (LBC) in the mucosa is a distinctive endoscopic finding that suggests an increased likelihood of detecting IM in the stomach. More precisely, blue-whitish patchy areas are often observed in NBI images of the antrum in patients with gastric IM. Uedo *et al*^[16] tested NBI-ME on 34 patients with atrophic gastritis and found that the LBC appearance was frequently observed in gastric antrum as blue-white lines visible on the epithelial surface. These authors demonstrated that the appearance of LBCs correlated with histological evidence of IM, with a sensitivity of 89% (95%CI: 83-96), a specificity of 93% (95%CI: 88-97), a positive predictive value of 91% (95%CI: 85-96), a negative predictive value of 92% (95%CI: 87-97) and an accuracy of 91% (95%CI: 88-95).

Despite these promising findings, the added value of NBI-ME in the stomach, and more precisely in detecting premalignant and malignant gastric lesions, requires confirmation from controlled longitudinal trials. Furthermore, a uniform and validated classification system is needed. Thus, the aim of our study was to define the value of NBI-ME for identifying gastric IM in an unselected population through the visualisation of LBC appearance. A secondary aim was to provide a semiquantitative evaluation of LBC appearance and to verify a correlation with the histological measurement of IM percentage.

MATERIALS AND METHODS

Subjects

This prospective, blinded study included unselected consecutive patients presenting to the endoscopy unit of the University Hospital of Genoa, Italy. Patients underwent an upper endoscopy for various indications, such as epigastric discomfort/pain, anaemia, gastro-oesophageal reflux disease, suspicion of peptic ulcer disease, or chronic liver diseases. Exclusion criteria included a previous gastrectomy or partial gastric resection; treatment with antiplatelet medication, anticoagulant medication or non-steroidal anti-inflammatory drugs; and the presence of haemorrhagic diseases. The study protocol was approved by the local Ethics Committee and was performed according to the Declaration of Helsinki. All patients pro-

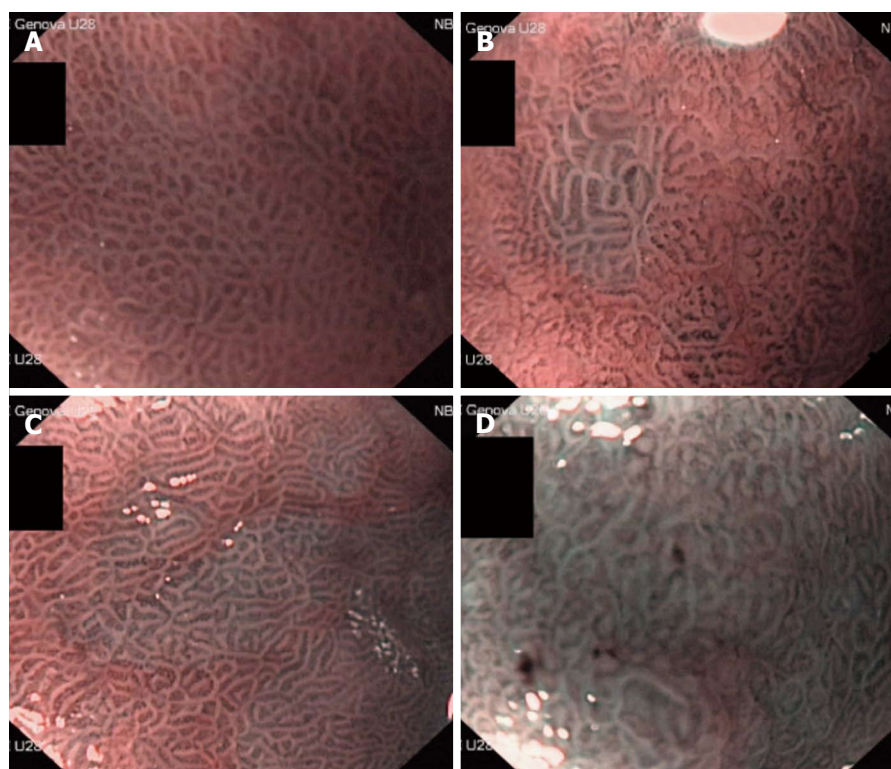


Figure 1 Normal mucosal aspects. A: Narrow-band-imaging with magnification endoscopy examination and the degree of light blue crest appearance; B: Light blue crest (LBC) +, less than 20%; C: LBC ++, 20%-80%; D: LBC +++, 80% or more.

vided written informed consent before the start of the study.

Study protocol

All subjects who agreed to participate in our investigation underwent a thorough physical and clinical examination, a careful collection of their medical history (including current medications, tobacco use, and alcohol and coffee consumption) and an upper gastric intestinal (GI) endoscopy with the NBI-ME system to determine the presence or absence of gastric IM. Five biopsies (2 antrum, 1 angulus and 2 corpus) were collected from all patients according to the updated Sydney classification^[17].

Endoscopic procedure

The endoscopic examinations were performed by experienced endoscopists (Corbo M, Savarino E, and Dulbecco P) who were blinded to the conditions and medical histories of the patients. Before starting the study, each endoscopist underwent training to obtain good expertise in the detection of IM using NBI-ME. Each endoscopist performed > 300 NBI-ME gastroscopies in patients selected independently of a previous diagnosis of IM and compared their NBI-ME findings with those obtained after a histologic evaluation. At the end of this training, the overall ability to detect histologic IM using NBI-ME was very high (92%). The procedure was conducted with a magnifying endoscope (GIF-Q160Z; Olympus Medical Systems, Tokyo, Japan; 10.9 mm outer diameter insertion tube with a 2.8 mm channel diameter; optical magnifica-

tion $\times 115$). A disposable attachment was fitted to the scope tip to maintain focal distance from the mucosa for magnifying observation (approximately 2 mm). A conscious sedation was performed at the request of the patient. Before the procedure, all patients ingested 66.6 mg of simethicone diluted in 40 mL of water (Istituto Biochimico Italiano Giovanni Lorenzini S.p.A., Aprilia, Italy). If poor visualisation persisted, the gastric mucosal surface was rinsed with an additional 30-60 mL of the simethicone solution. After white light examination, the gastric antrum and body were examined by NBI for blue-whitish patchy areas and then by NBI-ME observation at the maximum magnification, as LBC was frequently observed in these areas. If no blue-whitish areas were observed, NBI-ME was conducted at the proximal and distal portions of the following areas: the anterior wall, the lesser curvature, the posterior wall, and the greater curvature, as previously described^[16]. During NBI-ME observation, we assessed for the presence or absence of LBCs, defined as a fine, blue-white lines on the crests of the epithelial surface that are similar in appearance to the light reflected from a mirror. The patient was defined as LBC-negative if LBCs were not observed in any of the image fields and as LBC-positive if LBCs were observed in any of the image fields. Moreover, we quantified the degree of LBC appearance as less than 20% (+), 20%-80% (++) and more than 80% (+++) of an image field (Figure 1). In each examination, six pictures were taken and stored (one during NBI and two during NBI-ME observation, both in the antrum and in the corpus).

Table 1 Demographic and clinical characteristics of unselected patients ($n = 100$) included in the study n (%)

Demographic and clinical features	Patients
Age, yr (mean \pm SD)	67 \pm 12
Sex	
Male	42 (42)
Female	58 (58)
<i>H. pylori</i> infection	
Positive	31 (31)
Negative	69 (69)
Smoking	
Non-smoker	68 (68)
Current smoker	16 (16)
Former smoker	16 (16)
Alcohol consuming	
Non-drinker	75 (75)
Current drinker	25 (25)
Medication	
PPI users	67 (67)
Light blue crests	
Present	33 (33)
Absent	67 (67)

Data are expressed as absolute numbers (percentages) or mean \pm SD. *H. pylori*: *Helicobacter pylori*; PPI: Proton-pump inhibitors.

A five biopsy set (2 antrum, 1 angulus, and 2 corpus) of LBC-positive areas was collected in all LBC-positive examinations; biopsy samples were taken from the non-LBC mucosa in the LBC-negative patients.

Histological assessment

All biopsy specimens were immersed in formalin and then embedded in paraffin. Sections cut from the paraffin blocks were stained with haematoxylin and eosin, Alcian blue PAS and Giemsa. The histological findings were evaluated by two expert pathologists who specialised in GI pathology; the pathologists were unaware of the medical history of the patients and of the endoscopic findings regarding LBC appearance. Inflammation, atrophy, metaplasia and dysplasia were classified according to the updated Sydney system^[17] and the revised Vienna classification^[18]. Intestinal metaplasia was also expressed as the percentage of metaplastic glands on the entire antral or oxyntic mucosa specimens. Moreover, each sample was evaluated for the presence of *H. pylori*.

Statistical analysis

Based on prior studies reporting a sensitivity of more than 80% and a specificity of more than 90%, we determined that a cohort of 100 patients was needed to detect an effect with a confidence interval of 95%, a sensitivity of 80% (interval width of 17%), and a specificity of 90% (interval width of 13%) (calculated with the Clopper-Pearson method using PASS 11; NCSS, LLC, Kaysville, Utah, United States; www.ncss.com). The sensitivity, specificity, positive and negative predictive values and accuracy were calculated and expressed as percentages and 95%CI. Pearson correlation analysis was used to compare the LBC grading with the percentage of histologically as-

Table 2 Relationship between narrow-band-imaging with magnification endoscopy ranking and the percentage of gastric intestinal metaplasia detected by histological assessment

Intestinal metaplasia	LBC (n)				Total (n)
	Negative	+	++	+++	
0%	59	6	0	0	65
$\leq 5\%$	7	8	2	0	17
5%-45%	1	6	5	2	14
45%-80%	0	0	2	1	3
$\geq 80\%$	0	0	0	1	1

LBC: Light blue crest.

sessed metaplasia.

RESULTS

The study included 100 consecutive patients (58 F/42 M, mean age 67 \pm 12 years) who presented at our endoscopy unit between December 2010 and February 2012. The demographic and clinical characteristics of the patients are shown in Table 1. The patients were referred to our endoscopy unit mainly because of gastro-oesophageal reflux disease and/or dyspepsia symptoms (46%); 34% underwent the endoscopic procedure for cancer screening, anaemia, a positive faecal occult blood test or suspicion of a peptic ulcer; 9% for chronic liver disease; 6% for suspected celiac disease and the remaining patients for other indications. The prevalence of *H. pylori* infection on the biopsies was 31%; in addition, 67% of the patients used proton pump inhibitors, 16% were current smokers, and 25% of the patients were alcohol consumers.

Endoscopic and histological findings

During the upper NBI-ME procedure, we observed LBC appearance in the gastric antrum of 33 patients (33%); 20 of the LBCs were classified as LBC+, 9 as LBC++, and 4 as LBC+++. Moreover, we detected LBC appearance in the gastric body in only 6 patients (6%), and 5 of the patients also had LBC appearance in the antrum; of these, 3 cases were classified as LBC+, 2 as LBC++, and 1 as LBC+++.

Intestinal metaplasia in the biopsy specimens (2 antrum, 1 angulus and 2 corpus) obtained during the endoscopy from both LBC-positive and -negative patients was classified as complete and incomplete based on special staining. Gastric IM was histologically detected in 35 (35%) patients, and 27 cases were LBC-positive. In five patients, histological IM was found in both the antrum and body, and this finding correlated with LBC appearance at both sites. A percentage of IM greater than 20% was observed in 13 (13%) of the cases.

Accuracy of LBC presence for diagnosing gastric intestinal metaplasia

For the diagnosis of IM, compared to histological assessment, the NBI-ME findings had an accuracy of 93%

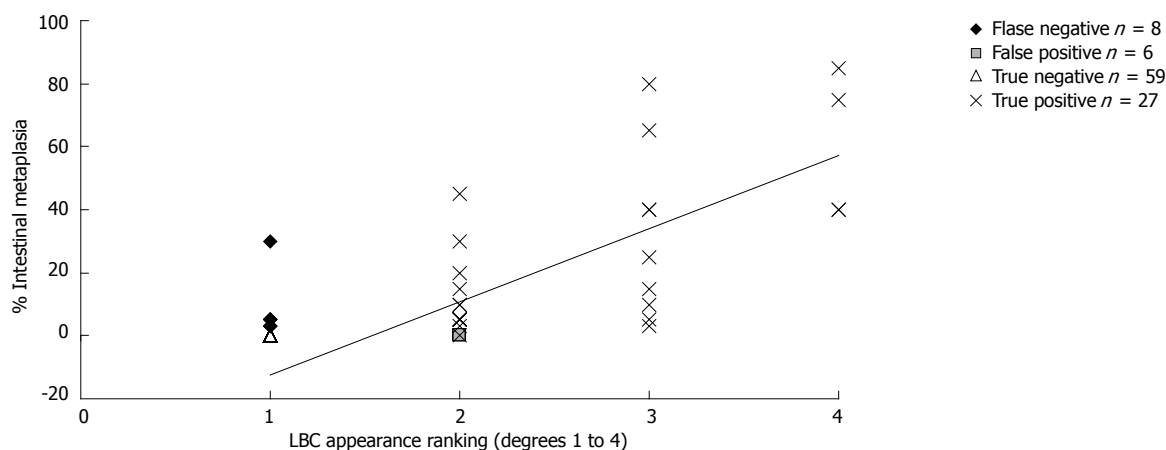


Figure 2 Graphic representation of the correlation between the degree of light blue crest appearance during narrow-band imaging with magnifying endoscopy examination and the percentage of gastric intestinal metaplasia detected by histological examination. LBC: Light blue crest.

(95%CI: 90-97), a sensitivity of 80% (95%CI: 67-92), a specificity of 96% (95%CI: 93-99), a positive predictive value of 84% (95%CI: 73-96), and a negative predictive value of 95% (95%CI: 92-98).

Interestingly, in our study, the NBI-ME examination overlooked the presence of gastric IM in 8 cases (false negative cases), and 7 out of the 8 cases had an IM that was less than 5%. Moreover, in the 6 false positive cases, the histological examination showed the presence of reactive gastropathy (4 cases) or *H. pylori* active chronic gastritis (2 cases).

The semiquantitative correlation between the grades of LBC appearance and the percentage of histological IM is shown in Table 2 and Figure 2; the correlation index was 79% ($P < 0.01$).

DISCUSSION

Intestinal metaplasia has been widely studied as the main mucosal background for the development of oesophageal and gastric adenocarcinomas. However, to date, no endoscopic patterns that clearly define IM or dysplasia in the stomach have been found. Kaminishi *et al*^[19] evaluated the accuracy of endoscopic findings for diagnosing chronic gastritis and found that ash-coloured nodular changes were highly specific (98% \pm 99%) but had a very poor sensitivity (6% \pm 12%) for detecting histological IM; the authors concluded that conventional endoscopy is not really useful for diagnosing gastric IM. Later, Dinis-Ribeiro *et al*^[20] observed that magnifying chromoendoscopy with methylene blue staining had good sensitivity (76%) and specificity (87%) for detecting gastric IM. Moreover, methylene blue chromoendoscopy has been found to be useful for assessing the extent of IM^[21]. However, this latter technique is time consuming and has some limitations, including the need for preparation with mucolytic agents, dye spraying and vigorous irrigation of the mucosal surface; furthermore, a recent report suggests that methylene blue chromoendoscopy induced questionable oxidative DNA damage in a patient with Barrett's

mucosa^[22]. Therefore, collecting biopsies for subsequent histological evaluation remains the current gold standard for diagnosing IM. However, the fact that premalignant gastric lesions may occur multifocally represents a limitation of random biopsy sampling.

Several new imaging techniques have been developed recently to improve the diagnostic yield of endoscopy. The NBI-ME system has the practical advantage of not requiring complex preparation procedures, the use of any staining technique or a long duration. The additional value of NBI-ME in the detection of gastric pre-malignant lesions is still unclear, especially in countries with a low gastric cancer incidence^[7].

Recently, Capelle *et al*^[23] compared the yield of NBI alone to that of white light endoscopy (WLE) in the surveillance of 43 patients with IM and dysplasia. The authors concluded that NBI alone considerably increased the detection of premalignant gastric lesions compared to routine WLE and appeared to be superior to WLE for the surveillance of patients with these advanced gastric lesions. However, a combination of NBI and magnification is likely to provide the best alternative to current endoscopic practice. Previous studies have already demonstrated a high correlation between the microvascular patterns found with NBI-ME and the diagnosis of gastric cancer^[24,25]. Moreover, the combined optical technology of NBI with ME has been shown to identify with great accuracy the presence of gastric IM through the visualisation of LBC appearance on the epithelial surface^[16]. However, the main limitations of the above-mentioned studies are that they were all conducted in patients with already known diagnoses of intestinal metaplasia/dysplasia/gastric cancer or in a population at a high risk for developing gastric cancer. Other limitations included the small sample sizes and the limited assessment of the gastric antrum and angulus with the exclusion of the gastric body.

Therefore, no prospective data are available on the diagnostic utility and accuracy of NBI-ME for detecting IM in an unselected population presenting at an endos-

copy unit in routine clinical practice. Our study showed that NBI-ME allowed us to detect gastric IM areas with an accuracy of 93%, a sensitivity of 80%, a specificity of 96%, a positive predictive value of 84% and a negative predictive value of 95%. These results are similar to those recently published by Pimentel-Nunes *et al.*^[26], who found a sensitivity of 68% and a specificity of 87% using the same method and a simplified classification system for NBI in the diagnosis of gastric lesions. In our investigation, NBI-ME underestimated the presence of IM in 8 patients; for 7 of these patients, metaplasia was histologically estimated to be less than 5%. The fact that NBI-ME has its major limitation in identifying gastric IM lower than 5% when performed by experienced endoscopists represents a new finding about the diagnostic yield of this technique; at this stage, we can assume that this value constitutes the sensitivity limit of this technique. The false positive cases (6 patients) presented with a histological diagnosis of reactive gastropathy (4 cases) or *H. pylori* active chronic gastritis (2 cases). Both of these conditions may sometimes represent a confounding factor because they show endoscopic features similar to those of gastric IM.

The prevalence of histologically observed IM in our cohort (35%) was slightly higher than the prevalence estimated in the general Western population (25%)^[5-9] and in other studies on the Italian population (20%-30%)^[5,8,11-13]. A possible explanation for our findings may be related to the higher mean age and prevalence of *H. pylori* infection in our patients, as it is well known that *H. pylori* infection, increased age and smoking (> 20 cigarettes/daily) are the main risk factors^[27] for the development of premalignant gastric lesions. However, the prevalence of IM in the general population is reported to be highly variable. Additionally, the majority of data come from studies on patients with dyspeptic symptoms and/or suspected *H. pylori* infections, while information derived from unselected patients (*i.e.*, consecutive patients enrolled independently of GI symptoms or clinical status) is limited.

The semiquantitative evaluation of LBC appearance in our study and its correlation with the histological measurement of IM percentage shows that the agreement was high (79%). Previously, Uedo *et al.*^[16] also found a significant correlation between the diffuse positivity of LBC (+++) mucosa and the presence of histological markers of IM, such as immunohistochemical staining for CD10 and Alcian blue/high iron diamine. However, these authors did not assess the correlation between LBC appearance and the percentage of histologic IM. Therefore, to the best of our knowledge, our study is the first to show a good correlation between the degree of IM detected by NBI-ME and the severity of IM identified by histological measurement in a series of consecutive patients without specific indications to perform an upper GI endoscopy.

In contrast to Uedo *et al.*^[16] who used NBI-ME and Capelle *et al.*^[23] who performed NBI alone, we opted to evaluate not only the antrum and the angulus of the stomach but also the body; including the body was re-

cently emphasised in the ESGE guidelines^[28], despite the significantly longer examination time. This measurement was included because our intention was to demonstrate the potential application of NBI-ME in a population-based screening and surveillance setting, and we thought that gastric assessment of only one region of the stomach was not adequate and fully informative. However, IM is predominantly found in the antrum, which was confirmed in our study. Therefore, limiting the endoscopic evaluation to the antrum permits an accurate observation and a shorter duration of the examination. Moreover, it has been shown that three biopsies (two antral and one angular) appear to be the best compromise between acceptable accuracy^[29] and ease of use in the detection of IM by endoscopy.

Our study had some limitations. First, the study included only a single centre. Further research in larger multicentre prospective studies is necessary to evaluate whether NBI-ME yields adequate results in the detection and grading of gastric IM. Second, the endoscopic procedure of WLE and NBI-ME was performed by the same endoscopist; thus, detection of IM by NBI-ME could possibly be biased by the previous WLE observations. Third, as a learning curve must still be defined, regular training is mandatory to improve our findings. Indeed, despite our prolonged training, we obtained a value of sensitivity that was lower than the value obtained by Uedo *et al.*^[16]. This difference was likely the result of differences in training between Japanese and Western gastroenterologists. Due to the higher gastric cancer incidence, Japanese endoscopists are trained to scrutinise gastric mucosal areas that are compatible with atrophy and early cancer and spend more time on a thorough mucosal examination than in Western countries. In addition, uniform criteria for this technique must be adopted in large controlled trials in Western or Eastern countries.

In conclusion, NBI-ME was shown to be a valid method for IM detection, except in those cases with IM lower than 5%, which may not be clinically relevant. In routine clinical practice, this technique can reliably target which patients should be biopsied to evaluate IM and those who do not need biopsies. Moreover, a semi-quantitative evaluation of LBC appearance was feasible as there was a good correlation with the histological assessment of IM percentage.

COMMENTS

Background

Gastric cancer is one of the most common neoplastic diseases in the Western world and has a poor prognosis and inconsistent signs and symptoms in the early phases. Therefore, adequate screening and prevention are needed to improve the diagnostic process and to identify this condition and its risk factors earlier. Recently, upper gastric intestinal endoscopy has been enhanced with the addition of the narrow band imaging with magnification endoscopy (NBI-ME) technique, which was found to identify precancerous lesions, such as gastric intestinal metaplasia (GIM).

Research frontiers

Light blue crests (LBCs) are light-blue patches or lines visible on gastric mucosa by NBI endoscopy. Several studies have demonstrated that LBCs are a sign

of underlying intestinal metaplasia, with a good correlation between endoscopic findings and histological assessments. Therefore, authors have assessed the predictive values of the NBI-ME technique to identify and estimate the extension of GIM to biopsies of the antrum, angulus and corpus in the stomach. Authors also studied the correlation between the appearance of LBCs and the histological detection of GIM in prospectively recruited consecutive unselected patients.

Innovations and breakthroughs

One hundred consecutive patients were studied. NBI-ME-aimed gastric bioptic samples were collected (2 in the antrum, 1 in the angulus, and 2 in the corpus) from each patient and compared with the presence or absence of LBCs as stated by skilled endoscopists; the biopsies were assessed according to the pathologists' expertise in the upper digestive tract. They determined a correlation of 79% between the appearance of LBCs and histologically assessed GIM. Moreover, authors observed that the appearance of LBCs correlated with the histological evidence of intestinal metaplasia, with a sensitivity of 80%, a specificity of 96%, a positive predictive value of 84%, a negative predictive value of 95% and an overall accuracy of 93%. Their results are similar, if not superior, to those of previously published studies in selected populations.

Applications

NBI-ME is a good technique to identify areas suggestive of GIM in the stomach and can be used to take adequate biopsies from the stomach to provide the pathologist with a useful sample. This technique is easy to learn and provides an accuracy of greater than 90% for diagnosing GIM.

Terminology

NBI-ME is an endoscopic technique that uses narrow band imaging to produce superior image contrast combined with a magnification of the image up to $\times 115$; this technique enables a more accurate observation of the gastric epithelium. Gastric intestinal metaplasia is a condition characterised by the replacement of gastric epithelial cells with cells of intestinal morphology. GIM is a pre-cancerous condition that can progress towards gastric cancer, especially the intestinal-type according to the Lauren classification. This progress varies from 0% to 10% according to different studies, with a prevalence of more than 20% in Western countries, and is considered to be the "breaking point" in the Correa sequence of carcinogenesis. Identifying this condition could halt or reverse the neoplastic progression.

Peer review

This research was performed by endoscopists who belong to single medical center in Italy. This article should be helpful for understanding the efficacy of NBI-magnifying endoscopy in detecting the presence of intestinal metaplasia of stomach.

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Clinical effects of adalimumab treatment with concomitant azathioprine in Japanese Crohn's disease patients

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Abstract

AIM: To assess adalimumab's efficacy with concomitant azathioprine (AZA) for induction and maintenance of clinical remission in Japanese Crohn's disease (CD) patients.

METHODS: This retrospective, observational, single-center study enrolled 28 consecutive CD patients treated with adalimumab (ADA). Mean age and mean disease duration were 38.1 ± 11.8 years and 11.8 ± 10.1 years, respectively. The baseline mean Crohn's disease activity index (CDAI) and C-reactive protein were 177.8 ± 82.0 and 0.70 ± 0.83 mg/dL, respectively. Twelve of these patients also received a concomitant stable dose

of AZA. ADA was subcutaneously administered: 160 mg at week 0, 80 mg at week 2, followed by 40 mg every other week. Clinical response and remission rates were assessed *via* CDAI and C-reactive protein for 24 wk.

RESULTS: The mean CDAI at weeks 2, 4, 8, and 24 was 124.4, 120.2, 123.6, and 135.1, respectively. The CDAI was significantly decreased at weeks 2 and 4 with ADA and was significantly suppressed at 24 wk with ADA/AZA. Overall clinical remission rates at weeks 4 and 24 were 66.7% and 63.2%, respectively. Although no statistically significant difference in C-reactive protein was demonstrated, ADA with AZA resulted in a greater statistically significant improvement in CDAI at 24 wk, compared to ADA alone.

CONCLUSION: Scheduled ADA with concomitant AZA may be more effective for clinical remission achievement at 24 wk in Japanese Crohn's disease patients.

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Key words: Crohn's disease; Adalimumab; Immuno-modulator; Azathioprine; Inflammatory bowel disease

Core tip: In this study, the authors found that adalimumab (ADA) treatment with concomitant azathioprine usage significantly suppressed the Crohn's disease activity index and increased the remission rate at 24 wk after the initiation of therapy, compared with ADA treatment alone.

Ishida K, Inoue T, Fujiwara K, Sakanaka T, Narabayashi K, Nouda S, Okada T, Kakimoto K, Kuramoto T, Kawakami K, Abe Y, Takeuchi T, Murano M, Tokioka S, Umegaki E, Higuchi K. Clinical effects of adalimumab treatment with concomitant azathioprine in Japanese Crohn's disease patients. *World J Gastroenterol* 2013; 19(17): 2676-2682 Available from: URL: <http://www.wjgnet.com>

INTRODUCTION

Tumor necrosis factor- α (TNF- α) is a proinflammatory cytokine implicated in the pathogenesis of inflammatory bowel disease (IBD)^[1-3]. Thus far, a variety of therapeutic approaches have been used to inhibit TNF- α in patients with IBD, including infliximab (IFX), which was the first biologic agent approved for the treatment of IBD^[4]. IFX is a mouse and human chimeric immunoglobulin G1 (IgG1) monoclonal antibody directed against TNF- α ; it binds to both the soluble and transmembrane forms of TNF- α and is effective as both an induction and maintenance therapy in patients with Crohn's disease (CD), including patients who have draining fistulas^[5-8]. However, IFX has a 25% murine fraction that has potent immunogenicity and can cause the formation of human anti-chimeric antibodies (HACA)^[9]. Furthermore, this potent immunogenicity may lead to the loss of efficacy, hypersensitivity reactions, or infusion reactions^[10].

Adalimumab (ADA) is the second biologic agent approved for use in the treatment of IBD. It is a subcutaneously administered, recombinant, fully human, IgG1 monoclonal antibody that binds with a high affinity and specificity to TNF- α and was found to be effective in refractory CD patients who were either naïve to or previously treated with IFX^[11-15]. Combination therapy with IFX and an immunosuppressant demonstrated increased efficacy compared with nonconcomitant use of immunosuppressants^[16,17]. Nonetheless, limited data are available for ADA. In the pivotal registration study of ADA (Crohn's trial of the fully Human antibody ADA for Remission Maintenance, CHARM), post-hoc analysis did not detect an impact of coadministration of immunosuppressants on the remission rates achieved at 1 year^[12]. Ardizzone *et al.*^[18] reported that ADA with concomitant immunosuppressant treatment was not associated with the loss of response or the need for dose escalation. However, Reenaers *et al.*^[19] reported that there may be a benefit from using immunosuppressive drugs during the first semester of initiating ADA treatment, with a slight decrease in ADA failure and lower need for ADA dosage escalation.

In Japan, ADA was approved for the treatment of CD; however, only limited data have been collected on its optimal usage. Therefore, this study aimed to assess the impact of ADA with concomitant azathioprine (AZA) therapy on the rate of clinical remission during maintenance with ADA in CD patients at the Osaka Medical College Hospital during routine clinical practice.

MATERIALS AND METHODS

Study participants

Between November 2010 and March 2012, about 28 CD

patients who were diagnosed by clinical, endoscopic, and histological criteria and who received ADA at the Osaka Medical College Hospital, Osaka, Japan, were consecutively enrolled in this retrospective, observational, single-center study. The patients were informed about the potential risks and benefits of ADA therapy and were provided and signed informed consent forms before all procedures. The medical records of all participating patients were reviewed by two senior investigators (Ishida K and Inoue T) for the following information: demographics; disease duration; disease location; prior surgical history; perianal disease presence; smoking behavior; previous IFX treatment and response to IFX treatment; Crohn's disease activity index (CDAI); C-reactive protein (CRP) levels; and concomitant medications (corticosteroids, AZA, mesalazine, and elemental diet).

All patients received 160 mg of ADA subcutaneously at week 0, 80 mg at week 2, followed by 40 mg every other week. A clinical response was defined as a Δ CDAI > 70 points, whereas a clinical remission was defined as a CDAI < 150 points. The loss of response to IFX was defined as a loss of therapeutic efficacy after > 2 IFX infusions.

Statistical analysis

Categorical data were compared by the χ^2 test or the Fisher's exact test, and continuous variables were compared by the Student *t* test. The results were expressed as the mean \pm SD. *P* values less than 0.05 were considered to be statistically significant. All calculations were made using the StatView system (SAS Institute, Cary, NC, United States).

RESULTS

Patient characteristics

ADA was administered to 28 consecutive patients with CD from November 2011 to March 2012 at the Osaka Medical College Hospital (male-to-female ratio: 22/6; age at presentation: 38.1 ± 11.8 years; and disease duration: 11.8 ± 10.1 years). The patients' baseline characteristics and demographics are listed in Table 1. According to the Montreal classification, patients presented with CD in the following locations: about 10.7% (3/28) in an isolated ileal location; 17.9% (5/28) in an isolated colonic location; and 71.4% (20/28) in an ileocolonic location. Of the patients, 60.7% (17/28) had a history of bowel resection, 57.1% had perianal disease, and 17.9% had an associated fistulizing disease. Two patients (7.1%) were current smokers.

Seventeen (60.7%) patients had previously taken IFX, and 6 patients did not have a response to IFX, including no primary response in one patient and emergent allergic reaction in another patient. Twelve of the 28 patients were treated with ADA and a concomitant stable dose of AZA throughout the observational period. The mean CDAI and mean CRP at baseline were 177.8 ± 82.0 and 0.70 ± 0.83 mg/dL, respectively.

Table 1 Baseline characteristics of all patients who received adalimumab for treatment of Crohn's disease *n* (%)

	Adalimumab <i>n</i> = 16	Combination <i>n</i> = 12	Total <i>n</i> = 28
Gender			
Male	13 (81.3)	9 (75.0)	22 (78.6)
Female	3 (18.7)	3 (25.0)	6 (21.4)
Age at presentation ¹ (yr)	43.1 ± 11.8	31.4 ± 8.3 ^a	38.1 ± 11.8
Disease duration ¹ (yr)	13.6 ± 11.5	9.3 ± 7.6	11.8 ± 10.1
Location of disease			
Isolated ileal	2 (12.5)	1 (8.3)	3 (10.7)
Isolated colonic	4 (25.0)	1 (8.3)	5 (17.9)
leocolonic	10 (62.5)	10 (83.3)	20 (71.4)
Previous resection	10 (62.5)	7 (58.3)	17 (60.7)
Perianal disease	10 (62.5)	6 (50)	16 (57.1)
Current smokers	1 (6.3)	1 (8.3)	2 (7.1)
Previous anti-infliximab treatment	10 (62.5)	7 (58.3)	17 (60.7)
Primary nonresponse	0 (0.0)	1 (8.3)	1 (3.6)
Secondary loss of response	8 (50.0)	6 (50.0)	14 (50.0)
Allergic reaction	1 (6.3)	0 (0.0)	1 (3.6)
Others	1 (6.3)	0 (0.0)	1 (3.6)
CDAI at initiation of adalimumab therapy ¹	195.4 ± 89.2	152.2 ± 65.8	177.8 ± 82.0
CDAI > 150 at initiation of adalimumab therapy, <i>n</i>	9	7	16
CRP at initiation of adalimumab therapy ¹	0.72 ± 0.92	0.66 ± 0.73	0.70 ± 0.83

¹Data are expressed as mean ± SD. CDAI: Crohn's disease activity index.^a*P* < 0.05 *vs* adalimumab.

Clinical efficacy of ADA therapy

The overall clinical response and remission rates are shown in Table 2. The rates of clinical remission at weeks 2, 4, 8, and 24 were 60%, 66.7%, 69.6%, and 63.2%, respectively. The mean CDAI of all patients at weeks 2, 4, 8, and 24 was 124.4 ± 60.2, 120.0 ± 66.8, 123.6 ± 73.5, and 135.1 ± 74.4, respectively. The CDAI was significantly decreased commencing 2 wk after the initiation of ADA treatment and remained low for 24 wk (Table 2).

Impact of ADA/AZA on the clinical remission compared to ADA maintenance

Regarding the concomitant use of immunosuppressive agents, 12 patients (42.9%) were treated with a stable dose of AZA (50.0 ± 21.3 mg/d, 1.0 ± 0.5 mg/kg/d) before the initiation of ADA. The concomitant use of AZA was well tolerated, with only minor side effects. Although there were no statistically significant differences seen until 12 wk into ADA and AZA treatment (compared with ADA treatment), combinational therapy with ADA and AZA significantly increased the clinical remission rate (Figure 1) and reduced the CDAI, compared with nonconcomitant use at week 24 (Figure 2A).

Sixteen of the 28 patients were found to have increased CRP serum levels (> 0.25 mg/dL) before starting ADA therapy. The mean serum CRP of all of the patients at weeks 2, 4, 8, and 24 were 0.27 ± 0.40, 0.28 ± 0.51, 0.46 ± 0.69, and 0.51 ± 0.94 mg/dL, respectively.

ADA significantly reduced the CRP levels at weeks 2 and 4 after the initiation of treatment. The concomitant use of AZA tended to maintain low CRP levels for 24 wk, even though the result was not statistically significant (CRP levels at weeks 0 and 24 were 0.66 ± 0.73 and 0.27 ± 0.44 mg/dL, respectively; *P* = 0.09, Figure 2B).

DISCUSSION

The effectiveness of AZA in CD patients treated with IFX has been clearly reported by many investigators. However, few reports have demonstrated the benefits of ADA and AZA combination therapy. In this study, we analyzed the use of ADA and AZA in patients with mild to moderate CD at our clinical practice and found that scheduled ADA with concomitant use of AZA was more effective in inducing and maintaining remission for 24 wk after the initiation of ADA treatment, compared with ADA monotherapy. Although ADA monotherapy was also able to induce and maintain clinical remission from 2 to 24 wk compared to the baseline, the effects of the combination therapy were better. Since previous IFX nonresponse and previous treatment with an anti-TNF agent were reported to be associated with a decreased clinical efficacy and a loss of response^[20], we also compared the CDAI between anti-TNF-naïve patients and anti-TNF-treated patients in this study, but there was no significant difference.

ADA is a human monoclonal IgG1 antibody that has demonstrated efficacy in the induction and maintenance of clinical remission in patients with moderate to severe CD and is useful in patients who have lost responsiveness to IFX^[11-15]. IFX, a chimeric anti-TNF-α antibody, has potent immunogenicity; repeated administration of IFX could result in the development of antibodies that may lead to the loss of response^[21]. Although no consensus has yet been established on the appropriateness of concomitant immunomodulators with anti-TNF-α antibody therapy for CD^[22-24], immunosuppressants were shown to inhibit the development of HACA^[10]. Moreover, since the clearance of IFX is affected by the presence of HACA, IFX serum levels were significantly higher in patients with concomitant immunosuppressive therapy, as this concomitant usage reduces the frequency of HACA formation^[4,25]. Sokol *et al.*^[17] assessed the efficacy of immunosuppressants with scheduled IFX in patients with IBD and reported that IBD flare-ups, perianal complications, and switching to ADA were less frequently observed in patients with combined immunosuppressant and biologic agent use than in those patients who were not concomitantly treated with immunosuppressants^[22]. Furthermore, in the SONIC study, Colombel *et al.*^[16] compared the efficacy and safety of IFX monotherapy and IFX plus AZA combination therapy for CD and showed that the combination therapy provided a significantly greater benefit than that of IFX monotherapy at weeks 26 and 50. These studies suggest

Table 2 Clinical efficacy of adalimumab therapy

Week	0	2	4	8	24
<i>n</i>	27	25	24	23	19
CDAI	177.8 ± 82.0	124.4 ± 60.2 ^a	120.0 ± 66.8 ^a	123.6 ± 73.5 ^a	135.1 ± 74.4 ^a
Remission	37%	60%	66.70%	69.60%	63.20%

Remission: Crohn's disease activity index (CDAI) < 150 points. ^a*P* < 0.05 *vs* week 0.

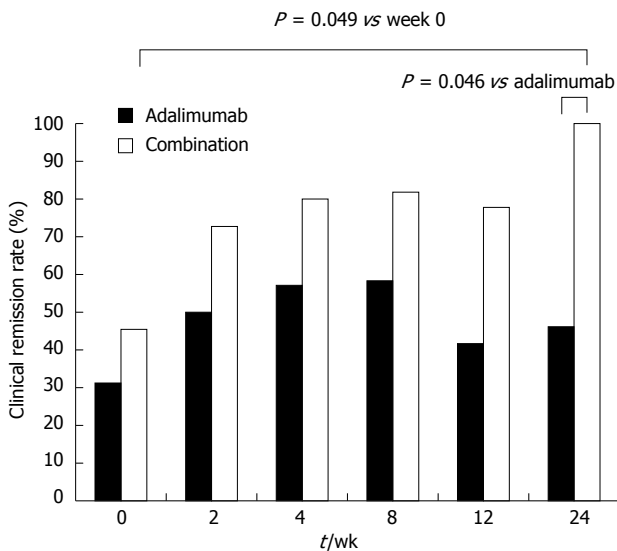


Figure 1 Clinical remission rate. Combinational therapy with adalimumab and azathioprine significantly increased the clinical remission rate vs nonconcomitant use at week 24.

that combination therapy with IFX and AZA may have an added benefit in maintaining the remission of CD. With regard to ADA, a substantial proportion of patients, including previously treated anti-TNF- α patients and anti-TNF- α treatment-naïve patients, needed dose escalation during ADA treatment^[12,26]. However, concomitant immunosuppressive therapy was not associated with a loss of response or a need for dose escalation and did not influence the development of ADA antibodies^[27-29]. Recently, Reenaers *et al.*^[19] reported that usage of ADA with immunosuppressive drugs, such as AZA, during the first semester of initiating ADA demonstrated a slight decrease in ADA failure and a lower need for ADA dose escalation. Moreover, the presence of anti-ADA antibodies and a low serum ADA concentration have been reported to be associated with a diminished clinical response in rheumatoid arthritis patients, and the concomitant use of an immunomodulator significantly suppresses the concentration of anti-ADA antibodies^[30,31]. Interestingly, in this study, AZA in the presence of ADA provides better efficacy in patients it has previously failed. However, some patients with IFX had not early received concomitant AZA. Therefore, we consider that ADA and AZA combination therapy may be more effective than ADA monotherapy via the inhibition of the development of anti-ADA antibodies. Prospective

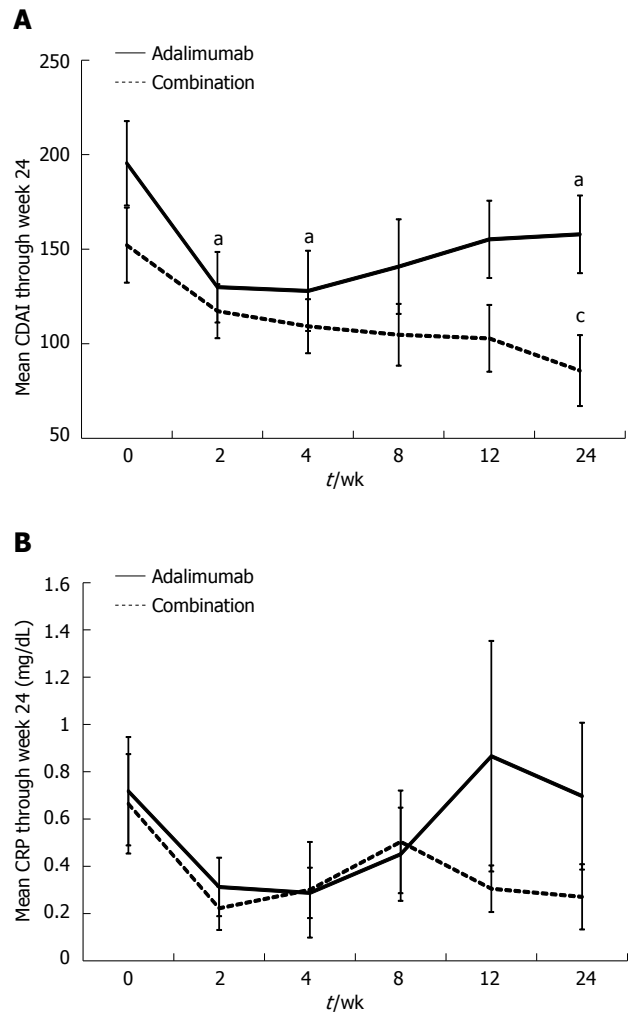


Figure 2 Mean Crohn's disease activity index and C-reactive protein through week 24. A: Combinational therapy with adalimumab (ADA) and azathioprine (AZA) significantly reduced the Crohn's disease activity index (CDAI), compared with nonconcomitant use at week 24. ^a*P* < 0.05 *vs* week 0; ^c*P* < 0.05 *vs* adalimumab; B: The concomitant use of AZA tended to maintain low C-reactive protein (CRP) levels for 24 wk (CRP levels at weeks 0 and 24 were 0.66 ± 0.73 and 0.27 ± 0.44, respectively, *P* = 0.09).

studies, combined with anti-ADA antibody and ADA trough level measurements, are required^[19].

In this study, we found that ADA treatment with concomitant AZA usage significantly suppressed the CDAI and increased the remission rate at 24 weeks after the initiation of therapy, compared with ADA treatment alone. Furthermore, these results were not consistent with previous reports showing that the efficacy of ADA is independent of the use of concomitant immunosup-

pression^[27-29]. One possible explanation for this discrepancy is that these previous studies assessed the efficacy of ADA in patients with moderate to severe CD, whereas the present study included mild to moderate CD patients. Notably, the CDAI at the beginning of this study was relatively low (177.8 ± 82.0). Since the CDAI scores were gradually increasing, some patients were switched from IFX treatment to ADA treatment, even though their CDAI scores were still in remission (CDAI < 150 at the initiation of ADA treatment). Indeed, Zheng *et al.*^[32] evaluated the efficacy of AZA in controlling the relapse of disease in patients with CD for at least 6 months and showed that AZA treatment decreased the CDAI from 187.3 ± 23.4 to 96.1 ± 13.5 . Actually, the finding of a significant efficacy with ADA plus AZA combination therapy compared to ADA monotherapy is revealed only after week 24 in this study. Generally, thiopurines require a lag time of approximately 6 mo before expressing full activity. Taken together, we consider that the combination of ADA and AZA may have an added benefit in inducing long-term remission compared with ADA alone, even though ADA has shown less immunogenicity compared with IFX.

Our study had some major limitations. The patients were not randomized between ADA monotherapy and ADA plus AZA combination therapy. Also, the mean age was significantly different between these two groups due to the nature of retrospective study. Therefore, these two groups were not strictly comparable. In addition, the patient numbers were limited, as we only analyzed the data in our own hospital. Moreover, recent studies have revealed that Japanese and Caucasian CD patients genetically differ, as Japanese CD patients lack the polymorphism in the *NOD2* gene^[33-36]. Genetic differences might have affected the results in this study. An additional, larger, long-term multicenter study should be conducted to determine the effect of combination therapy with ADA and AZA for patients with CD.

So far, there have been few reports demonstrating that CD patients could achieve a better clinical remission with ADA and immunomodulator combination therapy, compared with ADA monotherapy^[19,37]. This study may help physicians decide whether to use combination therapy. In conclusion, scheduled ADA with concomitant AZA is more effective for clinical remission achievement at 24 wk in Japanese CD patients. Although further long-term studies evaluating the efficacy of combination therapy with ADA and AZA are required, our data suggest that ADA with concomitant AZA therapy may provide clinical benefit in inducing long-term remission.

COMMENTS

Background

The benefits of azathioprine (AZA) in Crohn's disease (CD) with infliximab as scheduled anti-tumor necrosis factor- α maintenance therapy have been established but remain unclear with adalimumab (ADA).

Research frontiers

So far, there have been few reports demonstrating that CD patients could achieve better clinical remission with ADA and immunomodulator combination therapy, compared with ADA monotherapy.

Innovations and breakthroughs

This is the first study reported in Asia on the effect of ADA with concomitant AZA for induction and maintenance of clinical remission in CD patients. The results of this study showed that ADA treatment with concomitant AZA usage significantly suppressed the Crohn's disease activity index and increased the remission rate at 24 wk after the initiation of therapy, compared with ADA treatment alone.

Applications

Although further long-term studies evaluating the efficacy of combination therapy with ADA and AZA are required, this study suggests that ADA with concomitant AZA therapy may provide clinical benefit in inducing long-term remission. The results of this study may help physicians decide whether to use combination therapy.

Peer review

This is an interesting small retrospective, observational, study assessing the effect of effects of ADA treatment with concomitant AZA in CD patients.

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Dietary polyphenols and colorectal cancer risk: The Fukuoka colorectal cancer study

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Abstract

AIM: To investigate the associations between dietary intake of polyphenols and colorectal cancer.

METHODS: The study subjects were derived from the Fukuoka colorectal cancer study, a community-based case-control study. The study subjects were 816 cases of colorectal cancer and 815 community-based controls. The consumption of 148 food items was assessed by a computer-assisted interview. We used the consumption of 97 food items to estimate dietary intakes of total, tea and coffee polyphenols. The Phenol-Explorer database was used for 92 food items. Of the 5 foods which were not listed in the Phenol-Explorer Database, polyphenol contents of 3 foods (sweet potatoes, satoimo and daikon) were based on a Japanese study and 2 foods (soybeans and fried potatoes) were estimated by ORAC-based polyphenol contents in the United States Department of Agriculture Database. Odds ratios (OR) and 95%CI of colorectal cancer risk according to quintile categories of intake were obtained by using logistic regression models with adjustment for age, sex, residential area, parental history of colorectal cancer, smoking, alcohol consumption, body mass index 10 years before, type of job, leisure-time physical activity and dietary intakes of calcium and n-3 polyunsaturated fatty acids.

RESULTS: There was no measurable difference in total or tea polyphenol intake between cases and controls, but intake of coffee polyphenols was lower in cases than in controls. The multivariate-adjusted OR of colorectal cancer according to quintile categories of coffee polyphenols (from the first to top quintile) were 1.00 (referent), 0.81 (95%CI: 0.60-1.10), 0.65 (95%CI: 0.47-0.89), 0.65 (95%CI: 0.46-0.89) and 0.82 (95%CI: 0.60-1.10), respectively ($P_{\text{trend}} = 0.07$). Similar, but less pronounced, decreases in the OR were also noted for the third and fourth quintiles of total polyphenol intake. Tea polyphenols and non-coffee polyphenols showed no association with colorectal cancer risk. The site-specific analysis, based on 463 colon cancer cases and 340 rectal cancer cases, showed an inverse association between coffee polyphenols and colon cancer. The multivariate-adjusted OR of colon cancer for the first to top quintiles of coffee polyphenols were 1.00 (referent), 0.92 (95%CI: 0.64-1.31), 0.75 (95%CI: 0.52-1.08), 0.69 (95%CI: 0.47-1.01), and 0.68 (95%CI: 0.46-1.00), respectively ($P_{\text{trend}} = 0.02$). Distal colon cancer showed a more evident inverse association with coffee polyphenols than proximal colon cancer. The association between coffee polyphenols and rectal cancer risk was U-shaped, with significant decreases in the OR at the second to fourth quintile categories. There was also a tendency that the OR of colon and rectal cancer decreased in the intermediate categories of total polyphenols. The decrease in the OR in the intermediate categories of total polyphenols was most pronounced for distal colon cancer. Intake of tea polyphenols was not associated with either colon or rectal cancer. The associations of coffee consumption with colorectal, colon and rectal cancers were almost the same as observed for coffee polyphenols. The trend of the association between coffee consumption and colorectal cancer was statistically significant.

CONCLUSION: The present findings suggest a decreased risk of colorectal cancer associated with coffee consumption.

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Key words: Colorectal cancer; Colon cancer; Rectal cancer; Polyphenols; Coffee; Tea

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INTRODUCTION

Colorectal cancer is one of the most common cancers in

the world and the second most common cause of cancer death in developed countries^[1]. In Japan, the incidence rate of colorectal cancer, especially of colon cancer, has increased dramatically until the 1990s^[2]. Lifestyle factors such as physical inactivity, obesity and alcohol use are known to confer increased risk of colorectal cancer. Of dietary factors, high intake of red meat has been related to increased risk of colorectal cancer, and fiber-containing plant foods and calcium have been considered to be protective^[3].

Polyphenols are the most abundant antioxidants in foods and have drawn much interest as potentially anticarcinogenic compounds. The flavonoids have been identified as potential cancer-preventive components of fruits and vegetables^[4]. Catechins and tea polyphenols inhibit tumorigenesis at initiation, promotion, and progression stages in animal experiments^[5]. Coffee is the major source of polyphenol intake in populations in which coffee is commonly consumed^[6], and phenolic compounds in coffee such as chlorogenic acids also have anticarcinogenic effects in animal models^[7]. Epidemiological studies have suggested that polyphenol-rich foods such as fruits and vegetables^[3,8] and beverages such as tea^[9-13] and coffee^[14,15] may be protective against colorectal cancer. Experimental studies on colorectal cancer cell lines also support a protective role of polyphenols in carcinogenesis by exerting antioxidant and anti-proliferative effects and by inducing cell cycle arrest^[16]. In the study reported here, we addressed the association of dietary intake of polyphenols with colorectal cancer risk using data from a community-based case-control study in Japan^[17].

MATERIALS AND METHODS

The present data were derived from the Fukuoka colorectal cancer study, a community-based case-control study in Fukuoka, Japan. The research protocol was approved by the ethics committee of Kyushu University and collaborating hospitals. Details of the design and conduct of the study have been described elsewhere^[17].

Study subjects

Cases were histologically confirmed incident cases of colorectal adenocarcinomas who were admitted for surgical treatment to one of the collaborating hospitals in Fukuoka City and three adjacent areas during the period from September 2000 to December 2003. Of 1053 eligible cases, a total of 840 cases (80%) participated in the interview; the eligible cases were aged between 20-74 years at the time of diagnosis; lived in the study area; had no prior history of partial or total removal of the colon, familial adenomatous polyposis or inflammatory bowel disease; and were mentally competent to give informed consent and to complete the interview. Research staff visited each hospital regularly, determined the eligibility of cases by referring to admission logs and medical records, and interviewed him/her if written informed consent was obtained.

Eligibility criteria for controls were the same as described for the cases except that they had no history of colorectal cancer. A total of 1500 control candidates were randomly selected by a two-stage random sampling on the basis of a resident registry with the frequency by sex and 10-year age class matched to expected sex- and age-specific frequencies of cases. Recruitment was initiated by a letter of invitation, which was followed by phone calls if available. After exclusion of 113 who were found to be ineligible and 5 who were diagnosed with colorectal cancer after the interview, 833 (60%) participated in the interview.

In the analysis, we excluded those who were in the top 1% or bottom 1% of total energy intake within each stratum of sex and age class (< 55, 55-64, and ≥ 65 years of age). A total of 816 cases and 815 controls remained.

Lifestyle questionnaire

Cases and controls were interviewed in person regarding smoking, alcohol use, physical activity and other factors using a uniform questionnaire. The index date was taken as the date of onset of symptoms or the screening leading to the diagnosis of colorectal cancer for cases and the date of interview for controls. Anthropometric questions inquired about height (cm) and body weight (kg) at the time of interview and 10 years earlier. Body mass index (kg/m^2) 10 years earlier was used in the analysis because the current body mass index was unrelated to the risk^[18]. For 4 cases and 11 controls, body weight 10 years before was not ascertained, and the current body weight was used for substitution. The amount of alcohol was expressed using the conventional Japanese unit; one go (180 mL) of sake, one large bottle (633 mL) of beer and half a go (90 mL) of shochu were each expressed as one unit; and one drink (30 mL) of whisky or brandy and one glass (100 mL) of wine were each converted to half a unit. Regarding smoking, ever smokers reported years of smoking and the numbers of cigarettes per day for each decade of life, and we calculated the cumulative exposure to cigarette smoking until the beginning of the previous decade of age. Types of job and non-job physical activities 5 years before were ascertained. The amount of non-occupational physical activity was expressed as a sum of metabolic equivalents (MET) multiplied by the hours of weekly participation in each activity, *i.e.*, MET-hours per week. History of parental colorectal cancer also was obtained.

Dietary assessment

Consumption frequencies and portion sizes of 148 food and beverage items were ascertained by a computer-assisted interview. Details of the dietary interview have been described elsewhere^[19]. Participants were asked to report their usual consumption during the past 12 mo. Intakes of nutrients were calculated based on the food composition tables in Japan^[20]. To estimate dietary intake of polyphenols, we used 97 food items, including cereals, soybeans, vegetables, fruits, beverages and condiments.

Table 1 Characteristics of colorectal cancer cases and controls

Variables	Cases (<i>n</i> = 816)	Controls (<i>n</i> = 815)
Male	60%	62%
Age (yr), mean ± SD	60.5 ± 9.1	58.9 ± 10.7
Dietary intake, median (IQR) ¹		
Total polyphenols (mg/d)	1025 (698-1487)	1047 (736-1431)
Tea polyphenols (mg/d) ²	432 (226-576)	397 (215-509)
Coffee polyphenols (mg/d) ³	187 (0.2-619)	260 (39-643)

¹Polyphenols and food intakes were energy-adjusted to 2000 kcal/d;

²Included green tea, black tea and oolong tea; ³Included coffee infusion, instant coffee and coffee drinks. IQR: Interquartile range.

Polyphenols for 92 food items were derived from the Phenol-Explorer Database, which is a compilation of polyphenol contents of 452 daily foods and beverages based on 638 published analytical studies^[21]. As for the remaining 5 foods which were not listed in the Phenol-Explorer Database, polyphenol contents of 3 foods (sweet potatoes, satoimo and daikon) were based on a Japanese study^[22], and 2 foods (soybeans and fried potatoes) were estimated by ORAC-based polyphenol contents in the United States Department of Agriculture Database^[23].

Statistical analysis

Dietary intakes of the nutrients and polyphenols were transformed to a natural log-scale and were adjusted to a total energy intake of 2000 kcal/d by the regression residual method^[24]. Subjects were divided into quintile categories according to intakes of polyphenols among controls. Logistic regression analysis was used to estimate odds ratios (OR) and 95%CI of colorectal cancer for each category with the lowest quintile category as the referent group. We also calculated the OR according to coffee consumption in terms of the number of cups per week or day, with an assumption that one cup corresponded to 150 g of coffee infusion.

Confounding variables under consideration were age, sex, residential area (Fukuoka City or others), parental history of colorectal cancer, smoking (0, 1-399, 400-799 or > 800 cigarettes/year), alcohol consumption (0, 0.1-0.9, 1.0-1.9 or ≥ 2 units per day), body mass index 10 years before (< 22.5, 22.5-24.9, 25.0-27.4 or ≥ 27.5 kg/m^2), type of job (sedentary or non-sedentary), leisure-time physical activity (0, 1-5.9 or ≥ 6 MET-h/wk) and dietary intakes of calcium and n-3 polyunsaturated fatty acids (PUFA). Calcium and n-3 PUFA intakes were related to decreased risk of colorectal cancer in the study population^[25-29]. Trends of the associations were assessed with ordinal scores assigned to quintile categories of intake. Statistical significance was declared with the two-sided $P < 0.05$. Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, United States).

RESULTS

Demographic and lifestyle characteristics of colorectal

Table 2 Association of dietary polyphenol intakes with colorectal cancer risk

Nutrient (unit)	Quintile of intake					<i>P</i> _{trend}
	Q1 (low)	Q2	Q3	Q4	Q5 (high)	
Total polyphenols						
Median (mg/d)	534	812	1047	1335	2104	
Cases/controls	190/163	170/163	142/163	138/163	176/163	
OR (95%CI) ¹	1 (reference)	0.85 (0.62, 1.16)	0.72 (0.52, 0.98)	0.74 (0.54, 1.02)	0.95 (0.70, 1.31)	0.52
OR (95%CI) ²	1 (reference)	0.85 (0.62, 1.16)	0.72 (0.52, 1.00)	0.74 (0.54, 1.03)	0.97 (0.70, 1.33)	0.59
Tea polyphenols						
Median (mg/d)	65	224	397	475	853	
Cases/controls	128/163	151/163	184/163	157/163	196/163	
OR (95%CI) ¹	1 (reference)	1.14 (0.82, 1.58)	1.37 (0.99, 1.89)	1.13 (0.81, 1.58)	1.37 (0.99, 1.90)	0.09
OR (95%CI) ²	1 (reference)	1.13 (0.81, 1.57)	1.36 (0.98, 1.88)	1.16 (0.83, 1.62)	1.38 (0.99, 1.92)	0.08
Coffee polyphenols						
Median (mg/d)	0	82	260	541	1287	
Cases/controls	220/163	167/163	133/163	129/163	167/163	
OR (95%CI) ¹	1 (reference)	0.80 (0.59, 1.08)	0.65 (0.48, 0.90)	0.64 (0.46, 0.87)	0.83 (0.60, 1.13)	0.09
OR (95%CI) ²	1 (reference)	0.81 (0.60, 1.10)	0.65 (0.47, 0.89)	0.65 (0.46, 0.89)	0.82 (0.60, 1.12)	0.07
Polyphenols other than coffee						
Median (mg/d)	280	466	631	790	1104	
Cases/controls	131/163	158/163	181/163	166/163	180/163	
OR (95%CI) ¹	1 (reference)	1.17 (0.85, 1.63)	1.29 (0.93, 1.79)	1.13 (0.81, 1.58)	1.23 (0.88, 1.72)	0.35
OR (95%CI) ²	1 (reference)	1.19 (0.86, 1.66)	1.31 (0.94, 1.82)	1.20 (0.85, 1.69)	1.31 (0.93, 1.84)	0.19

¹Adjusted for age, sex, residence area, parental history of colorectal cancer, smoking, alcohol drinking, body mass index 10 years before, type of job and leisure-time physical activity; ²Further adjusted for calcium and n-3 polyunsaturated fatty acids. OR: Odds ratio.

cancer cases and controls were previously described^[25-29]. The two groups did not differ much with respect to sex, age, residence area and most other factors. Body mass index was greater and a history of parental colorectal cancer was slightly more frequent in the cases than in the controls. There was no measurable difference in total or tea polyphenol intake between cases and controls, but intake of coffee polyphenols was lower in cases than in controls (Table 1). In the whole sample, intake of total polyphenols derived from tea and coffee was 38% and 34%, respectively.

The associations of intakes of total, tea and coffee polyphenols with colorectal cancer are shown in Table 2. Statistically significant decreases in the OR were observed in those with high intake of coffee polyphenols (the third and fourth quintile categories) compared with the lowest quintile, but the OR for the top quintile did not decrease further, showing an upward tendency. Similar, but less pronounced, decreases in the OR were also noted for the third and fourth quintiles of total polyphenol intake. Tea polyphenols and non-coffee polyphenols showed no clear association with colorectal cancer risk regardless of adjustment for the dietary factors.

Multivariate-adjusted ORs in the subsite-specific analysis are summarized in Table 3. Cases of colon and rectal cancers numbered 463 and 340, respectively. There were 188 cases who had proximal colon cancer only and 272 cases with distal colon cancer alone. The OR of colon cancer decreased with increasing intakes of coffee polyphenols; although the individual OR for the third to fifth quintiles were not significantly different from unity, a decreasing trend was statistically significant. Distal colon cancer showed a more evident inverse association with coffee polyphenols than proximal colon cancer. The

association between coffee polyphenols and rectal cancer was U-shaped, with significant decreases in the OR at the second to fourth quintile categories. Again, there was a tendency for the OR of colon and rectal cancer to decrease in the intermediate categories of total polyphenols. The decrease in the OR in the intermediate categories of total polyphenols was most pronounced for distal colon cancer. Intake of tea polyphenols was not associated with either colon or rectal cancer.

Additionally, we analyzed the associations of coffee consumption with colorectal cancers (Table 4). The associations of coffee consumption with colorectal, colon, and rectal cancers were almost the same as observed for coffee polyphenols. Nonetheless, the trend of the association between coffee consumption and colorectal cancer was statistically significant. The trend of an inverse association with coffee was also statistically significant for distal colon cancer. The OR of proximal colon cancer tended to decrease with increasing consumption of coffee, but the trend failed to reach statistical significance.

DISCUSSION

The present study showed protective associations of coffee polyphenols and coffee consumption with the risk of colorectal cancer, especially of colon cancer. Non-coffee polyphenols showed no measurable association with the overall or site-specific risk of colorectal cancer. Thus, a modestly decreased risk of colorectal cancer associated with total polyphenol intake was probably ascribed to coffee polyphenols.

The association between coffee consumption and colorectal cancer has been examined in many studies in different populations. A meta-analysis of 12 cohort stud-

Table 3 Multivariate-adjusted odds ratio (95%CI) of colon and rectal cancers according to polyphenols intake

Nutrient	Quintile of intake					<i>P</i> _{trend}
	Q1 (low)	Q2	Q3	Q4	Q5 (high)	
Total polyphenols						
Colon						
Cases/controls	111/163	110/163	76/163	80/163	86/163	
OR (95%CI) ¹	1 (reference)	0.90 (0.63, 1.29)	0.66 (0.45, 0.96)	0.73 (0.50, 1.07)	0.81 (0.55, 1.18)	0.13
Proximal colon						
Cases/controls	40/163	45/163	34/163	38/163	31/163	
OR (95%CI) ¹	1 (reference)	0.91 (0.55, 1.52)	0.78 (0.46, 1.33)	0.91 (0.53, 1.54)	0.79 (0.45, 1.38)	0.44
Distal colon						
Cases/controls	70/163	64/163	42/163	42/163	54/163	
OR (95%CI) ¹	1 (reference)	0.86 (0.56, 1.31)	0.57 (0.36, 0.91)	0.61 (0.38, 0.98)	0.79 (0.50, 1.23)	0.11
Rectum						
Cases/controls	77/163	60/163	62/163	56/163	85/163	
OR (95%CI) ¹	1 (reference)	0.73 (0.48, 1.10)	0.73 (0.48, 1.11)	0.70 (0.46, 1.07)	1.08 (0.72, 1.62)	0.74
Tea polyphenols						
Colon						
Cases/controls	64/163	92/163	103/163	98/163	106/163	
OR (95%CI) ¹	1 (reference)	1.37 (0.91, 2.05)	1.50 (1.00, 2.23)	1.34 (0.89, 2.03)	1.42 (0.95, 2.14)	0.18
Proximal colon						
Cases/controls	21/163	33/163	44/163	41/163	49/163	
OR (95%CI) ¹	1 (reference)	1.38 (0.75, 2.54)	1.77 (0.98, 3.19)	1.42 (0.77, 2.61)	1.82 (1.01, 3.30)	0.08
Distal colon						
Cases/controls	43/163	58/163	58/163	56/163	57/163	
OR (95%CI) ¹	1 (reference)	1.36 (0.85, 2.19)	1.32 (0.82, 2.12)	1.26 (0.77, 2.06)	1.18 (0.72, 1.92)	0.74
Rectum						
Cases/controls	62/163	57/163	80/163	56/163	85/163	
OR (95%CI) ¹	1 (reference)	0.89 (0.57, 1.36)	1.25 (0.83, 1.88)	0.91 (0.59, 1.42)	1.27 (0.84, 1.93)	0.25
Coffee polyphenols						
Colon						
Cases/controls	128/163	103/163	82/163	75/163	75/163	
OR (95%CI) ¹	1 (reference)	0.92 (0.64, 1.31)	0.75 (0.52, 1.08)	0.69 (0.47, 1.01)	0.68 (0.46, 1.00)	0.02
Proximal colon						
Cases/controls	52/163	41/163	34/163	32/163	29/163	
OR (95%CI) ¹	1 (reference)	0.89 (0.55, 1.44)	0.75 (0.45, 1.25)	0.76 (0.45, 1.27)	0.70 (0.41, 1.20)	0.15
Distal colon						
Cases/controls	76/163	61/163	47/163	42/163	46/163	
OR (95%CI) ¹	1 (reference)	0.89 (0.58, 1.35)	0.72 (0.46, 1.12)	0.62 (0.39, 0.98)	0.66 (0.42, 1.04)	0.02
Rectum						
Cases/controls	90/163	62/163	48/163	53/163	87/163	
OR (95%CI) ¹	1 (reference)	0.65 (0.44, 0.98)	0.50 (0.33, 0.77)	0.55 (0.36, 0.84)	0.93 (0.63, 1.37)	0.51

¹Adjusted for age, sex, residence area, parental history of colorectal cancer, smoking, alcohol drinking, body mass index 10 years before, type of job, leisure-time physical activity, calcium and n-3 polyunsaturated fatty acid. OR: Odds ratio.

ies found a relative risk (RR) of 0.91 (95%CI: 0.81-1.02) of colorectal cancer for the highest versus lowest coffee consumption^[15]. A slightly pronounced decrease in the RR of colorectal cancer for the highest versus lowest consumption was noted in a combined analysis of 3 Japanese cohort studies (RR = 0.83, 95%CI: 0.62-1.10)^[15]. On the other hand, a decrease in the risk of colorectal cancer associated with coffee use was more pronounced in a meta-analysis of 24 case-control studies^[14]. The pooled OR for the highest versus non/low consumption was 0.70 (95%CI: 0.60-0.81) for colorectal cancer, 0.75 (95%CI: 0.60-0.81) for colon cancer and 0.87 (95%CI: 0.75-1.00) for rectal cancer^[14]. A weaker association in the cohort studies is probably ascribed to a different time of exposure under consideration^[14]. Coffee consumption in the recent past was assessed in most case-control studies. In the cohort studies, a follow-up shorter than 10 years was more likely to result in an inverse association between

coffee consumption and colorectal cancer risk than a longer follow-up^[15]. In a recent large cohort study^[30], coffee consumption was associated with a decreased risk of proximal colon cancer, but not of distal colon cancer. The present study showed no such site-specific association with coffee.

Coffee polyphenolic compounds include chlorogenic, caffeic, ferulic and cumaric acids, and diterpenes (cafestol and kahweol) have been shown to possess anticarcinogenic properties^[7]. Possible mechanisms by which coffee polyphenols are protective against colorectal cancer include reduction in bile acid secretion^[31], reduction in the synthesis of bile by down-regulation of the expression of bile acid homeostatic genes^[32] and increase in colonic motility^[33].

The present study did not provide any evidence for a decreased risk of colorectal cancer associated with dietary intake of tea polyphenols. Tea polyphenols have been

Table 4 Association of frequency of coffee consumption with colorectal cancer risk

	Frequency of coffee consumption					<i>P</i> _{trend}
	< 1 cup/wk	1-3 cups/wk	4-6 cups/wk	1-3 cups/d	≥ 4 cups/d	
Colorectal						
Cases/controls	245/181	93/83	66/78	265/317	147/156	
OR (95%CI) ¹	1 (reference)	0.88 (0.61, 1.26)	0.66 (0.45, 0.99)	0.65 (0.50, 0.85)	0.82 (0.59, 1.13)	0.01
Colon						
Cases/controls	145/181	62/83	40/78	141/317	75/156	
OR (95%CI) ¹	1 (reference)	1.04 (0.69, 1.57)	0.75 (0.47, 1.18)	0.64 (0.47, 0.87)	0.78 (0.53, 1.14)	0.01
Proximal colon						
Cases/controls	60/181	22/83	19/78	62/317	25/156	
OR (95%CI) ¹	1 (reference)	0.84 (0.47, 1.50)	0.88 (0.48, 1.62)	0.69 (0.45, 1.06)	0.69 (0.39, 1.21)	0.08
Distal colon						
Cases/controls	84/181	40/83	21/78	78/317	49/156	
OR (95%CI) ¹	1 (reference)	1.16 (0.72, 1.89)	0.65 (0.37, 1.15)	0.58 (0.40, 0.85)	0.82 (0.52, 1.29)	0.02
Rectum						
Cases/controls	98/181	29/83	26/78	118/317	69/156	
OR (95%CI) ¹	1 (reference)	0.63 (0.38, 1.04)	0.55 (0.32, 0.93)	0.63 (0.45, 0.88)	0.82 (0.54, 1.23)	0.10

¹Adjusted for age, sex, residence area, parental history of colorectal cancer, smoking, alcohol drinking, body mass index 10 years before, type of job, leisure-time physical activity, calcium and n-3 polyunsaturated fatty acid. OR: Odds ratio.

shown to have anti-cancer properties in numerous *in vitro* and *in vivo* studies^[34,35], but epidemiologic findings are inconsistent as regards tea and colorectal cancer risk. In China, regular green tea consumption was associated with a reduced risk of colorectal cancer in male non-smokers, but not in male smokers^[9] and in women^[10]. On the other hand, in cohort studies of Chinese in Singapore^[11] and Japan^[13], green tea consumption was unrelated to colorectal cancer risk in men and women combined. A pooled analysis of 13 cohort studies in Western countries showed an increased risk associated with tea consumption; the RR for a consumption of > 900 g in liquid/d versus no consumption was 1.28 (95%CI: 1.02-1.61) in men and women combined^[12]. A combined analysis of two Japanese prospective studies found no association between green tea consumption and colorectal cancer risk^[13].

We examined the associations with polyphenol and coffee for proximal and distal colon cancer separately, because these two cancers have distinct molecular mechanisms in carcinogenesis^[36]. It was recently suggested that molecular changes in colorectal cancer were continuous, rather than dichotomous, from rectum to ascending colon^[37]. The number of colorectal cancer cases was not large enough to perform a detailed subsite analysis in the present study. It should be noted that the difference in the molecular changes among the detailed subsites of proximal colon was less marked as compared with the difference between proximal and distal colon cancer.

In addition to a large sample size and the use of community controls, the detailed dietary assessment was a notable strength in the present study. The dietary interview was conducted as an in-person interview with typical dishes and serving sizes shown on a computer display. The present study had some weaknesses to be mentioned. The retrospective assessment of diet is a problem inherent to case-control studies. Dietary intakes in the past year may not have captured a habitual consumption

relevant to the development of colorectal cancer. Additionally, the participation rate was lower in the controls (60%) than in the cases (80%), and this may have caused a selection bias.

In conclusion, a case-control study in Japan showed protective associations of coffee polyphenols and coffee consumption with the risk of colorectal cancer, especially of colon cancer.

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COMMENTS

Background

Polyphenols are the most abundant antioxidants in foods and have drawn much interest as potentially anticarcinogenic compounds. Epidemiological studies have suggested that polyphenol-rich foods and beverages may be protective against colorectal cancer.

Research frontiers

The Phenol-Explorer Database has enabled researchers to study the association between polyphenol intake and cancer risk. Evidence from meta-analyses

indicates that coffee may be protective against colorectal cancer.

Innovations and breakthroughs

This was the first Japanese epidemiological study reporting that coffee polyphenol intake was associated with a decreased risk of colorectal cancer, particularly of colon cancer.

Applications

Understanding the role for coffee polyphenols in colorectal carcinogenesis may advance a future strategy in the prevention of colorectal cancer.

Terminology

The flavonoids are plant antioxidative compounds and possess anticarcinogenic properties. Tea catechins are a class of flavonoids, and are shown to inhibit tumorigenesis in animal experiments. Coffee is the major source of polyphenol intake such as chlorogenic and caffeic acids.

Peer review

The present study is a very timely and well performed study that investigates the associations between intake of dietary polyphenols and colorectal cancer. The authors have done a tremendous job of designing and executing the project, and the data analysis and interpretations are very logical. I think these data are very important and provide another dimension and validity to the growing awareness of diet and its link to colorectal cancer.

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Coinfection with hepatitis C virus and schistosomiasis: Fibrosis and treatment response

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Abstract

AIM: To assess whether schistosomiasis coinfection with chronic hepatitis C virus (HCV) influences hepatic fibrosis and pegylated-interferon/ribavirin (PEG-IFN/RIB) therapy response.

METHODS: This study was designed as a retrospective analysis of 3596 chronic HCV patients enrolled in the Egyptian National Program for HCV treatment with PEG-IFN/RIB. All patients underwent liver biopsy and anti-schistosomal antibodies testing prior to HCV treatment. The serology results were used to categorize the patients into group A (positive schistosomal serology) or group B (negative schistosomal serology). Patients in group A were given oral antischistosomal treatment

(praziquantel, single dose) at four weeks prior to PEG-IFN/RIB. All patients received a 48-wk course of PEG-IFN (PEG-IFN α 2a or PEG-IFN α 2b)/RIB therapy. Clinical and laboratory follow-up examinations were carried out for 24 wk after cessation of therapy (to week 72). Correlations of positive schistosomal serology with fibrosis and treatment response were assessed by multiple regression analysis.

RESULTS: Schistosomal antibody was positive in 27.3% of patients (15.9% females and 84.1% males). The patients in group A were older ($P = 0.008$) and had a higher proportion of males ($P = 0.002$) than the patients in group B. There was no significant association between fibrosis stage and positive schistosomal serology ($P = 0.703$). Early virological response was achieved in significantly more patients in group B than in group A (89.4% vs 86.5%, $P = 0.015$). However, significantly more patients in group A experienced breakthrough at week 24 than patients in group B (36.3% vs 32.3%, $P = 0.024$). End of treatment response was achieved in more patients in group B than in group A (62.0% vs 59.1%) but the difference did not reach statistical significance ($P = 0.108$). Sustained virological response occurred in significantly more patients in group B than in group A (37.6% vs 27.7%, $P = 0.000$). Multivariate logistic regression analysis of patient data at treatment weeks 48 and 72 showed that positive schistosomal serology was associated with failure of response to treatment at week 48 (OR = 1.3, $P = 0.02$) and at week 72 (OR = 1.7, $P < 0.01$).

CONCLUSION: Positive schistosomal serology has no effect on fibrosis staging but is significantly associated with failure of response to HCV treatment despite anti-schistosomal therapy.

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Key words: Hepatitis C virus; Schistosomiasis; Coinfection; Fibrosis; Treatment response

Core tip: Both hepatitis C virus (HCV) and schistosomiasis are highly endemic in Egypt and coinfection is frequently encountered. The effect of such coinfection on hepatic fibrosis and response to pegylated-interferon and ribavirin therapy (PEG-IFN/RIB) remains unclear. Our study aimed to assess the impact of schistosomiasis on hepatic fibrosis and response to PEG-IFN/RIB therapy in chronic HCV Egyptian patients. Antischistosomal antibody was positive in 27.3% of 3596 chronic HCV patients. Findings suggest positive schistosomal serology has no effect on fibrosis stage but is significantly associated with failure of response to HCV treatment despite antischistosomal therapy.

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INTRODUCTION

The hepatitis C virus (HCV) is a major public health problem and a leading cause of chronic liver disease^[1]. In fact, Egypt has the largest epidemic of HCV in the world with an overall serum positive prevalence of 14.7% as reported by the Egyptian demographic health survey^[2]. It also is a major cause of liver fibrosis, which is associated with significant morbidity and mortality^[3].

Schistosomiasis is also of significant concern as it is endemic in Egypt^[4,5]. *Schistosoma haematobium* is endemic in Upper Egypt (7.8% prevalence), while *Schistosoma mansoni* has greater prevalence in Lower Egypt (36.4%)^[6]. The presence of both HCV and *Schistosoma* spp. is of significant concern as patients with coinfections have been shown to have higher HCV RNA titers, increased histological activity, greater incidence of cirrhosis/hepatocellular carcinoma, and higher mortality rates than patients suffering from single infections^[7]. In addition, patients diagnosed with hepatosplenic schistosomiasis have increased opportunities for additional infections and medical abnormalities. These may include up to a 10-fold opportunity for coinfection with hepatitis B virus (HBV) (compared to healthy counterparts), chronic hepatitis on liver biopsy, persistent antigenemia, and increased frequency of liver failure^[8].

The aim of this study was to evaluate the impact of schistosomiasis on hepatic fibrosis and on response to pegylated-interferon combined with ribavirin (PEG-IFN/RIB) therapy in Egyptian patients with chronic HCV.

MATERIALS AND METHODS

Patient characteristics and study design

This retrospective study included 3596 Egyptian patients

with chronic HCV treated with PEG-IFN/RIB at Cairo-Fatemic Hospital (Cairo, Egypt). Study enrollment inclusion and exclusion criteria are listed in Table 1.

All patients received PEG-IFN α 2a (180 μ g/wk dose) or PEG-IFN α 2b (1.5 μ g/kg/wk dose) via subcutaneous injection and oral RIB (800-1200 mg/d) for 48 wk as genotype 4 causes approximately 90% of HCV infections in Egypt^[9]. Patients were followed for 24 wk after cessation of therapy (to week 72).

The study was approved by the ethical committee of the Ministry of Health (Cairo, Egypt), and all patients consented to blood sampling and data usage in future research. Anti-schistosomal antibody testing was completed for all patients. Patients were stratified according to their schistosomal serological status; group A, HCV patients with positive schistosomal serology; group B, HCV patients with negative schistosomal serology. Study participants with positive schistosomal serology were given praziquantel (PZQ) therapy (oral, 40 mg/kg, single dose) at four weeks prior to initiation of the PEG-IFN/RIB therapy. Liver biopsies were performed for all patients to determine the grade of necroinflammation and stage of fibrosis (Table 2) (based on the METAVIR scoring system^[10]).

Quantitative real-time reverse transcription-polymerase chain reaction was used to detect HCV RNA (detection limit \geq 50 IU/mL) at baseline and weeks 12, 24, 48 and 72 after initiation of anti-viral therapy. Clinical and laboratory follow-up examinations were carried out to identify the presence of adverse side effects and treatment response. A total of 845 patients were lost to follow-up, so that 2751 participants completed the follow-up to week 72. Patients with early virologic response (EVR) continued follow-up to identify subsequent non-responders. Patients with detectable HCV-RNA at week 24 or those with less than 2 log decrease in viral load at week 12 were designated as treatment failure. HCV therapy was discontinued prematurely in those patients.

Statistical analysis

Quantitative data were described by averaging and calculating the SD. Intergroup differences were assessed by the Student's *t*-test, χ^2 or Fisher's exact tests were used for comparisons when appropriate. Multivariate logistic regression was performed with failure of treatment set as the dependent variable. In all tests, a *P*-value of < 0.05 was considered as the threshold for significance.

RESULTS

No statistically significant differences were found in the baseline characteristics of the two groups of patients, with the exceptions of age and sex (Table 3). HCV patients with positive schistosomal serology were older than the negative schistosomal group ($P = 0.008$) and showed a higher rate of males ($P = 0.002$). Of the 27.3% of the patients with positive schistosomal serology, 15.9% were

Table 1 Inclusion criteria and exclusion criteria

Inclusion criteria
Age ≥ 18 yr and ≤ 60 yr
Positive HCV antibodies and detectable HCV RNA by PCR
Positive liver biopsy for chronic hepatitis with F1 METAVIR score and elevated liver enzymes or F2/F3 METAVIR score
Naïve to treatment with PEG-IFN and RIB
Hepatitis B surface antigen negativity
Normal complete blood count, normal thyroid function, prothrombin concentration $\geq 60\%$, normal bilirubin, α -fetoprotein < 100 (ng/mL) and antinuclear antibody titer $< 1/160$
Exclusion criteria
Serious co-morbid conditions such as severe arterial hypertension, heart failure, significant coronary heart disease, poorly controlled diabetes (hemoglobin A1C $> 8.5\%$), chronic obstructive pulmonary disease
Major uncontrolled depressive illness
Solid transplant organ (renal, heart, or lung)
Untreated thyroid disease
History of previous anti-HCV therapy
Body mass index (BMI) > 35 kg/m ²
Known human immunodeficiency virus (HIV) coinfection
Hypersensitivity to one of the two drugs (PEG-IFN, RIB)
Concomitant liver disease other than hepatitis C (chronic hepatitis B, autoimmune hepatitis, alcoholic liver disease, hemochromatosis, α -1 antitrypsin deficiency, Wilson's disease)
Liver biopsy showing severe steatosis ($> 66\%$) and steatohepatitis, decompensated cirrhosis, hepatocellular carcinoma or METAVIR score F4

HCV: Hepatitis C virus; PCR: Polymerase chain reaction; PEG-IFN: Pegylated-interferon; RIB: Ribavirin.

Table 2 Necroinflammatory activity and fibrosis staging scale

Feature	
Grade A0	No histologic necroinflammatory activity
Grade A1	Mild activity
Grade A2	Moderate activity
Grade A3	Severe activity
Stage F0	No fibrosis
Stage F1	Portal fibrosis without septa
Stage F2	Portal fibrosis with rare septa
Stage F3	Numerous septa without cirrhosis
Stage F4	Cirrhosis

Cited from reference 10. A: Activity; F: Fibrosis.

females and 84.1% were males.

There were no significant differences between groups regarding fibrosis staging (Figure 1) or end of treatment response (ETR) (Table 4). However, the EVR and virological response at week 24 were significantly higher in patients with negative schistosomal serology ($P = 0.015$ and $P = 0.024$, respectively).

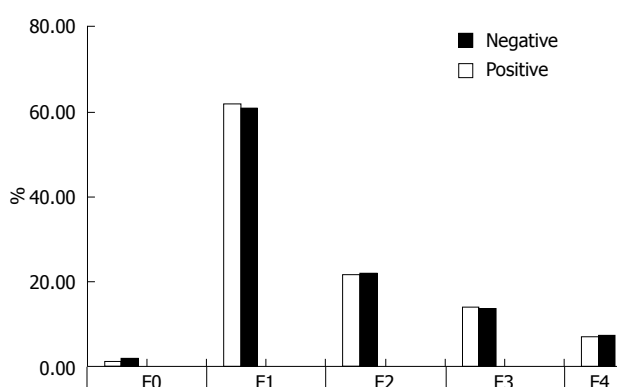
Of the 2751 patients that were followed-up to week 72, those with negative schistosomal serology had achieved a higher sustained virological response (SVR) than the other group (37.6% *vs* 27.7%, $P = 0.000$).

Multivariate logistic regression analyses indicated that patients with positive schistosomal serology were more likely to fail treatment (OR = 1.3, $P = 0.02$) at week 48 and (OR = 1.7, $P < 0.01$) at week 72 (Table 5).

Table 3 Baseline characteristics

	Schisto + ve (<i>n</i> = 983)	Schisto - ve (<i>n</i> = 2613)	<i>P</i> value
Age	42.59 \pm 9.21	41.62 \pm 9.81	0.008
Albumin (ref.: 3.5-5.5 mg/dL)	4.20 \pm 0.47	4.20 \pm 0.47	0.251
AST (ref.: 40 IU/L)	55.91 \pm 33.91	57.07 \pm 46.00	0.412
ALT (ref.: 40 IU/L)	63.19 \pm 41.68	63.38 \pm 43.30	0.908
HCV RNA, IU/mL	$1.4 \times 10^6 \pm 6.9 \times 10^6$	$9.5 \times 10^5 \pm 7 \times 10^6$	0.083
BMI	28.12 \pm 4.09	28.27 \pm 4.4	0.387
Male/female	5.3/1	3.88/1	0.002

Data are expressed as mean \pm SD. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; BMI: Body mass index.

**Figure 1** Fibrosis stages in relation to schistosomal serology.

DISCUSSION

This study was undertaken to determine a correlation between HCV and schistosomiasis infection in relation to hepatic fibrosis stages and antiviral treatment response.

Our findings showed a correlation of positive schistosomal serology in reference to sex, with the predominance involving males. HCV patients with positive schistosomal serology were also found to be older than those with negative serology. This finding is suspected to be due to the reservoir of HCV infection in Egypt, for which intravenously administered tartar emetic was used as a primary treatment^[11].

While this study showed no significant difference between the two groups in terms of fibrosis staging, other studies report HCV/schistosomiasis coinfecting patients have more rapid progression of hepatic fibrosis than those with HCV mono-infection^[12], as evidenced by increased fibrosis scores for the liver biopsies taken at 96.0 \pm 4.6 mo of follow-up. Moreover, another study demonstrated that serum transforming growth factor- β (TGF- β) and tumor necrosis factor- α (TNF- α) levels are higher in coinfecting groups^[13].

Ahmad *et al.*^[14] showed that schistosomiasis coinfection with HCV and/or non-alcoholic steatohepatitis had no significant impact on fibrosis stage. Results involving differences in fibrosis stages may be explained by several

Table 4 Virological response at weeks 12, 24, 48 in relation to schistosomal serology *n* (%)

	Anti-schisto antibody			<i>P</i> value
	Negative (<i>n</i> = 2613)	Positive (<i>n</i> = 983)	Total (<i>n</i> = 3596)	
Responders at week 12 (EVR)	2335 (89.4)	850 (86.5)	3185 (88.6)	0.015
Responders at week 24	1768 (67.7)	626 (63.7)	2394 (66.6)	0.024
Responders at week 48 (ETR)	1621 (62.0)	581 (59.1)	2202 (61.2)	0.108

EVR: Early virologic response; ETR: End of treatment response.

factors. Genetic predisposition may play a role, whereby only a minority of the individuals infected with *Schistosoma mansoni* may develop hepatic fibrosis or be more sensitive to infection(s). Moreover, frequency of exposure is directly correlated with the presence and amount of fibrosis^[15]. In addition, several clinical and pathological studies have shown that schistosomal hepatopathy is a reversible condition and that resolution of the schistosomiasis disease is accompanied by subsequent fibrosis resorption^[16,17].

Moreover, HCV patients with evidence of coinfection or previous exposure to schistosomiasis received oral antischistosomal treatment of PZQ prior to starting the anti-viral therapy. PZQ is believed to exert antifibrotic effects by affecting (decreasing) activation of hepatic stellate cells through inhibition of profibrotic gene expression^[18]. Morcos *et al*^[19] demonstrated that PZQ treatment could induce resolution of schistosoma-induced pathology, showing partial reversal of liver fibrosis in *Schistosoma mansoni* infected mice. Improvements and/or resolutions of schistosomal-induced periportal thickening/fibrosis in PZQ treated models have also been demonstrated by Berhe *et al*^[20] and Frenzel *et al*^[21]. It is theorized that the beneficial effects are likely related to the clearance of schistosomal worms and subsequent reduction of egg deposition.

Other limiting issues for the use of liver biopsy as a clinical tool for assessing fibrosis in schistosomiasis include ethical considerations and the risk of sampling errors, which may be especially evident for small-volume biopsies^[22].

In our current study, the EVR, virological response at week 24, and SVR were significantly higher in patients with negative schistosomal serology. This finding may be attributed to coinfecting patients with a down-regulated immune response to HCV leading to reduced IFN γ , interleukin (IL)-4 and IL-10 secreted by HCV-specific T cells. Early reports by Kamal *et al*^[23] using standard IFN in the treatment of chronic HCV patients reported that Egyptian patients with coinfections have higher HCV RNA titers, more advanced liver disease, more hepatic complications and greater mortality rates than those infected with HCV alone.

Table 5 Multivariate logistic regression analysis in which the failure of treatment is the dependent variable at weeks 48 and 72

Factors	OR	<i>P</i> value	95%CI	
			Lower	Upper
Week 48				
Age of > 50 yr	1.15	0.245	0.91	1.50
Female	0.65	0.000	0.51	0.80
Viremia, > 600 × 10 ³ IU/mL	1.78	0.000	1.41	2.30
IFNα2b	1.21	0.050	1.01	1.50
Activity, A2, A3	0.68	0.001	0.54	0.90
Fibrosis, > F2	1.69	0.000	1.35	2.10
BMI, > 30 kg/m ²	0.86	0.009	0.76	0.96
Positive schisto status	1.29	0.015	1.10	1.60
Week 72				
Age of > 50 yr	1.0	0.990	0.7	1.3
Female	0.7	0.006	0.5	0.9
Viremia, > 600 × 10 ³ IU/mL	1.6	0.001	1.2	2.2
Activity, A2, A3	0.5	< 0.01	0.4	0.7
Fibrosis, > F2	1.9	< 0.01	1.5	2.5
Positive schisto status	1.7	< 0.01	1.3	2.1

IFN: Interferon; BMI: Body mass index.

In light of the previous real time PCR findings from Bahgat *et al*^[24], soluble egg antigen (SEA) should be considered as a potential stimulatory factor for HCV RNA that may have influenced the early detection of HCV RNA as SEA can stimulate viral replication. The higher morbidity that is observed in patients coinfecting with schistosomiasis and HCV is related, at least in part, to direct stimulation of viral replication by SEA^[25].

It is interesting to consider that Derbala *et al.* found no significant difference in the treatment responses of patients treated with and without bilharziasis^[26]. This finding might be explained by phenotypic variations in Egyptian patients infected with HCV genotype 4, whereby some patients may mount HCV-specific T cell responses, both CD4+ and CD8+, despite the prevalence of concomitant schistosomiasis^[27].

A major limitation of this study was the need to diagnose schistosomiasis in patients by using an antischistosomal serology approach with a commercially available indirect hemagglutination test (IHAT). While the IHAT is sensitive in detecting bilharziasis, it cannot differentiate between past and current infection status nor between *Schistosoma mansoni* and *Schistosoma haematobium* species. While rectal snips are the preferred method of schistosomiasis diagnosis, this approach was not possible in our study population due to the large number of participants. Finally, genotyping for HCV was not performed on the patients in our study, since approximately 90% of infections in Egypt are due to genotype 4^[9] and the Egyptian National Committee for Control of Viral Hepatitis does not recommend routine genotyping.

In conclusion, positive schistosomal serology has no effect on fibrosis stage but it is significantly associated with failure of response to HCV treatment despite antischistosomal therapy.

COMMENTS

Background

Hepatitis C virus (HCV) is a major public health problem and is the primary cause of liver fibrosis and chronic liver disease worldwide. Both HCV and schistosomiasis are highly endemic in Egypt and cases of coinfection are frequently encountered. Intriguingly, HCV prevalence shows a direct correlation to the amount of intravenous tartar emetic used to control schistosomiasis in some geographic regions of Egypt. Moreover, patients with hepatosplenic schistosomiasis show a higher susceptibility to coinfection with hepatitis B virus and HCV than healthy individuals.

Research frontiers

HCV/Schistosomiasis coinfecting patients are characterized by higher HCV RNA titers, histological activity, incidence of cirrhosis and hepatocellular carcinoma, as well as higher mortality rates than monoinfected patients. This research aimed to assess the influence of schistosomiasis on hepatic fibrosis and to evaluate the response to pegylated-interferon/ribavirin (PEG-IFN/RIB) therapy in patients with chronic HCV infection.

Innovations and breakthroughs

Results showed that 27.3% of the patients had positive schistosomal serology, with a prevalence towards males (15.9% female and 84.1% male). The extent of fibrosis was not significantly different between patients with HCV/schistosomiasis coinfection and patients with chronic HCV mono-infection. Patients with HCV/schistosomiasis coinfection showed lower rates of early virologic response and virological response at week 24 of antiviral treatment, as well as lower rates of end-treatment response and sustained virological response. *Schistosomiasis* appears to be significantly associated with failure to respond to HCV treatment despite antischistosomal therapy.

Applications

Schistosomiasis appears to be significantly associated with failure to respond to HCV treatment. Authors propose that mandatory schistosomal serology should be considered for chronic HCV patients prior to initiating PEG-IFN/RIB therapy. In those patients with positive schistosomal serology, administration of antischistosomal therapy (praziquantel at 40 mg/kg single dose) four weeks prior to antiviral therapy (PEG-IFN/RIB) may decrease the effect of schistosomiasis, reduce subsequent complications and improve response to treatment.

Terminology

Early virological response is defined as a ≥ 2 log reduction or complete absence of serum HCV RNA at week 12 of therapy, as compared to the baseline level. End-of-treatment response is defined as an undetectable level of virus (by polymerase chain reaction) in serum at the end of a 48-wk course of therapy (for patients infected with HCV genotype 4). Sustained virological response is defined as an undetectable level of HCV RNA in serum at 24 wk after the discontinuation of therapy. Virological breakthrough refers to the reappearance of HCV RNA during the ongoing course of therapy.

Peer review

This study analyzes the impact of schistosomiasis on hepatic fibrosis and response to PEG-IFN/RIB therapy in patients with chronic HCV. The results suggest that positive schistosomal serology has no effect on fibrosis stage, but is significantly associated with failure to respond to HCV treatment.

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Increased expression of DLX2 correlates with advanced stage of gastric adenocarcinoma

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Abstract

AIM: To investigate the expression of distal-less homeobox 2 (DLX2) in gastric adenocarcinoma and its clinicopathological significance.

METHODS: Gastric adenocarcinoma tissues were obtained from gastrectomy specimens of 129 patients from the Department of Surgery and Pathology, the Second Affiliated Hospital of Kunming Medical University. Sixty cases of normal gastric tissues were collected from gastrectomy specimens of adjacent gastric cancer margins greater than 5 cm. Patient diagnosis was established pathologically, and no patient had received chemotherapy or radiotherapy before surgery. All tissue specimens were formalin-fixed and paraffin-embedded. Immunohistochemistry was carried out to investigate the expression of DLX2 in 129 gastric adenocarcinoma tissues and 60 adjacent normal tissues. The immunos-

taining reaction was semiquantitatively evaluated based on the proportion of positive cells and the median staining intensity in normal gastric epithelial cells or tumor cells. All patients had follow-up records for more than 5 years. Correlations of DLX2 expression with clinicopathological features and prognosis of patients with gastric adenocarcinoma were analyzed. All statistical analyses were performed using the SPSS 17.0 software.

RESULTS: The positive expression of DLX2 was detected in 68 (52.7%) cases of 129 gastric adenocarcinoma tissues and 14 (23.3%) cases of 60 adjacent normal tissues. The difference in DLX2 expression between gastric adenocarcinoma tissues and adjacent normal tissues was statistically significant ($\chi^2 = 14.391$, $P < 0.001$). Moreover, high expression of DLX2 was detected in 48 (37.2%) cases of 129 human gastric cancer tissues, but not in adjacent normal tissues. The expression of DLX2 correlated with the size of tumor ($P = 0.001$), depth of invasion ($P = 0.008$), lymph node metastasis ($P = 0.023$) and tumor-node-metastasis stages ($P = 0.020$), but was not correlated with age, gender, histological differentiation and distant metastasis. The Kaplan-Meier survival analysis revealed that survival time of patients with high DLX2 expression was significantly shorter than that with low DLX2 expression. However, the multivariate analysis showed that invasion depth ($P < 0.001$), lymph nodes metastasis ($P = 0.001$) and distant metastasis ($P < 0.001$) were independent prognostic factors for patients with gastric adenocarcinoma, but DLX2 expression, tumor location and tumor size were not.

CONCLUSION: These results suggest that increased expression of DLX2 may correlate with the advanced stage of gastric adenocarcinoma, and it may contribute to tumor development.

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Key words: Gastric adenocarcinoma; Distal-less homeobox 2; Immunohistochemistry; Invasion; Metastasis; Prognosis

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INTRODUCTION

Gastric cancer is one of the leading causes of cancer-related death worldwide due to its frequency, poor prognosis and limited treatment options^[1]. Although the incidence of gastric cancer has been declining for several decades in most Western countries, it remains a crucial public health problem in developing countries^[2,3]. In China, gastric cancer is the second most common malignancy and was the third leading cause of death from cancer in 2007, representing a major disease burden on health services^[4]. Several studies have shown that various genetic and epigenetic alterations are involved in the course of carcinogenesis and progression of gastric cancer^[5-8]. However, the molecular mechanism involved in the development of gastric cancer remains unclear.

The distal-less homeobox (*DLX*) gene family, a homolog of *Drosophila* distal-less, comprises six *DLX* genes in humans, of which three exist as bigene clusters: *DLX-1/DLX-2*, *DLX-3/DLX-4*, *DLX-5/DLX-6*^[9]. The *DLX* gene family has crucial roles in regulating embryonic development, tissue homeostasis, lymphocyte development, cell cycle and apoptosis^[10-14]. However, the role of the *DLX* gene family in tumor development has only recently been explored. As a member of *DLX* gene family, the abnormal expression of *DLX2* has been reported in many human hematological malignancies and solid tumors, including acute lymphoblastic leukemia, acute myeloid leukemia, melanoma, glioma, breast, lung, prostate, ovarian and colon cancer^[9,14-17]. A recent study showed that the expression of *DLX2* plays a critical role in shifting transforming growth factor β (TGF- β) from its tumor suppressive to its tumor-promoting functions^[13]. Moreover, abnormal TGF- β expression is involved in tumor progression, metastasis, angiogenesis and poor survival of gastric cancer^[18,19]. These studies led us to investigate the possible role of *DLX2* in the development of gastric adenocarcinoma.

In the present study, we assessed the expression of *DLX2* in gastric adenocarcinoma tissues and adjacent normal tissues by immunohistochemistry. Correlations of *DLX2* expression with clinicopathological features and survival of gastric adenocarcinoma patients were then analyzed.

MATERIALS AND METHODS

Patients and tissue samples

Gastric adenocarcinoma tissues were obtained from gastrectomy specimens of 129 patients from the Depart-

ment of Surgery and Pathology, the Second Affiliated Hospital of Kunming Medical University. Sixty samples of normal gastric tissues were collected from gastrectomy specimens of adjacent gastric cancer margins greater than 5 cm and served as controls. All operations were performed between January 2001 and June 2007. Patient diagnosis was established pathologically, and no patient had received chemotherapy or radiotherapy prior to surgery. All tissue specimens were formalin-fixed and paraffin-embedded. There were 87 males and 42 females (mean age, 57.6 years; range, 26-84 years). The age and gender of patients, tumor size, tumor location, histological differentiation, depth of invasion, status of lymph node metastasis and distant metastasis were obtained from histopathology records. The stage was determined according to the 7th edition of the AJCC Tumor Staging Manual and Japanese Classification 2011 in gastric cancer^[20,21]. Forty-three cases were categorized as stage I, 43 were stage II, 34 were stage III and nine were stage IV. All patients had follow-up records for more than 5 years. The follow-up deadline was July 2012. The survival time was determined from the date of surgery to the follow-up deadline or date of death, which was mostly caused by recurrence or metastasis. The hospital's ethics committee approved this study.

Immunohistochemistry

Immunohistochemical analysis was used to investigate *DLX2* expression in 129 cases of gastric adenocarcinoma tissues and 60 cases of adjacent normal tissues. According to protocol^[22,23] for immunohistochemistry on paraffin-embedded tissue sections, paraffin-embedded blocks were sectioned at about 4 μ m thickness. Slides were baked at 60 °C for 2 h, deparaffinized with xylene and rehydrated using an alcohol gradient (100% alcohol, 95% alcohol, 80% alcohol, and 70% alcohol). After microwave pretreatment in citrate buffer (pH 6.0) for antigen retrieval, sections were treated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Sections were incubated with 1% bovine serum albumin to block nonspecific binding, and then incubated overnight at 4 °C with the polyclonal antibody against *DLX2* (Epitomics, Inc., California, United States) at a dilution of 1:100. Phosphate buffer solution (PBS) was used as a negative control. After rinsing 3 \times 3 min with PBS, tissue sections were treated with peroxidase-linked secondary antibody (Maixin-Bio, Inc., Fuzhou, China) for 30 min at room temperature. Staining was carried out with diaminobenzidine chromogen (Maixin-Bio, Inc., Fuzhou, China) and counterstained with hematoxylin. All slides were then dehydrated using an alcohol gradient, and mounted with a coverslip.

Scoring of immunohistochemical staining

The results of immunostaining were reviewed and scored independently by two observers in a blinded fashion without knowledge of clinical and pathological information. To avoid artifactual effects, the cells on the margins

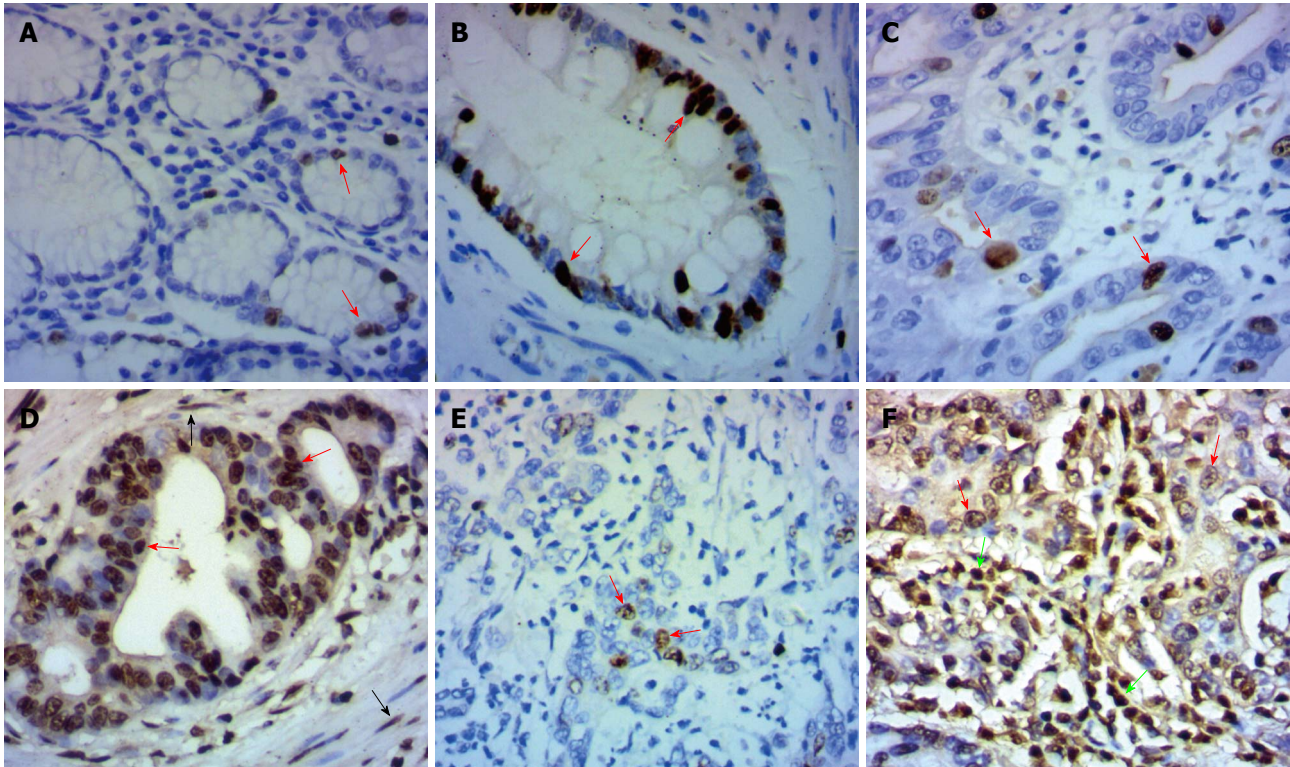


Figure 1 Immunohistochemical staining for distal-less homeobox 2 in gastric adenocarcinoma tissues and adjacent normal gastric tissues. A: Low expression of distal-less (DLX2) in normal gastric mucosa; B: High expression of DLX2 in intestinal metaplasia cells; C: Low expression of DLX2 in gastric adenocarcinoma tissue with well differentiation; D: High expression of DLX2 in gastric adenocarcinoma tissue with well differentiation; E: Low expression of DLX2 in gastric adenocarcinoma tissue with poor differentiation; F: High expression of DLX2 in gastric adenocarcinoma tissue with poor differentiation. DLX2 staining was detected mainly in nucleus of normal gastric epithelial cells (Figure 1A, red arrows) or tumor cells (Figure 1C-F, red arrows). Besides, increased expression of DLX2 was detected in intestinal metaplasia cells (Figure 1B, red arrows), fibroblasts (Figure 1D, black arrows) and inflammatory cells (Figure 1F, green arrows) around tumor cells. Original magnification, $\times 200$.

of sections and areas with poorly presented morphology were not counted. Five fields ($\times 400$ magnification) per tissue section, chosen at random, were counted. The immunostaining reaction was semiquantitatively evaluated based on the proportion of positive cells and the median staining intensity in normal gastric epithelial cells or tumor cells. The proportion of positive cells was scored as follows: 0, $\leq 5\%$; 1, 6%-25%; 2, 26%-50% and 3, $\geq 51\%$. The sections were considered to be positively stained when there were more than 5% of observed cells with immunostaining. Staining intensity was graded according to the following criteria: 0, no staining; 1, weak staining, light yellow; 2, moderate staining, yellow brown; and 3, strong staining, brown. The immunoreactive score was calculated based on the proportion score multiplied by the staining intensity score. All immunoreactive scores were less than 4 in adjacent normal tissues; therefore, the results of immunostaining in tumor tissues were divided into two groups, low expression (immunoreactive score ≤ 3) and high expression (immunoreactive score ≥ 4).

Statistical analysis

All statistical analyses were performed using the SPSS 17.0 software. Correlation of DLX2 expression with clinicopathological parameters was calculated by Pearson χ^2 test, χ^2 test with continuity correction and Spearman's rank

correlation test, respectively. Univariate survival analysis was assessed by the Kaplan-Meier method and the difference in survival curves was analyzed by the log-rank test. The Cox proportional hazards regression model was used to analyze independent prognostic factors. All reported *P* values were two-sided and *P* < 0.05 was considered statistically significant.

RESULTS

Expression of DLX2 in gastric cancer and adjacent normal tissues

In the present study, immunohistochemical analysis was carried out to investigate the DLX2 expression in 129 gastric adenocarcinoma tissues and 60 adjacent normal tissues. Positive expression of DLX2 was detected in 68 (52.7%) cases of 129 gastric cancer tissues and in 14 (23.3%) cases of 60 adjacent normal tissues. The difference of DLX2 expression between gastric cancer tissues and adjacent normal tissues was statistically significant ($\chi^2 = 14.391$, *P* < 0.001). Moreover, high expression of DLX2 was detected in 48 (37.2%) cases of 129 human gastric cancer tissues, but not in adjacent normal tissues. DLX2 staining was detected mainly in the nuclei of normal gastric epithelial cells (Figure 1A) or tumor cells (Figure 1C-F). In addition, increased expression of

Table 1 Relationship of distal-less homeobox 2 expression with clinicopathological features of gastric adenocarcinoma *n* (%)

Clinicopathological features	Cases	DLX2 expression		χ^2 test	<i>P</i> value
		Low	High		
Age (yr)				0.954	0.329
< 60	69	46 (66.7)	23 (33.3)		
≥ 60	60	35 (58.3)	25 (41.7)		
Gender				1.989	0.158
Female	42	30 (71.4)	12 (28.6)		
Male	87	51 (58.6)	36 (41.4)		
Tumor size (cm)				11.518	0.001
< 5	68	52 (76.5)	16 (23.5)		
≥ 5	61	29 (47.5)	32 (52.5)		
Tumor location				2.335	0.506
Upper	14	10 (71.4)	4 (28.6)		
Middle	20	14 (70.0)	6 (30.0)		
Lower	84	52 (61.9)	32 (38.1)		
Diffuse	11	5 (45.5)	6 (54.5)		
Histologic differentiation				3.186	0.364
Well	16	11 (68.8)	5 (31.3)		
Moderately	28	21 (75.0)	7 (25.0)		
Poorly	74	42 (56.8)	32 (43.2)		
Other	11	7 (63.6)	4 (36.4)		
Depth of invasion				11.940	0.008
T1	28	24 (85.7)	4 (14.3)		
T2	24	16 (66.7)	8 (33.3)		
T3	58	28 (48.3)	30 (51.7)		
T4	19	13 (68.4)	6 (31.6)		
Lymph node metastasis				9.577	0.023
N0	69	50 (72.5)	19 (27.5)		
N1	17	12 (70.6)	5 (29.4)		
N2	25	11 (44.0)	14 (56.0)		
N3	18	8 (44.4)	10 (55.6)		
Distant metastasis				0.012	0.914
M0	120	76 (63.3)	44 (36.7)		
M1	9	5 (55.6)	4 (44.4)		
TNM stages				9.849	0.020
I	43	35 (81.4)	8 (18.6)		
II	43	24 (55.8)	19 (44.2)		
III	34	17 (50.0)	17 (50.0)		
IV	9	5 (55.6)	4 (44.4)		

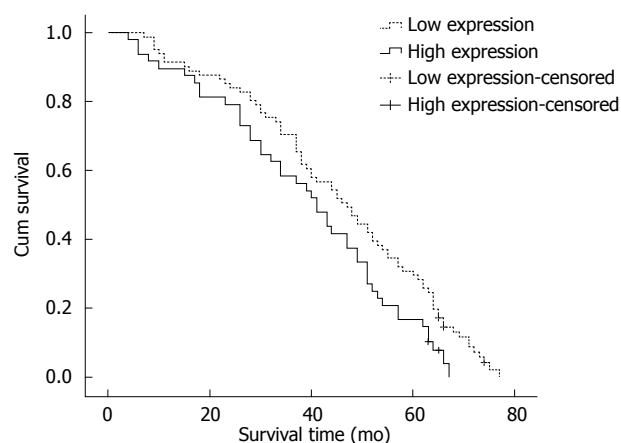
DLX2: Distal-less homeobox 2.

DLX2 was detected in intestinal metaplasia cells (Figure 1B), fibroblasts (Figure 1D) and inflammatory cells (Figure 1F) around tumor cells.

Expression of DLX2 and clinicopathological features

The expression of DLX2 in gastric adenocarcinoma was significantly correlated with tumor size ($P = 0.001$), depth of invasion ($P = 0.008$), lymph node metastasis ($P = 0.023$) and TNM stages ($P = 0.020$), but was not correlated with age, gender, histological differentiation and distant metastasis ($P > 0.05$, Table 1). Spearman's rank correlation test showed that DLX2 expression was positively related to tumor size ($P < 0.001$), depth of invasion ($P = 0.010$), lymph node metastasis ($P = 0.013$) and TNM stages ($P = 0.004$).

To investigate whether the ranks of percentage or staining intensity of DLX2 expression was more prominent for the immunoreactive assessment, the statistical analysis of the correlation between clinicopathological parameters and the proportion score or staining intensity

**Figure 2** Kaplan-Meier curves with univariate analysis (log-rank) for patients with low distal-less homeobox 2 expression vs high distal-less homeobox 2 expression.

score of DLX2 expression was calculated separately. As shown in Table 2, the proportion score of DLX2 expression was significantly correlated with tumor size ($P = 0.002$), depth of invasion ($P = 0.016$) and lymph node metastasis ($P = 0.002$). The staining intensity score was significantly correlated with tumor size ($P = 0.029$) and lymph node metastasis ($P = 0.044$). These results suggest that the ranks of percentage of DLX2 expression in gastric cancer tissues may be more prominent than staining intensity for the immunoreactive assessment.

Correlation between DLX2 expression and patient prognosis

The Kaplan-Meier survival analysis revealed that survival time of patients with high DLX2 expression was significantly shorter than for those with low DLX2 expression ($\chi^2 = 4.986$, $P = 0.026$; Figure 2). The mean survival time of the former was only 39.216 mo (95%CI: 34.030-44.402), whereas the mean survival time of latter was 45.669 mo (95%CI: 41.426-49.912). For patients with high DLX2 expression, the cumulative 3- and 5-year survival rates were 58.3% and 16.7%, respectively, which was significantly lower than those for patients with low DLX2 expression (70.4% and 29.6%, respectively).

Additionally, the clinicopathological features for possible prognostic effects in gastric cancer were analyzed by Cox regression analysis. The following six clinicopathological features were selected for evaluation: tumor size, tumor location, depth of invasion, lymph nodes metastasis, distant metastasis and DLX2 expression (all $P < 0.05$ in univariate survival analysis). The multivariate analysis showed that invasion depth ($P < 0.001$), lymph nodes metastasis ($P = 0.001$) and distant metastasis ($P < 0.001$) were independent prognostic factors for patients with gastric adenocarcinoma, but DLX2 expression, tumor location and tumor size were not independent prognostic factors (Table 3).

DISCUSSION

In the present study, DLX2 expression levels were in-

Table 2 Relationship of proportion score or staining intensity of distal-less homeobox 2 expression with clinicopathological features of gastric adenocarcinoma

Clinicopathological features	Cases	Proportion score			χ^2 test	P value	Staining intensity			χ^2 test	P value
		0	1	2, 3			0	1, 2	3		
Age (yr)					5.637	0.060				3.924	0.141
< 60	69	39	7	23			29	26	14		
≥ 60	60	22	12	26			16	25	19		
Gender					2.583	0.275				1.785	0.410
Female	42	22	8	12			18	15	9		
Male	87	39	11	37			27	36	24		
Tumor size (cm)					12.975	0.002				7.115	0.029
< 5	68	42	9	17			30	26	12		
≥ 5	61	19	10	32			15	25	21		
Histological differentiation					3.302	0.192				0.286	0.867
Well and moderately	44	24	8	12			16	16	12		
Poorly and other	85	37	11	37			29	35	21		
Invasion depth					8.291	0.016				3.724	0.155
T1, T2	52	31	9	12			22	21	9		
T3, T4	77	30	10	37			23	30	24		
Lymph node metastasis					17.511	0.002				9.815	0.044
N0	69	39	11	19			30	20	19		
N1	17	6	6	5			4	11	2		
N2, N3	43	16	2	25			11	20	12		
TNM stages					5.982	0.050				0.753	0.686
I, II	86	43	16	27			32	32	22		
III, IV	43	18	3	22			13	19	11		

Table 3 Multivariate analysis for disease-related deaths (Cox regression model)

Variables	B	P value	Exp (B)	95%CI for Exp (B)
Tumor location	0.145	0.261	1.156	0.898-1.488
Tumor size (< 5 cm vs ≥ 5 cm)	0.176	0.421	1.193	0.777-1.832
Depth of invasion	0.726	< 0.001	2.067	1.570-2.722
Lymph node metastasis	0.303	0.001	1.354	1.126-1.629
Distant metastasis (no vs yes)	2.415	< 0.001	11.185	4.187-29.878
Distal-less homeobox 2 expression (low vs high)	-0.214	0.308	0.808	0.535-1.218

investigated in 129 gastric adenocarcinoma tissues and 60 adjacent normal tissues by immunohistochemistry. We showed that DLX2 expression was more frequent in gastric cancer tissues than in adjacent normal tissues. The expression of DLX2 in gastric cancer tissues was significantly associated with the size of the tumor, the depth of invasion, lymph node metastasis and TNM stages. Based on these results, we suggest that increased expression of DLX2 may correlate with the advanced stage of gastric adenocarcinoma.

In several investigations, it has been shown that the abnormal expression of DLX2 in cancer cells is associated with tumor progression. However, the mechanism of DLX2's involvement in tumor progression is not clear. Yilmaz *et al*^[14] showed that expression of DLX2 correlated significantly with advanced tumor progression and with the metastatic potential of melanoma, glioma, lung, and prostate cancers. In their research, they found that DLX2 counteracted TGF- β -induced cell-cycle arrest and apoptosis in mammary epithelial cells, and DLX2 ex-

pression supported experimental tumor growth and metastasis of B16 melanoma cells. These results established that DLX2 has an important role in shifting TGF- β from its tumor suppressive to its tumor-promoting functions. Additionally, Lee *et al*^[16] found that DLX2 expression was higher in breast and ovarian cancer tissues compared with the adjacent normal tissues. Furthermore, DLX2 expression was related to poor differentiation grade of ovarian cancer. DLX2 short hairpin RNA inhibited the metabolic stress-induced increase in propidium iodide-positive cell population and high mobility group box 1 and lactate dehydrogenase release. They concluded that DLX2 might be involved in tumor progression *via* the regulation of metabolic stress-induced necrosis.

In our research, high expression of DLX2 was detected in intestinal metaplasia, which is a risk factor for development of gastric cancer^[24,25], indicating that increased expression of DLX2 might contribute to an early event of gastric cancer development. In addition, we found that increased expression of DLX2 was detected in inflammatory cells around tumor cells. Recent studies have expanded the concept that inflammation is a critical component of tumor progression^[26]. Moreover, the mediators and cellular effectors of inflammation are important constituents of the local environment of tumors^[27]. These results further support the hypothesis that DLX2 is involved in the development of gastric adenocarcinoma.

In 2010, Morini *et al*^[9] found that expression of DLX2 was detected in 21.6% of the patients with breast cancer, and was significantly correlated with prolonged disease-free survival and reduced incidence of relapse. DLX5 expression was detected in 2.2% of all cases, displaying reduced disease-free survival and high incidence

of relapse. In all cases, they found mutually exclusive expression of DLX2 and DLX5. Their study suggested that *DLX* genes were involved in human breast cancer progression, and that *DLX2* and *DLX5* genes might serve as prognostic markers. In our research, the Kaplan-Meier survival analysis revealed that the survival times of gastric adenocarcinoma patients with high DLX2 expression were significantly shorter than those with low DLX2 expression. However, the multivariate analysis showed that DLX2 expression was not an independent prognostic factor in gastric adenocarcinoma. The multivariate analysis might mask DLX2's contribution to survival rate. Therefore, DLX2 expression might not be related with poor prognosis in patients with gastric adenocarcinoma.

In conclusion, our study demonstrates that increased expression of DLX2 may correlate with the advanced stage of gastric adenocarcinoma, and it may contribute to tumor development. These findings further support the hypothesis that, as a key regulator of embryogenesis, DLX2 may also play a critical role in tumor development. Consequently, further investigation is necessary to clarify the role of DLX2 in the development of gastric adenocarcinoma.

COMMENTS

Background

Gastric cancer is one of the leading causes of cancer-related death worldwide because of its frequency, poor prognosis and limited treatment options. Although the incidence of gastric cancer has been declining for several decades in most Western countries, it remains a crucial public health problem in developing countries. Several studies have demonstrated that various genetic and epigenetic alterations are involved in the course of carcinogenesis and progression of gastric cancer.

Research frontiers

The distal-less homeobox (*DLX*) gene family exerts an important role in regulating embryonic development, tissue homeostasis, lymphocyte development, cell cycle and apoptosis. However, the role of the *DLX* gene family in tumor development has only recently been explored. As a member of *DLX* gene family, the abnormal expression of distal-less homeobox 2 (*DLX2*) has also been reported in many human solid tumors and hematological malignancies.

Innovations and breakthroughs

This is the first study attempt to explore the expression of DLX2 in gastric adenocarcinoma and its clinicopathological significance. This study suggests that increased expression of DLX2 may correlate with the advanced stage of gastric adenocarcinoma, and it may contribute to tumor development.

Applications

By understanding the expression of DLX2 and its correlation with clinicopathological features of gastric adenocarcinoma, this study will form the basis for further research to explore the mechanism of tumor development, and may represent a potential therapeutic target of gastric adenocarcinoma.

Terminology

Homeobox genes encode transcription factors that play essential roles in controlling cell growth and differentiation during embryonic development. These genes are characterized by a highly conserved 61-amino acid homeodomain that binds DNA elements containing a TAAT core motif. Many homeobox genes are aberrantly expressed in a wide variety of solid tumors and hematological malignancies.

Peer review

The authors investigated the expression of DLX2 in gastric adenocarcinoma tissues and adjacent normal tissues by immunohistochemistry. Correlations of DLX2 expression with clinicopathological features and prognosis of patients with gastric adenocarcinoma were then analyzed. This study has shown that

increased expression of DLX2 may correlate with the advanced stage of gastric adenocarcinoma, and it may contribute to tumor development. These results are interesting and may represent a novel molecular mechanism of gastric adenocarcinoma development.

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Effects of medical adhesives in prevention of complications after endoscopic submucosal dissection

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Abstract

AIM: To evaluate the use of medical adhesive spray in endoscopic submucosal dissection (ESD).

METHODS: Patients who underwent ESD between January 2009 and June 2012 ($n = 173$) were enrolled in the prospective randomized study. Two patients undergoing surgery due to severe intraoperative hemorrhage and failed hemostasis were excluded, and the remaining 171 patients were randomly divided into two groups: group A (medical adhesive group, $n = 89$) and group B (control group, $n = 82$). In group A, a medical adhesive spray was evenly applied after routine electrocoagulation and hemostasis using hemostatic clip after ESD. Patients in group B only treated with routine wound management. Intraoperative and postoperative data were collected and compared.

RESULTS: In all 171 patients, ESD was successfully

completed. There was no significant difference in the average treatment time between groups A and B (59.4 min vs 55.0 min, respectively). The average length of hospital stay was significantly different between group A and B (8.89 d vs 9.90 d, respectively). The incidence of intraoperative perforation was 10.1% in group A and 9.8% in group B, and was not significantly different between the two groups. In all cases, perforations were successfully managed endoscopically and with conservative treatment. The incidence of postoperative delayed bleeding in group A was significantly lower than that in group B (0.00% vs 4.88%, respectively).

CONCLUSION: ESD is an effective minimally invasive treatment for gastrointestinal precancerous lesions or early-stage gastrointestinal cancer. Medical adhesive spray is effective in preventing delayed bleeding after ESD, and can thus reduce the average length of hospital stay.

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Key words: Endoscopic submucosal dissection; Medical adhesive; Early-stage gastrointestinal cancer; Postoperative delayed bleeding; Intraoperative hemorrhage

Core tip: This is the first report to use medical adhesive after endoscopic submucosal dissection (ESD), and results were exciting. Application of medical adhesive spray can prevent complications of ESD, especially the delayed bleeding, consequently reducing the average length of hospital stay, and avoiding additional health care expenditures.

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INTRODUCTION

In endoscopic submucosal dissection (ESD), special instrument and other ancillary equipment are used to resect and strip gastrointestinal precancerous lesions and early-stage cancer on the basis of endoscopic mucosal resection (EMR). ESD has been widely performed in clinical practice due to its advantages of complete resection and reduced recurrence rates. However, compared to EMR, ESD is associated with a high incidence of complications such as perforation and bleeding, which has limited the utility of ESD. Currently, ESD is only performed at some institutions in China. Medical adhesives (spray type) have been widely used in surgery and for the treatment of gastric varices because of their adhesive, reinforcement, and leak proofing functions^[1,2]. However, there has been no report of their use in gastrointestinal (GI) endoscopy. This study investigated the use of medical spray adhesives for the prevention of complications in patients undergoing ESD.

MATERIALS AND METHODS

Patients

Patients who underwent ESD at the department of digestive endoscopy of our hospital between January 2009 and June 2012 ($n = 173$) were enrolled. Two patients undergoing surgery due to severe intraoperative hemorrhage and inability to achieve adequate hemostasis were excluded. The remaining 171 patients completed the ESD treatment and were included in the analysis. There were 75 males and 96 females with an average age of 57.21 ± 12.22 years (range, 18-82 years). There were 37 cases of esophageal lesions, 110 cases of gastric lesions, and 24 cases of colorectal lesions diagnosed by routine preoperative endoscopy, endoscopic ultrasonography, and histopathological examination of biopsy specimens. There were 50 cases of mucosal or submucosal lesions (early-stage cancer or precancerous lesions) and 121 cases of muscularis propria lesions (stromal tumors). The pathological examination revealed 92 cases of leiomyoma, 61 cases of stromal tumor and 18 cases of early-stage cancer. The average size of lesions was 4.42 ± 1.28 cm.

Instruments and adhesives

An Olympus GIF-Q260J electronic gastroscope and CF-Q260 colonoscopy system were used. In addition, an NM-4L-1 injection needle, triangle-tip knife, FD-1U-1 hot biopsy forceps, snare, Poko hemostatic clip, HX-610-135 hemostatic clip, and ERBE ICC-200 high-frequency electric cutting device were also employed. The medical adhesive (Compont Medical Adhesive) is a spray-type adhesive and the primary ingredient is butyl α -cyanoacrylate. During ESD, a transparent cap was added at the end of the lens and carbon dioxide insufflation was adopted.

ESD

After conventional therapeutic steps, patients were ran-

domly divided into group A (medical adhesive group, $n = 89$) and group B (control group, $n = 82$) according to a computer-generated random number table. There was no significant difference in age, gender, or lesion type between the two groups (Table 1). Patients receiving anticoagulant drugs such as aspirin underwent ESD 5-7 d after drug withdrawal.

All patients underwent the following operations under intubation anesthesia or intravenous anesthesia: (1) Staining: During endoscopy, the lesions were identified and stained with methylene blue. After staining, the lesion boundary was obvious; (2) Marking: A needle knife or argon plasma coagulation (APC) was used to mark at the lesion edges; (3) Injection: An epinephrine/saline solution (1:10000) containing a small amount of methylene blue was injected submucosally at multiple places lateral to the marked points at the lesion edges; (4) Pre-cut: A needle knife was used to cut open the mucosa at the marked points at the lesion edges; (5) Cut: The TT knife was used to make a circular incision on the lesion edge along the marker; (6) Stripping: The TT knife was used to cut open the submucosa layer by layer, and the lesion was peeled off. For the muscularis propria lesions, the lesions were completely stripped from the nearby tissue after the lesion was exposed. In some cases, when the lesion was almost completely peeled off, the snare was used to trap the lesion root so that the lesion could be resected completely; and (7) Wound treatment: After resection of the lesion, small visible blood vessels were treated with argon plasma coagulation (APC) or hot biopsy forceps. The perforated wound surface was closed with metal hemostatic clips. In addition, medical adhesive was sprayed onto the wound surface *via* a spray catheter in group A patients.

Patients with severe intraoperative bleeding or perforation who were unable to undergo endoscopic treatment received surgical treatment. All the patients receiving ESD were maintained *non per os* (NPO) postoperatively and received a nasogastric tube and low flow suction. Antacids and necessary hemostatic drugs (Ethamsylate, PAMBA) were administered, together with the prophylactic antibiotics. Abdominal signs such as abdominal pain and distension were monitored closely. Endoscopy was repeated 1, 3, and 6 mo after surgery to examine wound healing, residual lesions, and recurrence.

The postoperative abdominal signs, recurrence, length of hospital stay, and incidence of complications including delayed bleeding, perforation and infection were compared between the two groups.

Statistical analysis

Categorical data were analyzed using the χ^2 test, and quantitative data were analyzed using the *t* test. SPSS 10.0 statistical software was used for analysis and the significance level α was set at 0.05.

RESULTS

A total of 173 patients underwent ESD treatment. There

Table 1 Patient clinical data

	All patients (<i>n</i> = 171)	Group A (medical adhesive group) (<i>n</i> = 89)	Group B (control group) (<i>n</i> = 82)	<i>P</i> value
Age ¹ (yr)	57.21 ± 12.22	56.47 ± 13.02	58.01 ± 11.31	NS
Gender (male/female)	75/96	39/50	36/46	NS
Lesion location				
Esophageal	37	21	16	NS
Gastric	110	58	52	NS
Colorectal	24	10	14	NS
Depth of lesion				
Mucosa or submucosa	50	23	27	NS
Muscularis propria	121	66	55	NS

¹Data are expressed as mean ± SD. NS: Not significant.

were two patients with muscularis propria lesions with a diameter of 4 or 5 cm, respectively, protruding toward the abdominal cavity. During ESD, the bleeding was difficult to control and these two patients received surgical treatment. The lesions were completely stripped off in the remaining 171 patients. Patient clinical data are shown in Table 1.

The average duration of ESD (from submucosal injection to complete stripping of lesions) was 59.44 min (range, 36–150 min) in group A and 55.00 min (range, 35–140 min) in group B and the difference was not statistically significant. The average length of hospital stay was 8.89 d (range, 6–15 d) in group A and 9.90 d (range, 5–21 d) in group B and the difference was statistically significant. In group B, four patients experienced delayed bleeding and were hospitalized for 19, 19, 20, and 21 d, respectively. Excluding those patients, the average length of hospital stay in group B was 9.40 d (ranged, 5–15 d), which was not significantly different compared with group A (Table 2).

All the 171 patients treated with ESD, with the exception of two patients who were lost to follow-up after treatment, underwent repeat endoscopy 1, 3, 6, and 12 mo after treatment. To date, no recurrence has been noted.

During ESD, bleeding (< 40 mL) occurred in all patients, and hemostasis was successful after electrocoagulation, APC, and application of the hemostatic clip. No delayed bleeding occurred in group A (0.00%, 0/89), whereas delayed bleeding occurred in four patients in group B (4.88%, 4/82), which was significantly different from that in group A. In group B, one patient with an esophageal lesion, two patients with gastric lesions, and one patient with colon lesions experienced bleeding 3, 5, 10 and 7 d after surgery. One patient had symptoms of shock including progressive drop in blood pressure and cold sweats, and the vital signs became stable after transfusion and active medical treatment. Gastric endoscopy and colonoscopy revealed active bleeding at the location of the ESD. After norepinephrine saline flush, APC, ap-

Table 2 Comparison of treatment duration and length of hospital stay

	Group A (medical adhesive group)	Group B (control group)	<i>P</i> value
Average duration of ESD treatment (min)	59.44 ± 18.46	55.00 ± 21.00	0.143
Average length of hospital stay (d)	8.89 ± 2.33	9.90 ± 3.30	0.021
Average length of hospital stay when patients with delayed bleeding were excluded (d)	8.89 ± 2.33	9.40 ± 2.47	0.172

Data are expressed as mean ± SD. ESD: Endoscopic submucosal dissection.

plication of hemostatic clips, and the endoscopic application of medical spray adhesive, hemostasis was successful. No patients underwent surgical intervention for the control of bleeding.

Perforation occurred in nine of 89 (10.1%) patients in group A. Of these patients, one had an esophageal lesion, seven had gastric lesions, and one patient had colon lesions. Perforation occurred in 8 (9.8%) of 82 patients in group B. Of these patients, two had esophageal lesions and six had gastric lesions. The incidence of perforation was not significantly different between the two groups. Among the nine patients who experienced perforation in group A, seven patients had muscularis propria lesions, one patient had ulcer scars, and the remaining patient had non-ulcer scar lesions. All of the eight patients who experienced perforation in group B had muscularis propria lesions, and none had ulcer scar formation or non-ulcer scar lesions. The diameter of perforation ranged from 0.2 to 2.0 cm, and the wound surface was clipped using hemostatic clips in all the cases. Abdominal paracentesis was performed to release gas in patients with obvious abdominal distension. All perforations resolved after fasting, placement of an indwelling nasogastric tube and gastrointestinal decompression in patients who received gastric procedures, absolute bed rest, and treatment with antibiotics. No patient underwent surgical treatment.

After ESD, 12 patients in group A and 10 patients in group B experienced varying degrees of abdominal distension and abdominal pain, and the incidence was 13.5% and 12.2%, respectively (*P* > 0.05). Eight of the 12 patients with abdominal distension and abdominal pain in group A experienced intraoperative perforation, and the remaining four patients had no intraoperative perforation. Six of the 10 patients with abdominal distension and abdominal pain in group B experienced intraoperative perforation, and the remaining four patients did not. The symptoms resolved in all patients within 1–3 d after conservative treatment including gastrointestinal decompression, fasting, antacids, and antibiotics. Anal pain during defecation occurred in two patients (2.25%) in group A 8–10 d after treatment, which was followed by the discharge of solid medical adhesive. A summary of complications is presented in Table 3.

Table 3 Complications

	Group A (medical adhesive group)	Group B (control group)	P value
Incidence of delayed bleeding	0.00%	4.88%	0.035
Incidence of perforation	10.10%	9.80%	0.938
Location of perforation (n)			
Esophageal	1	2	
Gastric	7	6	
Colorectal	1	0	
Perforated lesions			
Muscularis propria	7	8	
Scar formation	1	0	
Non-scar forming lesion	1	0	
Incidence of abdominal distension	13.50%	12.20%	0.802
With perforation	8	6	
Without perforation	4	4	
Difficulty excreting adhesive	2.25%	0.00%	

DISCUSSION

ESD is a technique that uses special instruments and other equipment to resect and strip gastrointestinal pre-cancerous lesions and early-stage tumors on the basis of EMR. ESD can be used for the one-time complete resection of lesions with a diameter greater than 2 cm, and the high *en bloc* resection rate can reduce residual lesions and the chances of recurrence, thus achieving a radical cure^[3-6]. The indications for ESD are still controversial; however, some scholars believe that as long as there is no lymphatic and blood vessel invasion or metastasis, the lesion can be resected using ESD regardless of lesion location and size^[7]. With improvement in the management of endoscopic complications, the depth of lesions treated with ESD has gradually increased and ESD has been used to treat some muscularis propria lesions and stromal tumors. Some authors have even proposed a concept of ESE^[8,9]. However, the incidence of major complications with ESD, including bleeding and perforation, is still relatively high which has limited the generalization of ESD to a larger extent.

The main ingredient of the medical adhesive used in this study is butyl α -cyanoacrylate. As an adhesive with special biomedical function, it has biomedical functions in addition to the common gluing function and mechanical function. It has been confirmed that butyl α -cyanoacrylate is non-toxic to the human body and not mutagenic, teratogenic, or carcinogenic, and will not cause an irritant reaction. Medical adhesives are widely used in surgery, and can rapidly solidify in the presence of anionic substances such as tissue fluid and blood. The adhesive used has a similar strength as tissues, and therefore is not likely to fall off during extension and flexion movement. The adhesive strength is greater than the physiological strength of human body; therefore, its tissue adhesive function is reliable^[1,2].

Manner *et al*^[10] reported an incidence of delayed bleeding after ESD of 6.5%, and Sugimoto *et al*^[11] in a

multi-center study reported an incidence of 3.7%. In our study, the incidence of delayed bleeding in the control group was 4.88%, similar to that previously reported. However, no delayed bleeding occurred in the medical adhesive group, and this was significantly different from the incidence in the control group.

Ono *et al*^[12] reported a perforation rate of 5% among 906 patients who were treated with ESD. Sugimoto *et al*^[11] reported a perforation rate of 10.3% in patients with scar lesions and 3.5% in patients without scar lesions. In our study, the perforation rates in groups A and B were 10.1% and 9.8%, and the difference was not statistically different. The incidence of perforation in our study was slightly higher than that in the report by Ono *et al*^[12], but it was similar to the incidence of perforation in patients with scar lesions in the study by Sugimoto *et al*^[11]. We analyzed the depth of the perforated lesions, and found that among the 17 cases of perforation in the two groups, there were 15 cases of muscularis propria lesions, one case of scar formation, and one case of a non-scar forming lesion. Therefore, the relatively high incidence of perforation in our study was due to relatively deep lesions. Generally, perforation was managed successfully with conservative treatment including fasting and antibiotics^[13].

As for other complications of ESD, 12 patients in group A (medical adhesive group) and 10 patients in group B (control group) experienced varying degrees of abdominal distension, and the incidence was 13.5% (12/89) and 12.2% (10/82), respectively, and was not different between the groups. Anal pain during defecation occurred in two patients in group A 8-10 d after treatment, which was followed by discharge of solid medical adhesive. Medical adhesive was not used in group B, so no difficulty in excreting the medical adhesive occurred.

The average duration of ESD in group A was 59.44 min (range, 36-150 min) and in group B was 55.00 min (range, 35-140 min) and the times were not statistically different. However, the average treatment time in group A was slightly more than that in group B, and we believe this is because of the extra time needed to apply the medical adhesive. Our results are consistent with a treatment time of 35-180 min reported by Sano *et al*^[14]. With improvement of techniques and accumulation of experience, the operation time will be gradually shortened, while treating larger and deeper lesions may increase the operation time.

The average length of hospital stay was 8.89 d (range, 6-15 d) in group A and 9.90 d (range, 5-21 d) in group B, and the differences were statistically significant. In group B, four patients experienced delayed bleeding and were hospitalized for 19, 19, 20 and 21 d, respectively. Excluding those patients, the average length of hospital stay in group B was 9.40 d (range, 5-15 d), and this was not significantly different compared with group A, indicating that delayed bleeding may significantly increase the length of hospital stay.

In summary, application of spray-type medical adhe-

sive may cause difficulty in excreting the medical adhesive in a few patients, but it does not affect the overall therapeutic effects and prognosis. Use of a spray-type medical adhesive can prevent complications of ESD, especially delayed bleeding. Application of a spray-type medical adhesive can reduce the average length of hospital stay, thereby avoiding additional health care expenditures.

COMMENTS

Background

Endoscopic submucosal dissection (ESD) is an effective minimally invasive treatment for gastrointestinal precancerous lesions or early-stage gastrointestinal cancer. Medical adhesive spray can be effective in preventing delayed bleeding after ESD, and can thus reduce the average length of hospital stay.

Research frontiers

ESD is associated with a high incidence of complications such as perforation and bleeding, which has limited the utility of ESD. Medical adhesives (spray type) have been widely adopted in surgery and for the treatment of gastric varices because of their adhesive, reinforcement, and leak proofing functions.

Innovations and breakthroughs

This is the first report to use medical adhesive after ESD, and results were exciting. Use of a spray-type medical adhesive can prevent complications of ESD, especially delayed bleeding. Application of a spray-type medical adhesive can reduce the average length of hospital stay, thereby avoiding additional health care expenditures.

Applications

Medical adhesive spray can be effective in preventing delayed bleeding after ESD, and can thus reduce the average length of hospital stay.

Terminology

ESD is an effective minimally invasive treatment for gastrointestinal precancerous lesions or early-stage gastrointestinal cancer.

Peer review

This is a good prospective randomized study in which authors analyzed the effect of medical adhesive spray in preventing delayed bleeding after ESD. The results are interesting and suggest that the use of medical adhesive spray may be a useful therapeutic approach in the prevention of delayed bleeding after ESD.

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Difference in *DRB1** gene polymorphisms between Han and Uyghur ulcerative colitis patients in China

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Abstract

AIM: To evaluate the association between *HLA-DRB1* alleles and Han and Uyghur ulcerative colitis (UC) patients residing in the Xinjiang Uyghur Autonomous Region of China.

METHODS: In this study, 102 UC patients (53 Han including 22 men and 31 women, and 49 Uyghur patients including 25 men and 24 women; aged 48.07 ± 15.83 years) and 310 age- and sex-matched healthy controls were enrolled in the Department of Gastroenterology, Xinjiang People's Hospital of China from January 2010 to May 2011. UC was diagnosed based on the clinical, endoscopic and histological findings following Lennard-Jones criteria. Blood samples were collected and genomic DNA was extracted by routine laboratory methods, and both polymerase chain reaction and gene sequencing were used to identify *HLA-DRB1* allele variants. The potential association between genetic varia-

tion and UC in Han and Uyghur patients was examined. There were no statistical differences in *HLA-DRB1* allele frequencies in Han UC patients.

RESULTS: There was no significant difference in the sex ratio between the controls and UC patients ($P = 0.740$). In Han patients with UC ($n = 53$), *HLA-DRB1* *03, *13 allele frequencies were lower than in healthy controls ($n = 161$), but not statistically significant, and *HLA-DRB1* *04*11*14 allele frequencies were higher than in healthy controls, but without statistical significance. Differences between Uyghur UC patients and the control group were observed for *HLA-DRB1* *04 and *HLA-DRB1* *13, both showed a greater frequency in UC patients (10.21% vs 2.69%, $P = 0.043$; 14.29% vs 4.03%, $P = 0.019$). *HLA-DRB1* *14 also showed a greater frequency in UC patients (14.29% vs 2.69%, $P = 0.006$). The frequencies of *DRB1* *04, *13*14 alleles were increased in Uyghur UC patients compared with normal controls. The frequency of *DRB1* *08 was decreased in Uyghur UC patients compared with normal controls. *HLA-DRB1* alleles showed no association with UC in Han patients. There were no statistical differences in *HLA-DRB1* allele frequencies in Han UC patients. The frequencies of *DRB1* *04, *13*14 alleles were increased in Uyghur UC patients compared with normal controls. The frequency of *DRB1* *08 was decreased in Uyghur UC patients compared with normal controls. Polymorphism of the *HLA-DRB1* gene may contribute to the clinical heterogeneity of UC between Han and Uyghur UC patients in China.

CONCLUSION: *HLA-DRB1* *04*13*14 and *DRB1* *08 may contribute to the clinical heterogeneity of UC between Han and Uyghur UC patients.

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Key words: Ulcerative colitis; *DRB1** gene polymorphisms; Han and Uyghur

Core tip: This study evaluated the association between

HLA-DRB1 alleles and Han and Uyghur ulcerative colitis (UC) patients residing in the Xinjiang Uyghur Autonomous Region of China. The authors found that polymorphism of the *HLA-DRB1** gene differed between the Han and Uyghur patients with UC. Polymorphism of the *HLA-DRB1* gene may contribute to the clinical heterogeneity of UC between Han and Uyghur UC patients in North-West China.

Aheman A, Gao F, Kuerbanjiang A, Li YX, Abuduhadeer M. Difference in *DRB1** gene polymorphisms between Han and Uyghur ulcerative colitis patients in China. *World J Gastroenterol* 2013; 19(17): 2709-2713 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2709.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2709>

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease are often grouped together as inflammatory bowel disease (IBD). IBD is the term used to describe idiopathic disorders associated with chronic inflammation of the gastrointestinal tract^[1,2]. Clinical features common to both disorders include abdominal pain, diarrhea, weight loss, and increased risk of developing colorectal cancer^[3,4]. The etiology of UC is still not known. However, underlying genetic, environmental, and lifestyle issues can affect an individual's predisposition to these diseases^[5,6]. Genetic factors involved in the regulation of the immune system are thought to play a significant role in the pathogenesis of UC^[7]. Human leukocyte antigens (HLA), located on chromosome 6, play an important role in the immune response and several immune-mediated diseases. Several studies have shown that HLA alleles are associated with UC^[8]. We previously compared the clinical characteristics of UC in the Han and Uyghur populations residing in the Xinjiang Uyghur Autonomous Region of China. We showed some differences between the Uyghur and Han populations living in the same region, which included a higher prevalence of UC, a younger age of onset, an increased prevalence of the chronic persistent and acute outbreak type, more moderate and severe forms, a higher complication rate, and an increased frequency of positive antineutrophilic cytoplasmic antibodies (ANCA) in the Uyghur population^[9]. However, to date, hardly a research shows that there is association between *HLA-DRB1* alleles and UC in the Uyghur population. It would be interesting to know what causes the clinical heterogeneity of UC between Han and Uyghur UC patients in China.

MATERIALS AND METHODS

Patients and controls

Consecutive patients with UC were recruited from the Department of Gastroenterology, Xinjiang People's Hospital of China. The diagnosis of UC was made based on clinical, endoscopic, and histological findings in accordance with Lennard-Jones criteria^[10]. The extent of

Table 1 The 5' amplification primer mix included the following primers

5'-CCACAGCACGTTCTTGGAGTACTCTA-3'
5'-CCAGTTCTTGTGGCAGCTTAAGTT-3'
5'-TCGTTCTGTGGCAGGGTAAGTATA-3'
5'-AGCCGTTTCTTGAAGCAGGATAAGTT-3'
5'-CCAAGCACGTTCTTGGAGGAGG-3'
5'-TCGTTCTGTGGCAGCCTAAGA-3'
5'-AGCCGTTTCTTGGAGCAGGTTAAAC-3'

disease was assessed by colonoscopy at initial diagnosis and at follow-up. Extensive colitis was defined as lesions located beyond the splenic flexure. Distal colitis was defined as lesions limited to the region distal to the splenic flexure^[11,12]. Age- and sex-matched healthy controls were also recruited from the Health Examination Center of Xinjiang People's Hospital. The study protocol was approved by the Ethics Committee of Xinjiang Uyghur Autonomous Region of China, and individuals selected from the populations sampled were Chinese and Uyghur UC patients from Urumqi, Xinjiang. Informed consent was obtained from all patients.

Genomic DNA was isolated using a QIAamp DNA Blood Mini Kit according to the manufacturer's instructions (Qiagen, Germany). DNA samples were quantified by *ultraviolet* measurements at A_{260} .

Polymerase chain reaction amplification

Polymerase chain reaction (PCR) amplifications were performed in a volume of 20 μ L consisting of 20 ng of genomic DNA, PCR buffer (1.5 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl pH 8.4, 50 mmol/L KCl, 0.1 mg/mL of Gelatin, 0.02% of NP-40), 200 mmol/L of each dNTP (Life Technologies, Rockville, MD, United States) and 1 unit of Taq Platinum polymerase (Life Technologies, Grand Island, NY, United States). Seven sense primers and 1 antisense primer were used to amplify *HLA-DRB1* alleles (Table 1).

The 3' amplification primer was 5'-CTGTTACCTC-GCCACTGCAC-3'. PCR amplification was performed in an ABI 9700. The DNA was amplified following initial denaturation at 95 $^{\circ}C$ for 120 s followed by 35 cycles at 95 $^{\circ}C$ for 30 s, 60 $^{\circ}C$ for 30 s and 72 $^{\circ}C$ for 60 s. PCR amplification was confirmed following electrophoresis on a 2.0% agarose gel. Samples were electrophoresed at 10 V/cm for 36 min. PCR products were purified using the High Pure PCR Purification Kit (Roche Diagnostics, Basel, Switzerland).

DNA sequencing and analysis

PCR products were sequenced using BigDye Terminator v3.1 kits (ABI, United States) and automated ABI 3730XL DNA sequencers. The 5' sequence primer was 5'-TTGCAATTCTTCAATGGGAC-3' and the 3' primer was 5'-ACCACCCGGTAGTTGTGTC-3'. The DNA was sequenced following initial denaturation at 95 $^{\circ}C$ for 120 s followed by 25 cycles at 95 $^{\circ}C$ for 10 s, 50 $^{\circ}C$ for 5 s and 60 $^{\circ}C$ for 60 s. The sequence within the 5' primer sites was included in the analysis to prevent the mistyping of

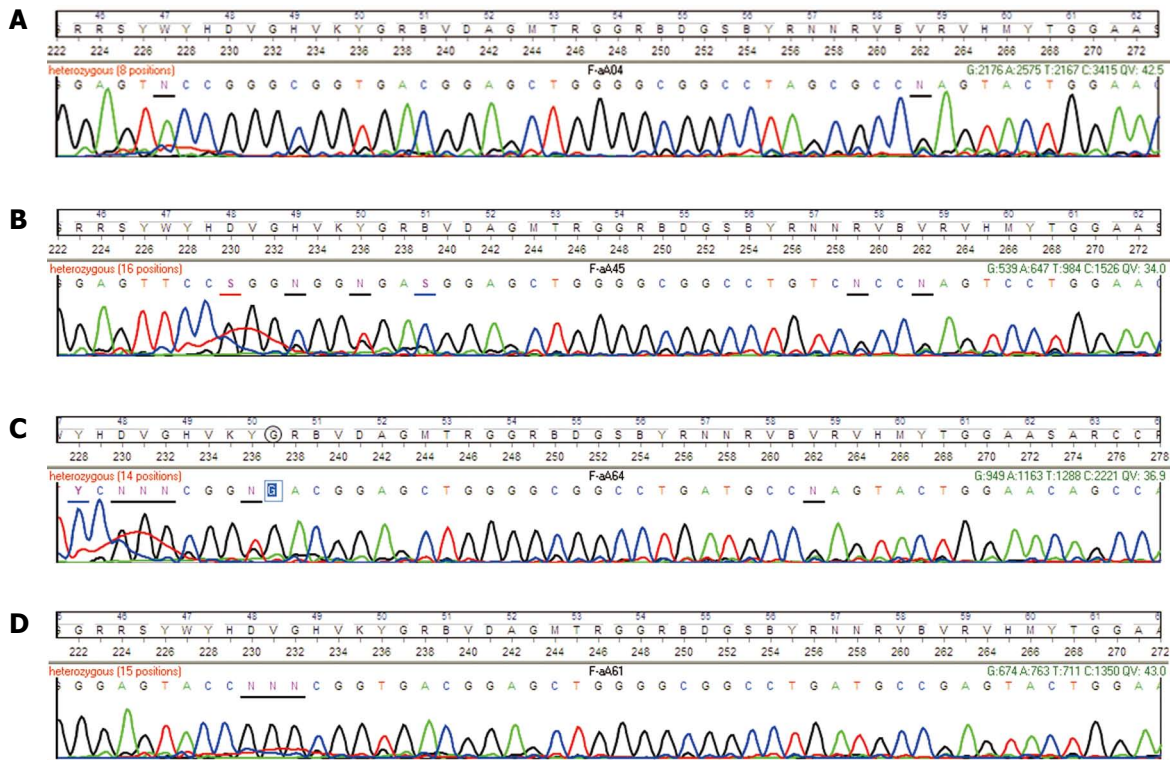


Figure 1 DRB1 genotyping was assigned using Qiagen SBTengine human leukocyte antigens typing software that compares an unknown sequence against a library of allele sequences. A: HLADRB1*08 in a Han patient; B: HLADRB1*12 in a Uyghur patient; C: HLADRB1*13 in a Han patient; D: HLADRB1*14 in a Uyghur patient.

Table 2 Clinical characteristics of the study subjects *n* (%)

Characteristics	UC patients	Controls	<i>P</i> value
Total number (<i>n</i>)	102	310	
Han	53	161	0.998
Uyghur	49	149	0.998
Gender, M/F	47/55	137/173	0.740
Age of onset ¹ , yr	48.07 ± 15.83	47.37 ± 14.49	0.801
Extensive colitis (Han)	20 (38)		
Extensive colitis (Uyghur)	30 (61)		
Distal colitis (Han)	33 (62)		
Mild and intermediate	47 (89)		
Severe	6 (11)		
Distal colitis (Uyghur)	19 (39)		
Mild and intermediate	40 (82)		
Severe	9 (18)		

¹Data are expressed as mean ± SD. UC: Ulcerative colitis.

novel alleles that may have arisen due to recombination within the first variable region. DRB1 genotyping was assigned using Qiagen SBTengine HLA Typing software that compares an unknown sequence against a library of allele sequences (Figure 1).

Statistical analysis

Hardy-Weinberg disequilibrium was assessed with χ^2 tests and clinical records including age were analyzed with *t* tests. χ^2 tests were used to compare genotype and allele frequencies between patients and normal controls. Fisher exact tests with 95%CI were used when the number of samples was less than 5. *P* values less than 0.05 were con-

sidered statistically significant.

RESULTS

Clinical characteristics of UC patients

DNA was obtained from 102 consecutive UC patients and 310 healthy individuals well matched for sex and age. The main clinical characteristics of the UC patients are summarized in Table 2. UC patients included 47 males and 55 females and the controls included 137 males and 173 females. The average age of UC patients and controls was 48.07 ± 15.83 years and 47.37 ± 14.49 years, respectively. There was no significant difference in the sex ratio between the controls and UC patients (*P* = 0.740, Table 2).

Comparison of DRB1*genotype variants between UC patients and healthy controls in the Han population

The HLA-DRB1 allele frequencies in the patients and controls are shown in Table 3. In Han patients with UC (*n* = 53), HLA-DRB1*03, *13 allele frequencies were lower than in healthy controls (*n* = 161), but not statistically significant (*P* > 0.05), and HLA-DRB1*04*11*14 allele frequencies were higher than in the healthy controls, but without statistical significance (*P* > 0.05) (Figure 2).

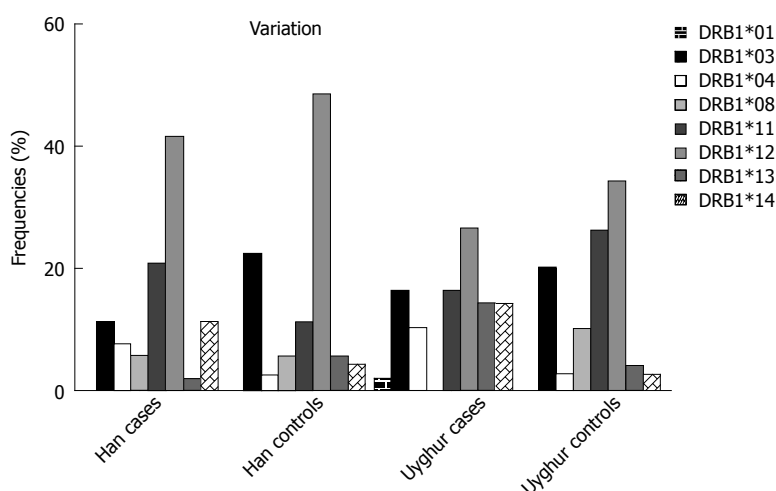
Comparison of DRB1*genotype variants between the UC patients and healthy controls in the Uyghur population

Differences between Uyghur UC patients and the control group were observed for HLA-DRB1*04 and HLA-

Table 3 Genotypic association between *DRB1** variants and ulcerative colitis in the Han and Uyghur population

	Han UC (n = 53)		Controls (n = 161)		χ^2	P value	Uyghur UC (n = 49)		Controls (n = 149)		χ^2	P value
	n (%)	Genotype frequencies	n (%)	Genotype frequencies			n (%)	Genotype frequencies	n (%)	Genotype frequencies		
<i>HLA-DRB1</i> *01	0	0	0	0			1 (2.04)	0.010	0	0	3.056	0.247
<i>HLA-DRB1</i> *03	6 (11.32)	0.058	36 (22.36)	0.119	3.081	0.110	8 (16.33)	0.085	30 (20.13)	0.106	0.345	0.678
<i>HLA-DRB1</i> *04	4 (7.55)	0.039	4 (2.49)	0.013	2.840	0.106	5 (10.21)	0.052	4 (2.69)	0.014	4.805	0.043
<i>HLA-DRB1</i> *08	3 (5.66)	0.029	9 (5.59)	0.028	0.000	1.000	0	0	15 (10.07)	0.052	5.337	0.024
<i>HLA-DRB1</i> *11	11 (20.76)	0.110	18 (11.18)	0.058	3.120	0.103	8 (16.33)	0.085	39 (26.18)	0.141	1.975	0.180
<i>HLA-DRB1</i> *12	22 (41.51)	0.235	78 (48.45)	0.282	0.771	0.429	13 (26.53)	0.143	51 (34.23)	0.189	0.999	0.380
<i>HLA-DRB1</i> *13	1 (1.89)	0.009	9 (5.59)	0.028	1.228	0.457	7 (14.29)	0.074	6 (4.03)	0.022	6.326	0.019
<i>HLA-DRB1</i> *14	6 (11.32)	0.058	7 (4.35)	0.023	3.398	0.093	7 (14.29)	0.074	4 (2.69)	0.014	9.458	0.006

UC: Ulcerative colitis.

**Figure 2** Comparison of the *DRB1** genotype variants between ulcerative.

*DRB1**13, both showed a greater frequency in UC patients (10.21% *vs* 2.69%, $P = 0.043$; 14.29% *vs* 4.03%, $P = 0.019$). *HLA-DRB1**14 also showed a greater frequency in UC patients (14.29% *vs* 2.69%, $P = 0.006$), however, *HLA-DRB1**08 showed a lower frequency in UC patients than in the controls (0% *vs* 10.07%, $P = 0.024$), Table 3.

DISCUSSION

UC was previously uncommon in China, however, in the last 10 years more cases have been identified and the incidence of this disease has increased^[13]. The clinical characteristics of UC in the Han and Uyghur populations residing in the Xinjiang Uyghur Autonomous Region of China were shown to be different^[14]. The genetic factors possibly associated with UC and its clinical characteristics in these two ethnic groups are unknown. A number of *HLA* alleles have been shown to be associated with variations in immune response diseases (e.g., celiac disease^[15], longitudinally extensive transverse myelitis^[16], and Behçet's disease^[17]). Several genome-wide scans have shown that the susceptibility locus of UC and Crohn's disease is on chromosome 16q (IBD1)^[18]. In the present study, we evaluated the association between the *HLA-DRB1* alleles and UC in Han and Uyghur populations from north-west China. We did

not find any association between *HLA-DRB1* alleles and UC in the Han population, and these results were consistent with previous Chinese studies. Lü *et al*^[19] showed that polymorphism of the *HLA-DRB1* gene did not have a strong association with UC in Chinese patients. Lee *et al*^[20] found that *HLA-DQA1c*, but not *HLA-DRB1* alleles, was associated with ANCA-positive UC in southern China.

We found differences between Uyghur UC patients and healthy controls, in whom *HLA-DRB1**04, *HLA-DRB1**13 and *HLA-DRB1**14 showed a greater frequency in UC patients than in controls. These results were consistent with previous Japanese studies^[21]. In contrast to previous Chinese studies *HLA-DRB1**08 showed a lower frequency in UC patients than in controls^[22]. Polymorphism of the *HLA-DRB1* gene may contribute to the clinical heterogeneity of UC between Han and Uyghur UC patients in China. The results in the present study were different probably due to the statistical methods used and the genetic heterogeneity of the ethnic populations. In future studies, we will continue to explore other *HLA* genes and amplify our sample size to identify the genes associated with UC in different ethnic groups in China and to provide more evidence for the genetic susceptibility of UC.

COMMENTS

Background

Previous studies have shown that human leukocyte antigens (HLA) alleles are associated with ulcerative colitis (UC). The clinical characteristics of UC in the Han and Uyghur populations residing in the Xinjiang Uyghur Autonomous Region of China were shown to be different. In this study, the authors examined whether polymorphism of the *HLA-DRB1** gene differed between Han and Uyghur patients with UC.

Research frontiers

Studies have found that disease distribution and phenotypic appearance differ significantly between ethnic groups and even within populations. The genetic and clinical heterogeneity of UC between the two ethnic groups, Chinese Han and Uyghur, were investigated.

Innovations and breakthroughs

This study found that polymorphism of the *HLA-DRB1** gene differed between Han and Uyghur patients with UC. Polymorphism of the *HLA-DRB1* gene may contribute to the clinical heterogeneity of UC between Han and Uyghur UC patients in north-west China.

Applications

The effects of different genotypes in normal controls and patients with UC are still unknown and likely represent a potentially productive area for future research to better understand the pathogenesis and treatment of UC.

Terminology

UC is a form of *inflammatory bowel disease*. It is a refractory, chronic, and non-specific disease which usually occurs in the rectum and the entire colon. HLA, located on chromosome 6, play an important role in the immune response and several immune-mediated diseases.

Peer review

The authors investigated the contribution of *DRB1** gene polymorphism to the genetic susceptibility and clinical heterogeneity of UC between Han and Uyghur patients. This manuscript contains potentially interesting findings.

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E-Editor Zhang DN



Isolated arteriportal fistula presenting with variceal hemorrhage

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Author contributions: Nookala A was the primary author in gathering information regarding the case, helped to coordinate the different authors in acquiring the information and figures, primary author in reviewing the literature and analyzing it as it pertained to the case and primary drafter of the manuscript; Saberi B assisted in critical revision of the manuscript for important intellectual content and review of the literature; Ter-Oganesyan R provided interventional radiological information on the case, Figure 1 images and legend, as well as assisting in critical review of the case; Kanel G provided pathological interpretation of liver biopsy, Figure 2 images and legend; Duong P assisted in gathering of information regarding the case and analyzing literature as it pertained to the case; Saito T assisted in the majority of critical revision of the manuscript for important intellectual content.

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setting. This is one of the first cases of adult-onset isolated APF who presented with portal hypertension and was successfully managed through endoscopic hemostasis and subsequent interventional radiological embolization.

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Key words: Arteriportal fistula; Pre-sinusoidal portal hypertension; Hepatic vein pressure gradient; Hepatic artery embolization

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INTRODUCTION

Portal hypertension is defined as the increase in porto-systemic resistance and/or flow^[1]. The causes of portal hypertension can be divided into pre-, intra-, and posthepatic causes. Moreover, intrahepatic portal hypertension can be further categorized into three groups: pre-, intra-, and postsinusoidal causes. The most common etiology of portal hypertension is due to liver cirrhosis which accounts for 90% of the cases in the United States^[2]. It is a cause of intrasinusoidal portal hypertension due to distortion of the hepatic lobular architecture and results in hyperdynamic splanchnic circulation. The second major cause of portal hypertension is due to extrahepatic portal vein thrombosis, accounting for 7% of cases. The remaining 3% of causes of portal hypertension encompasses a variety of rare etiologies, including our case of intrahepatic presinusoidal portal hypertension^[2]. While infrequent, the causes for presinusoidal portal hypertension comprise of schistosomiasis, myeloproliferative diseases, sarcoidosis, hepatic portal fibrosis, primary biliary cirrho-

Abstract

We report a case of life-threatening hematemesis due to portal hypertension caused by an isolated arteriportal fistula (APF). Intrahepatic APFs are extremely rare and are a cause of presinusoidal portal hypertension. Etiologies for APFs are comprised of precipitating trauma, malignancy, and hereditary hemorrhagic telangiectasia, but these were not the case in our patient. Idiopathic APFs are usually due to congenital vascular abnormalities and thus usually present in the pediatric

sis, arsenic toxicity, idiopathic portal hypertension^[3].

CASE REPORT

The patient is a 30 year old male who presented to Los Angeles County-University of Southern California Medical Center with a two day history of hematemesis. The physical examination on arrival showed vital signs of a blood pressure of 93/61 mmHg, a heart rate of 135 beats per minute with exam findings significant for splenomegaly and left femoral artery bruit, but otherwise negative for shifting dullness, spider angiomas, palmar erythema, asterixis, and jaundice. Pertinent laboratory values were as follows: alkaline phosphatase 175 units/L, total protein 6.3 g/dL, albumin 3.9 g/dL, total bilirubin 0.6 mg/dL, aspartate aminotransferase 39 units/L, alanine transaminase 72 units/L, prothrombin 14.5 s, INR 1.16, hemoglobin 13.5 g/dL, white blood cells 8.0 k/cumm, and a platelet count of 79×10^3 /cumm. Further testing was done and showed all viral hepatitis, autoimmune, and metabolic liver disease markers to be negative. No history of alcohol intake and no history of prescribed, over the counter or supplement medications. His family history was negative for liver disease.

Upon presentation, the patient underwent an emergent esophagogastroduodenoscopy, which showed four columns of esophageal varices with active bleeding. Band ligation of the varices was performed successfully. Subsequent abdominal ultrasound demonstrated splenomegaly (length 14.8 cm) but a normal size, smooth liver surface and homogeneous liver parenchyma, suggesting a non-cirrhotic etiology for the portal hypertension. A multi-phase computed tomography (CT) examination of the liver demonstrated the left portal vein was highlighted at the arterial phase, strongly suggesting the existence of a hepatic arteriportal communication (Figure 1A). Based upon this finding, it was speculated that the overflowing of the portal vein due to the shunt between the hepatic artery to the portal vein was causing presinusoidal portal hypertension. In order to further confirm this, a hepatic venous pressure gradient (HVPG) measurement was performed and indicated normal value; 2 mmHg (< 5 mmHg is normal). Along with the signatures of portal hypertension-variceal bleeding and splenomegaly-the normal HVPG was consistent with pre-sinusoidal portal hypertension. To further confirm this, an angiogram was performed. A brisk contrast opacification of the left portal vein was noted upon contrast injection into the left hepatic artery, which branched from left gastric artery, confirming arteriportal shunting (Figure 1B). The shunting was determined not to be direct, but passing through a network of small capillary-like “fuzz” prior to brisk drainage into the portal system (Figure 1C). Furthermore, angiography showed the right superficial femoral artery with pseudoaneurysm and fistula and this additional vascular abnormality support our diagnosis of arteriportal fistulas (APFs) of congenital etiology. Taken all together, it was concluded that the arteriportal fistula is the cause

of this patient’s non-cirrhotic portal hypertension. The arteriportal and femoral artery fistulas were also closed with catheter directed embolization utilizing the liquid embolic agent Onyx[®] (ev3 Endovascular Inc., Plymouth, MN, United States). Following the embolization, no further early portal vein enhancement was seen during left hepatic arteriogram (Figure 1D). Finally, a liver biopsy was performed showing focal portal venule dilatation with dilated outflow vessels, but was otherwise normal, compatible with the clinical diagnosis of presinusoidal portal hypertension secondary to the hepatic artery and portal vein shunt (Figure 2). Post-embolization recovery of the patient has been uneventful, and no additional episodes of upper gastrointestinal bleeding have been reported by the patient on two subsequent clinic visits during the last four months.

DISCUSSION

HVPG measurements often provide valuable information in identification of the site or cause of the portal hypertension^[4]. The HVPG is the difference between the wedged and free hepatic venous pressures, which has been shown to correlate well with actual portal vein pressure^[5]. Its increase is consistent with sinusoidal portal hypertension and is known to be a predictor of development of varices and ascites^[6]. Upon visualization of the hepatic artery and portal vein communication through CT, the measurement of HVPG was conducted. The classic pattern of the prehepatic or intrahepatic presinusoidal portal hypertension shows normal HVPG and free hepatic vein pressure (FHVP) along with the stigmata of portal hypertension. The pathophysiology consists of a presinusoidal block preventing the transmission of the elevated portal pressure to the sinusoid, thus resulting in a normal wedged hepatic vein pressure^[5]. In our case, the HVPG value was consistent to the pathophysiology of presinusoidal portal hypertension.

Most of the literature regarding APFs is focused on the following etiologies: precipitating trauma, hepatocellular carcinoma (HCC), congenital, and in the context of hereditary hemorrhagic telangiectasia (HHT)^[7]. Clinical presentation of APFs varies from asymptomatic to symptoms related to congestive heart failure (40%-60%), portal hypertension (20%-40%), and diarrhea with abdominal pain secondary to a “steal” phenomenon (20%)^[8]. Hepatic trauma is a common cause for APFs. It may be from penetrating trauma such as gunshot wounds, a complication of liver transplantation or from previous liver biopsies^[9,10]. HCC can cause APFs and two studies have shown that APFs may occur in up to 60% of patients with HCC^[10,11]. Congenital APFs are associated with portal hypertension, failure to thrive, and gastrointestinal hemorrhage in infancy or early childhood^[7,12]. In general, it is noted that less than 10% of all APFs are congenital^[13]. As for HHT, hepatic involvement has been reported in 8%-78% of cases in retrospective and prospective studies^[14]. Our patient did not have the any his-

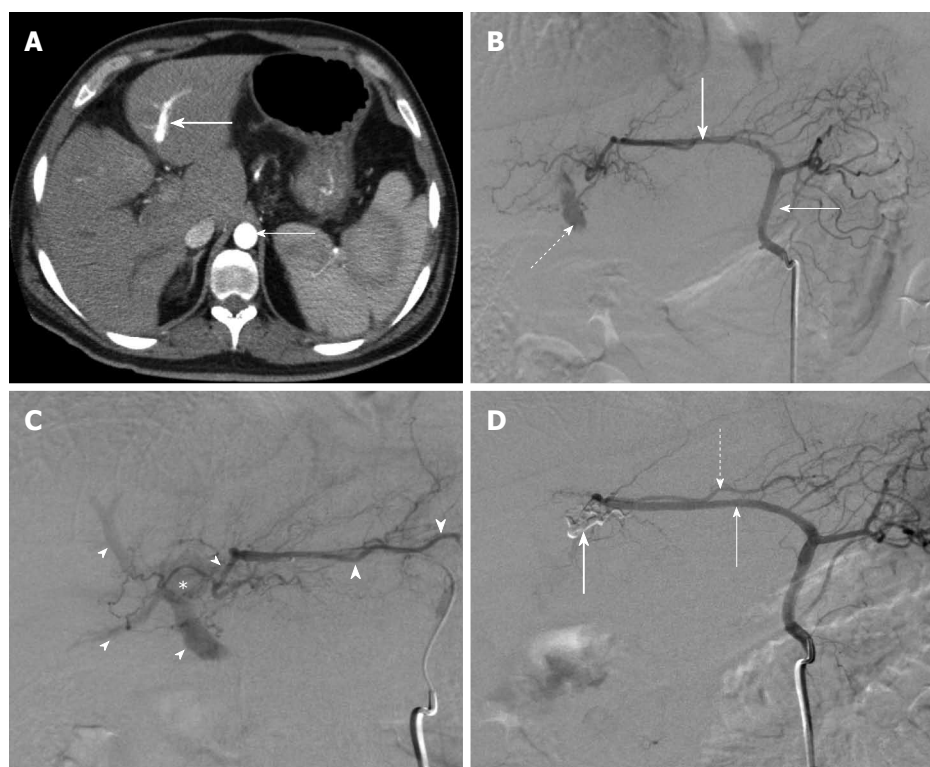


Figure 1 Intrahepatic communication between hepatic artery and portal vein. A: Contrast enhanced axial computed tomography of the abdomen demonstrating similar contrast opacification of the aorta (thin arrow) and the left portal vein (thick arrow); B: Left gastric arteriogram demonstrates gastrohepatic trunk (thin arrow) giving rise to aberrant left hepatic artery (thick arrow). Note early opacification of the portal vein (dashed arrow); C: Selective arteriogram with coaxial microcatheter in the left hepatic artery demonstrates medial branch of the left hepatic artery (large arrowheads) contributing to a parenchymal blush (star) and leading to opacification of the portal veins (small arrowheads). Note the normal appearance of the lateral branch of the left hepatic artery (thick arrow); D: Post-embolization arteriogram in the gastrohepatic trunk demonstrates opacification of the left hepatic artery (thin arrow). Onyx cast (thick arrow) is seen occupying the previously seen medial branch of the left hepatic artery. No further opacification of the portal veins is seen. Note the preserved lateral branch of the left hepatic artery (dashed arrow).

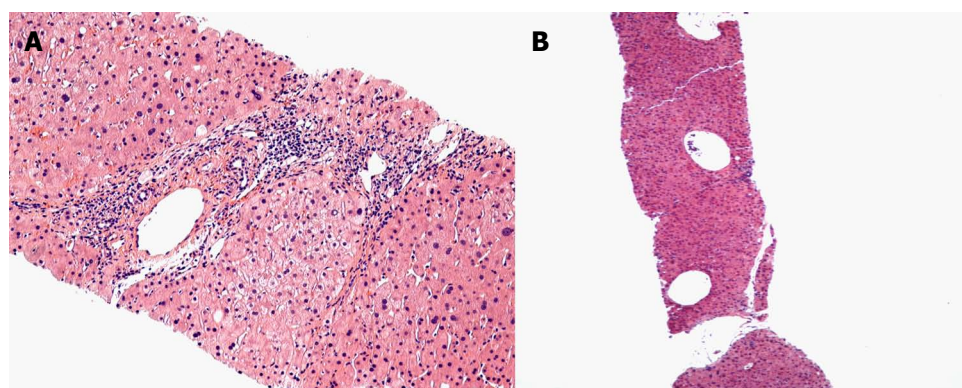


Figure 2 The hepatic histology of arterioportal fistula. A: This low power image shows a small portal tract in the center of the field that has a markedly dilated portal venule. The terminal hepatic (central) venules above and below the portal tract are markedly dilated; B: The portal venule is slightly dilated and there are increased numbers of small portal venous radicals.

tory suggestive of congenital APF or signatures of HHT. Furthermore, he had no abdominal trauma or surgery, malignancy and no previous liver biopsies. Moreover the incidental finding of the femoral arteriovenous fistula and unique variation of hepatic artery further imply the congenital etiology. Therefore, we believe that this is a case of adult onset congenital APF that likely did not present in infancy or childhood because his fistula is through a capillary connection and probably took multiple decades

to develop clinical manifestation. Based on our intensive literature search, this is one of the first cases reported to be adult onset portal hypertension due to congenital APFs.

Treatment of the APFs through shunt reduction is either surgical or minimally invasive through interventional radiology (IR) techniques. In the past, surgical ligation of the supplying artery was performed, however with the advancement of interventional radiology techniques, the trend has now shifted to endovascular catheter directed

therapy^[15]. IR directed therapy offers many advantages over the conventional surgical treatment and is now the preferred technique for treatment. These advantages include decreased morbidity and mortality, reduced risk of subsequent complications, and significant reduction in the time required for recovery. IR directed therapy is accomplished through hepatic artery embolization (HAE)^[16]. Embolization is usually performed with metal coils, detachable balloons, or gelfoam^[17,18]. However at selected centers such as ours, liquid embolic agents are also utilized. Types of liquid embolic agents include ethylene vinyl alcohol (Onyx[®]) and N-butyl cyanoacrylate, both of which are predominantly used in congenital APFs per the literature^[17,18]. HAE complications do occur, and include non-target embolization, hepatic infarction, and ischemic cholangitis. In a series of 15 patients studied by Chavan *et al*^[19] ischemic cholangitis and/or cholecystitis occurred in three patients and one patient died of hepatic necrosis leading to multi-organ failure. Nevertheless, due to the dual blood supply of the liver (hepatic artery and portal vein), infarctions are rare^[16]. In our limited experience with IR therapy of hepatic APFs, no such complications have occurred.

In summary, we described a case of lethal variceal bleeding due to an adult onset congenital APF. The patient was successfully treated through radiological interventional therapy and is being monitored regularly in our outpatient clinic without any further episodes of bleeding.

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Therapeutic efficacy of the Qing Dai in patients with intractable ulcerative colitis

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2 g/d of Qing Dai orally and continued taking other medications for UC as prescribed. Electron spin resonance was applied to explore the mechanisms of action of Qing Dai. After 4 mo of treatment with Qing Dai, the CAI score decreased from 8.3 ± 2.4 to 2.4 ± 3.4 (mean \pm SD; $P < 0.001$). Similarly, the endoscopic Matts grade decreased from 3.4 ± 0.5 to 2.2 ± 0.8 ($P = 0.02$). Six of 7 patients who were on prednisolone upon enrollment in the study were able to discontinue this corticosteroid. Electron spin resonance revealed that Qing Dai possesses strong hydroxyl radical scavenging activity. Qing Dai showed significant clinical and endoscopic efficacy in patients who failed to respond to conventional medications. Scavenging of hydroxyl radicals appears to be a potential mechanism through which Qing Dai acts, but the significance of the scavenging ability of Qing Dai with respect to the anti-inflammatory effect in UC patients warrants further investigation.

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Abstract

Ulcerative colitis (UC) is a chronic inflammatory bowel disease that may become intractable when treated with conventional medications such as aminosalicylates, corticosteroids, and azathioprine. The herbal medicine Qing Dai has traditionally been used in Chinese medicine to treat UC patients, but there is a lack of published data on the efficacy of Qing Dai in UC treatment. We report several cases of patients with intractable UC who take Qing Dai in a retrospective observational study. Furthermore, we explore the mechanisms of action of Qing Dai. Nine patients with active UC who received conventional medications but wished to receive Qing Dai as an alternative medication were included in our analysis. The UC severity level was determined based on the clinical activity index (CAI). Additionally, 5 of the 9 patients were endoscopically evaluated according to the Matts grading system. Each patient received

Key words: Qing Dai; Herbal medicine; Ulcerative colitis; Hydroxyl radical; Electron spin resonance

Core tip: Nine intractable ulcerative colitis patients using the herbal medicine Qing Dai were observed in a retrospective observational study. After 4 mo of treatment, clinical activity and endoscopic findings improved dramatically in most patients. Electron spin resonance revealed that Qing Dai has a strong radical scavenging effect.

Suzuki H, Kaneko T, Mizokami Y, Narasaka T, Endo S, Matsui H, Yanaka A, Hirayama A, Hyodo I. Therapeutic efficacy of the Qing Dai in patients with intractable ulcerative colitis. *World J Gastroenterol* 2013; 19(17): 2718-2722 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2718.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2718>

INTRODUCTION

The prevalence of ulcerative colitis (UC) is increasing worldwide and has reached a total of 130000 cases in Japan. Clinical practice guidelines issued by the Japanese Ministry of Health, Labor and Welfare recommend aminosalicylates, sulfasalazine, or 5-aminosalicylic acid (5-ASA) followed by corticosteroids [prednisolone (PSL)] or azathioprine (AZA) as the standard treatment for UC patients. Recently, infliximab (IFX) and tacrolimus have become available and are used clinically as novel medications for patients with UC. However, some patients do not respond to these pharmacological interventions; UC in such patients is called refractory or intractable. The herbal medicine Qing Dai is also known as Indigo naturalis and is extracted from plants such as *Strobilanthes cusia* and *Isatis tinctoria*. Qing Dai contains natural ingredients such as indigo, indirubin, isoindirubin, and nimbosterol. In China, Qing Dai has been traditionally used as an antipyretic, an antiphlogistic, and as a hemostatic remedy. Qing Dai enemas have been used for ulcerative proctitis and are currently described in Chinese medical guidelines for the treatment of UC. However, until now, no report has been published describing the efficacy of orally administered Qing Dai in UC patients.

CASE REPORT

Cases

Nine UC patients (7 men and 2 women, listed in Table 1) who voluntarily received Qing Dai between 2008 and 2011 were retrospectively evaluated. Patients who did not respond to 5-ASA and PSL or IFX were defined as “intractable”. One patient who was not intractable but refused to add PSL or IFX was included in the analysis. The average age of the patients was 34 years with a range of 16–75 years. All patients purchased Qing Dai from Seishinshoyakudo (Tokyo, Japan), a company that imports Qing Dai from China and sells it at a price of 4200 Japanese yen (US \$50) per 500 g. Qing Dai was provided as powder, and each patient took 1 g of Qing Dai orally twice a day without wrapping, according to the manufacturer’s recommendations. All 9 patients were on 5-ASA, and 7 patients were also on PSL. 5-ASA and PSL were taken at the recommended dose for at least 8 wk prior to the initiation of Qing Dai; however, unique cases include patient D, whose pharmacological regimen was changed from 3 g/d of Pentasa (Ferring Pharmaceuticals, Malmö, Sweden) to 3.6 g/d of Asacol (Warner Chilcott, Dublin, Ireland) 4 d before the start of Qing Dai and patient F, whose prescription was changed from 4 g/d of Pentasa to 3.6 g/d of Asacol 3 d before taking Qing Dai. The clinical activity index (CAI)^[1] of the patients before taking Qing Dai and 1, 2, and 4 mo after initially taking Qing Dai and the endoscopic Matts grade^[2] before and after treatment with Qing Dai were retrospectively collected from clinical records because those data had been routinely recorded for all UC patients.

Table 1 Patient characteristics including lesion type and ulcerative colitis duration and treatment

Patient	Sex	Age (yr)	Type of UC Lesion	Duration of UC (mo)	Ongoing treatment at time of Qing Dai initiation
A	M	16	Proctitis	18	5-ASA, PSL
B	M	36	Left-sided colitis	65	5-ASA, IFX
C	M	35	Proctitis	96	5-ASA, PSL, CAP
D	M	33	Pancolitis	60	5-ASA, PSL, AZA, IFX
E	M	31	Left-sided colitis	40	5-ASA, PSL, AZA
F	M	27	Left-sided colitis	32	5-ASA, PSL, CAP, IFX
G	F	26	Proctitis	6	5-ASA
H	F	29	Left-sided colitis	8	5-ASA, PSL
I	M	75	Left-sided colitis	74	5-ASA, PSL, AZA

Nine UC patients (7 men and 2 women) were included in this study. The type of colitis lesion, the duration of UC and the ongoing treatment at the time of Qing Dai initiation are shown. UC: Ulcerative colitis; M: Male; F: Female; 5-ASA: 5-aminosalicylates; PSL: Prednisolone; IFX: Infliximab; CAP: Cytopheresis; AZA: Azathioprine.

Electron spin resonance

To investigate the mechanisms of action of Qing Dai, we performed electron spin resonance (ESR) spectroscopy by using the spin-trapping reagent 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO)^[3]. Hydroxyl radicals were produced in an aqueous solution containing 50 μ L of 2 mmol/L H₂O₂ dissolved in 0.1 mol/L phosphate buffer. Fifty microliters of 8.9 mmol/L DMPO with or without 2.5, 25, or 250 μ g/mL Qing Dai was incubated for 60 s after the addition of 50 μ L of 0.2 mmol/L FeSO₄, and results were obtained using a JEOL-TE X-Band spectrometer (JEOL, Tokyo, Japan).

Ethical consideration

All 9 patients began taking Qing Dai voluntarily. Nonetheless, for this paper, written informed consent was obtained from all of the patients included in the analysis.

Statistical analysis

The data for CAI and Matts grade score were analyzed by a paired *t*-test using Excel 2010 (Microsoft, United States) with the add-in software Statcel 3 (OMS publishing Co., Saitama, Japan). Differences corresponding to a *P* value < 0.05 were considered statistically significant.

Clinical and endoscopic efficacy of Qing Dai

The average CAI score (mean \pm SD) decreased from 8.3 ± 2.4 to 2.4 ± 3.4 ($P < 0.001$) (Figure 1A). This effect persisted unless the patient withdrew from Qing Dai treatment. Patients A and C showed recurrence of UC after cessation of Qing Dai. However, their CAI score immediately recovered upon re-administration of Qing Dai.

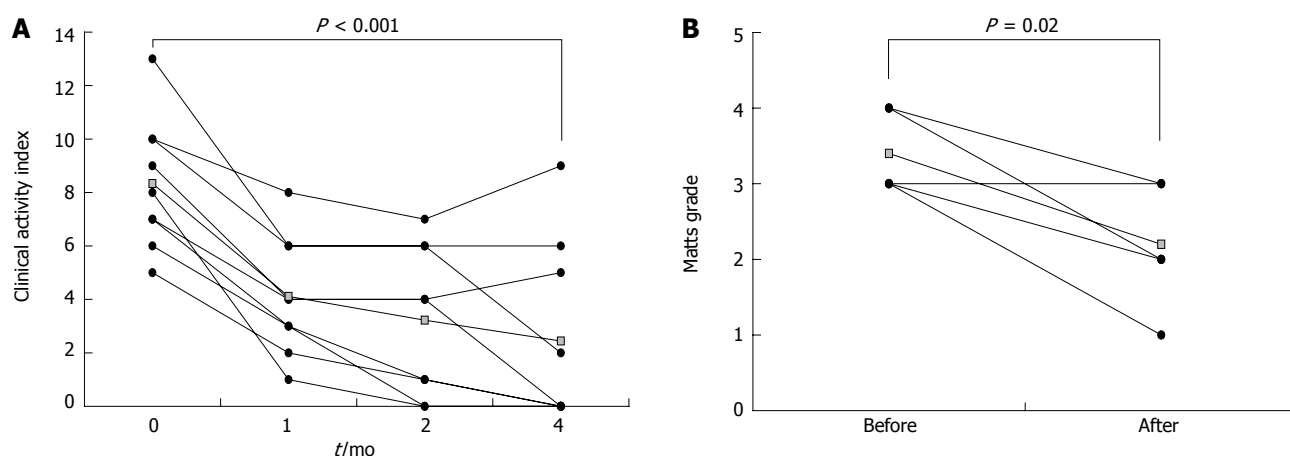


Figure 1 The clinical activity index score and endoscopic Matts grade before and after Qing Dai initiation. A: The score markedly improved from 8.3 ± 2.4 to 2.4 ± 3.4 (mean \pm SD; *t*-test, $P < 0.001$) after the initiation of Qing Dai. The line plotted in gray shows the average; B: The Matts grade significantly improved from 3.4 ± 0.5 to 2.2 ± 0.8 (mean \pm SD; *t*-test, $P = 0.02$) after the initiation of Qing Dai treatment. The line plotted in gray shows the average.

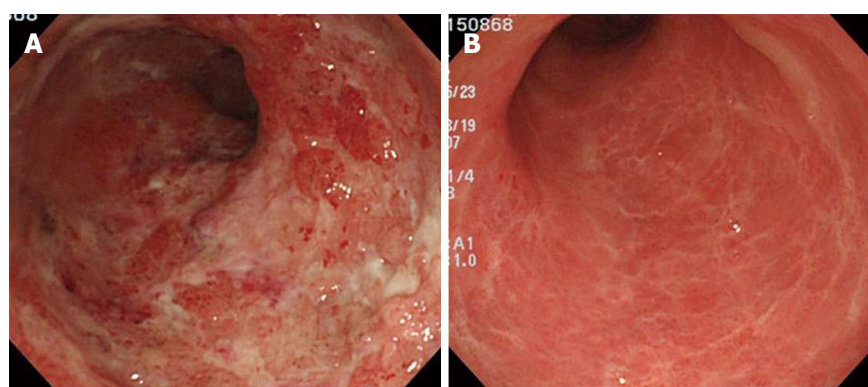


Figure 2 Endoscopic finding before and after treatment. A: Before the initiation of Qing Dai, endoscopic examination showed severe ulcers and erosions in the rectum; B: After 3 mo of Qing Dai treatment, the mucosal damage completely disappeared.

Five patients, including 3 patients with left-sided colitis, 1 patient with pancolitis and 1 patient with proctitis, were monitored by endoscopy. The average time interval between endoscopies was 7 mo (range 2–10 mo). The endoscopic Matts grade (Figure 1B) also decreased from 3.4 ± 0.5 to 2.2 ± 0.8 ($P = 0.02$). Six of the 7 patients (86%) who were also taking PSL were able to discontinue this corticosteroid.

Patient E, a 28-year-old man who presented with hematochezia was diagnosed as having left-sided UC and demonstrated remarkable benefit from Qing Dai therapy. Initially, patient E was prescribed 4 g/d sulfasalazine and 40 mg/d PSL for the treatment of UC, and the hematochezia rapidly disappeared. However, the hematochezia recurred as the patient reduced his intake of PSL. Thus, patient E required the dose of PSL to be increased several times, and treatment with AZA and cytopheresis failed to provide long-term improvement. Patient E met our criteria for refractory UC. After 3 years of treatment, patient E began to use Qing Dai of his own volition. One month after treatment with Qing Dai, hematochezia in patient E was resolved, and the CAI score decreased from 9 to 4. The serum C-reactive

protein level also decreased from 1.33 mg/dL to 0.11 mg/dL. The CAI score fell to 0 after 3 mo of treatment with Qing Dai, and patient E was withdrawn from PSL and AZA after 8 mo of Qing Dai treatment. Notably, this effect of Qing Dai therapy persisted for more than 2 years. The endoscopic findings are shown in Figure 2A and B. The ulcers completely disappeared and only erythema was observed. In contrast, patient D did not respond to Qing Dai, and required the administration of conventional medications.

Safety

Although no serious adverse side effects were reported in patients consuming Qing Dai, 2 patients developed mild headaches while taking Qing Dai. The headaches disappeared upon reduction of the dose of Qing Dai by half.

Hydroxyl radical scavenging effect of Qing Dai

As shown in Figure 3, without Qing Dai, the ESR spectrum agreed with CYPMPO-OH, a hydroxyl radical adduct trapped by CYPMPO. The reduction in the signal intensity of CYPMPO-OH reflected the hydroxyl radical scavenging ability of Qing Dai, and the results of treat-

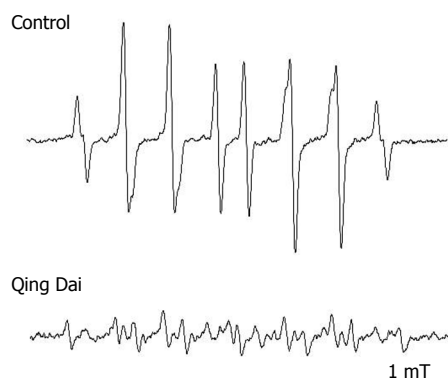


Figure 3 Electron spin resonance of Qing Dai. Representative electron spin resonance spectra of CYPMPPO-OH (for hydroxyl radical determination) obtained by the addition of solvent control or an ethanol extract of Qing Dai at a concentration of 25 $\mu\text{g/mL}$. The test was repeated in five independent trials, and a representative result is presented.

ment with 25 $\mu\text{g/mL}$ Qing Dai are shown in Figure 3. The 250 and 2.5 $\mu\text{g/mL}$ concentrations of Qing Dai showed a similar hydroxyl radical scavenging effect in a dose-dependent manner (data not shown).

DISCUSSION

Approximately 21% of patients with inflammatory bowel disease reportedly use alternative treatment outside of the medications typically prescribed to treat inflammatory bowel disease in a clinical setting^[4]. Consistent with this statistic, several herbal medications have shown promising efficacy and safety in patients with UC, including preparations that contain a sulfhydryl group in their structure^[5]. Evidence of the clinical efficacy of Qing Dai and its constituent substances has been previously reported. Yuan *et al*^[6], reported that Qing Dai enemas are associated with significant clinical efficacy in the treatment of chronic hemorrhagic radiation proctitis. Likewise, Xilei-san, which is a major ingredient in Qing Dai, has traditionally been used for the treatment of UC in China and, more recently, in Japan^[7,8]. Accordingly, in a double blind, randomized clinical trial setting, Fukunaga *et al*^[9] reported that suppositories of Xilei-san showed significant efficacy in patients with ulcerative proctitis refractory to conventional medications. In the current study, we found a good clinical response to Qing Dai with no serious adverse side effect in patients with intractable UC. Ferber has also reported very low toxicity for Qing Dai^[10].

There are few reports on the pharmacokinetics of Qing Dai. Because Qing Dai appears in the stool without significant digestion, the mucosal healing effect could be associated with the ability of Qing Dai to provide a protective coating to the injured mucosa. Qing Dai has been reported to have anti-inflammatory effects on human neutrophils based on its ability to suppress superoxide generation^[11]. The same authors reported efficacy of Qing Dai in patients with recalcitrant psoriasis in a randomized study^[12]. Indirubin, a constituent of Qing Dai, has been reported to produce anti-inflamma-

tory effects by suppressing interferon- α , interleukin-6^[13], and nuclear factor κB (NF- κB) production^[14]. Xilei-san was also reported to decrease the expression of toll-like receptor 4, NF- κB , and tumor necrosis factor- α in mice with oxazolone-induced colitis^[15]. Interestingly, cytaferesis, which has been used in patients with active UC as a highly effective therapeutic, possesses a similar mechanism of action by decreasing reactive oxygen-producing neutrophils and interleukin-6 secretion^[16]. In the present investigation, although we observed a strong hydroxyl radical scavenging effect in Qing Dai, further studies are warranted to fully understand the mechanisms of action in UC patients.

This study has several limitations. First, this was not a double-blind placebo-controlled trial. Second, basic experiments using animal models were not conducted. In light of these limitations, we are planning an *in vivo* study and a prospective multicenter double-blind placebo-controlled trial to investigate the effects of Qing Dai in patients with inflammatory bowel disease.

In conclusion, this investigation showed that oral Qing Dai is associated with significant clinical and endoscopic improvement of UC in patients who maintain active disease despite receiving conventional medications. The hydroxyl radical scavenging effect appears to be one mode of action of Qing Dai. Our results suggest that Qing Dai may represent a clinically relevant intervention for patients with inflammatory bowel disease. Additional controlled trials using larger cohorts of patients should be conducted to verify the findings of this investigation.

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Accurate hemostasis with a new endoscopic overtube for emergency endoscopy

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posed vessels resulted in success of hemostasis.

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Key words: Emergency endoscopic hemostasis; Right lateral decubitus position; Identification of exposed vessel; Newly developed inverted overtube; Clear endoscopic view

Core tip: The inverted overtube helped us obtain a clear view in patients who were laid in the right lateral position. Rapid identification of exposed vessels resulted in success of hemostasis.

Mori H, Kobara H, Fujihara S, Nishiyama N, Oryu M, Rafiq K, Masaki T. Accurate hemostasis with a new endoscopic overtube for emergency endoscopy. *World J Gastroenterol* 2013; 19(17): 2723-2726 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2723.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2723>

Abstract

Endoscopic hemostasis performed in the emergency room is difficult due to the presence of blood clots and food residue that makes obtaining a clear view of the bleeding vessel difficult. We experienced the efficacy of a newly developed inverted overtube to shorten the hemostatic time and obtain a clear endoscopic view with upper gastrointestinal bleeding patient who were transferred by ambulance car and required emergency endoscopy. The technique improved the endoscopic views and enabled us to perform the hemostatic procedures from the conventional standing position while freely and easily changing the patient's position. The presence of blood clots and food residue in the gastric fornix or upper gastric body makes identifying a bleeding exposed vessel impossible. This set-up significantly shortened the procedure time. The inverted overtube helped us obtain a clear view in patients who were laid in the right lateral position. Rapid identification of ex-

INTRODUCTION

Patients with upper gastrointestinal bleeding who are transferred by an ambulance commonly present in emergency rooms and require an emergency endoscopy to achieve hemostasis. Although the significance, indication and timing of emergency endoscopies are controversial^[1,2], in the case of esophageal or gastric varix or Dieulafoy's ulcer, exposed vessels with spurting bleeding require prompt hemostasis, and failure to achieve hemostasis may lead to a serious condition. Endoscopists are under pressure to perform these emergency endoscopic treatments, especially in patients with cardiovascular or cerebrovascular diseases who are being treated with antiplatelet and anticoagulant agents^[3] or in seriously ill patients who require rapid and reliable hemostasis^[4]. In almost all cases, once the bleeding site is visually identified

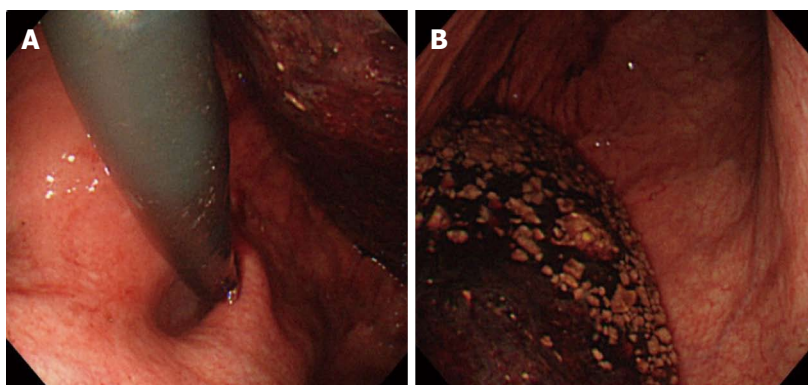


Figure 1 Emergency endoscopic view. A: The presence of large amounts of blood clots; B: The presence of food residue in the stomach complicated the observation of the region from the gastric fornix to the upper corpus.

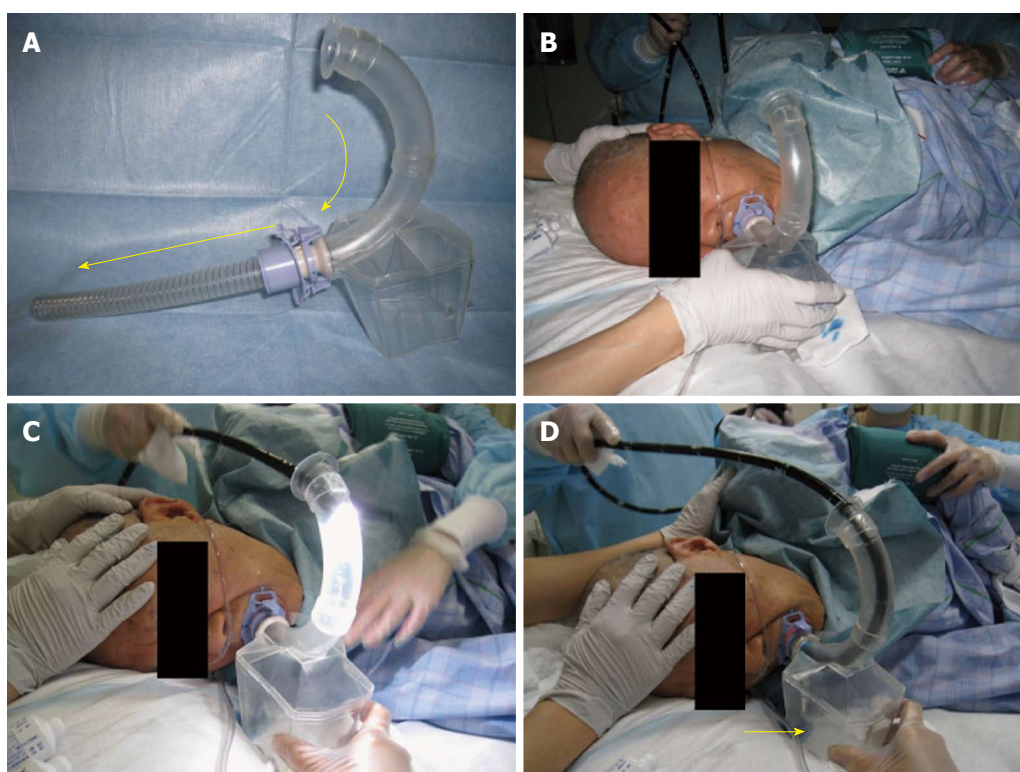


Figure 2 Outer appearance and procedures of using the inverted overtube. A: Outer appearance of the inverted overtube; B: Switching the patient from the conventional left lateral decubitus position to the right lateral decubitus position using the inverted overtube to dislodge blood clots during emergency endoscopic hemostasis; C: Insertion of the endoscope into the stomach through the inverted overtube in the right lateral decubitus position; D: Continuous aspiration from the bottom of the box (yellow arrow) enabled massive blood clots to be eliminated from the overtube, which maintained a clear endoscopic view during hemostasis.

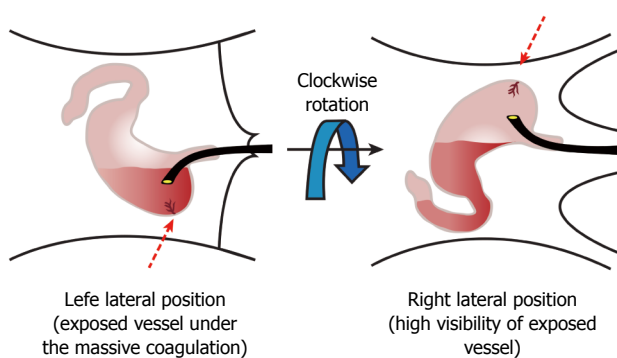


Figure 3 The schema of the visibility differences between 2 positions. We clockwise rotated the patient's position to a right lateral decubitus position to dislodge any massive clots and food residue.

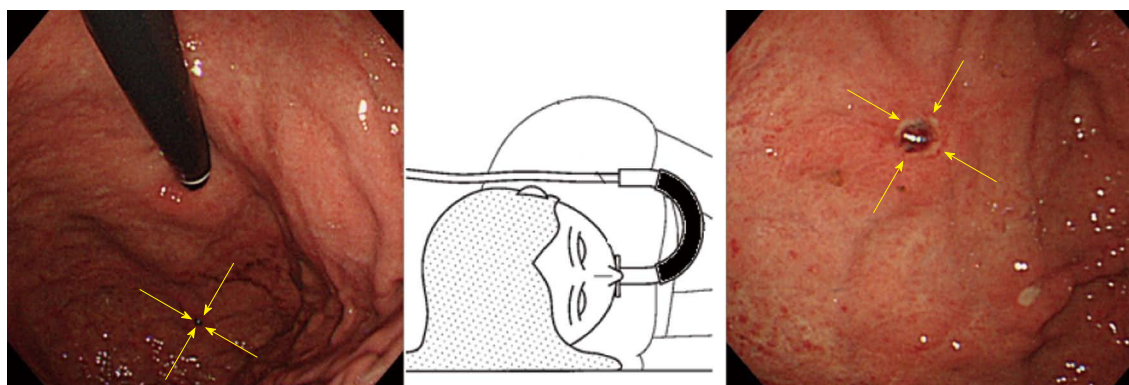


Figure 4 Obtaining a clear view of the gastric fornix and exposed vessels of a Dieulafoy's ulcer. Obtaining a clear view of the gastric fornix and upper gastric corpus, with all of the blood clots and food residue dislodged into the gastric antrum and duodenum. The exposed vessels of a Dieulafoy's ulcer were revealed (yellow arrows). The schema of the inverted overtube is shown in the center.

by endoscopy, hemostasis can be achieved using current endoscopic techniques and hemostatic forceps^[5]; however, if it cannot be visually identified, a surgical procedure is required^[6-8]. The presence of blood clots and food residue in the gastric fornix or upper gastric body makes identifying a bleeding exposed vessel impossible. In the case of gastric variceal bleeding, erythromycin or somatostatin has a beneficial effect on upper gastrointestinal bleeding by inducing rapid gastric emptying^[9,10]. However, even with such drugs, obtaining a clear endoscopic view to rapidly identify the bleeding exposed vessels in an emergency situation is still difficult. This case series demonstrates a method for shortening the hemostasis time and reducing stress for both patients and endoscopists.

CASE REPORT

A 55-year-old woman who was transported to our emergency room in shock after feeling nauseous and experiencing sudden massive hematemesis in June 2012. Although her family informed us that a blood test 6 mo prior had shown no signs of anemia, a blood examination revealed severe anemia (hemoglobin, 5.5 g/dL). Emergency endoscopic hemostasis and a blood transfusion were performed. Large amounts of blood and food residue were observed in the stomach. Because the patient appeared drowsy, an emergency endoscopy to detect the bleeding vessel was immediately performed in the areas that could be observed in the left lateral decubitus position. The bleeding vessel could not be identified in the duodenum or gastric antrum. As the amount of fresh blood increased, obtaining a clear endoscopic view to identify the bleeding vessel gradually increased in difficulty (Figure 1). Assuming that the bleeding vessel was located in either the upper gastric body or gastric fornix, which was not visible due to the blood clot, we immediately switched from the conventional observation position to the right lateral decubitus position using newly developed inverted overtube (Figure 2). The inverted overtube was approved by the Institutional Ethics Committee of Kagawa University Hospital, Kagawa, Japan. Additionally, the inverted overtube was approved by the

Japanese Pharmaceutical Law.

The blood clot and food residue in the gastric corpus and fornix were immediately dislodged to the right into the duodenum by gravity (Figure 3), allowing for the visual identification of an exposed blood vessel in the fornix (Figure 4). Up to this point, no spurting bleeding had been observed due to the decreased blood pressure. However, massive spurting bleeding occurred immediately after the exposed blood vessel was pinched with hemostatic forceps. The pinched vessel was then completely cauterized by coagulation mode to achieve hemostasis. No bleeding was observed thereafter.

DISCUSSION

Despite the dramatic progress made in endoscopic hemostatic techniques^[1,4], hematemesis from an esophageal or gastric varix or Dieulafoy's ulcer can lead to serious consequences if hemostasis is not achieved^[8]. Achieving accurate and reliable hemostasis is difficult without a clear view of the bleeding vessel. Although hemostasis *via* a laparotomy can be performed as a last resort, surgery in a patient with a poor systemic condition carries a high risk. Thus, emergency endoscopic hemostasis remains the first-line treatment of choice^[2,6].

During endoscopic hemostatic procedures performed in the emergency room, where pretreatment is not performed, the presence of blood clots and food residue makes obtaining a clear view of the bleeding vessel difficult^[5]. The removal of blood clots from the stomach has conventionally been achieved by gastric suction with a gastric tube and/or by manual removal of the clot with grasping forceps. However, the use of gastric suction and/or grasping forceps currently requires a great deal of time. Thus, endoscopists have adopted a procedure in which the patient's posture is rotated to the right lateral decubitus position to dislodge the blood clots and enable the identification of the bleeding vessel. Because most endoscopists perform conventional endoscopic examinations and treatment procedures with the patient lying in the left lateral decubitus position, they find that performing accurate hemostatic procedures from the opposite

side, with the patient lying in the right lateral decubitus position, to be difficult. Thus, the use of the inverted overtube is the best method to help endoscopists perform an emergency endoscopy with less stress because they are in their conventional standing position relative to patients who are rotated to the right lateral decubitus position, without changing the positions of the endoscopy unit and light source. This technique is the most effective way to dislodge blood clots and food residue by gravity in these patients. The present technique dramatically improved the clarity of the endoscopic views and enabled the endoscopist to perform the hemostatic procedures from the conventional standing position while freely and easily changing the patient's position. This set-up significantly shortened the procedure time. The inverted overtube, with its very simple structure, helped the endoscopist acquire a clear view in patients who were laid in the right lateral position. This clear view contributed to the rapid identification of the bleeding vessels and the subsequent rapid achievement of hemostasis.

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Meckel's diverticulum bleeding diagnosed with magnetic resonance enterography: A case report

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Abstract

Although the introduction of double-balloon enteroscopy has greatly improved the diagnostic rate, definite diagnosis of Meckel's diverticulum far from the ileocecal valve is still impossible in most cases. We explored the role of magnetic resonance (MR) enterography in detecting bleeding from Meckel's diverticulum that can not be confirmed *via* double-balloon enteroscopy. This study describes a case of male patient with bleeding from Meckel's diverticulum diagnosed with MR enterography of the small intestine. No bleeding lesion was found *via* colonoscopy, anal enteroscopy, or oral colonoscopy. MR enterography of the small intestine revealed an occupying lesion of 3.0 cm in the lower segment of the ileum. The patient was transferred to the Department of Abdominal Surgery of our hospital for surgical treatment. During surgery, a mass of 3 cm × 2 cm was found 150 cm from the ileocecal valve, in conjunction with congestion and edema of the corresponding mesangium. Intraoperative diagnosis was small bowel diverticulum with bleeding. The patient underwent partial resection of the small intestine. Postopera-

tive pathology showed Meckel's diverticulum containing pancreatic tissues. He was cured and discharged 7 d after operation. We conclude that MR enterography of the small intestine has greatly improved the diagnosis rate of Meckel's diverticulum, particularly in those patients with the disease which can not be confirmed *via* double-balloon enteroscopy.

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Key words: Meckel's diverticulum; Double-balloon enteroscopy; Magnetic resonance enterography

Core tip: This study describes a case of male patient with bleeding from Meckel's diverticulum diagnosed with magnetic resonance (MR) enterography of the small intestine. MR enterography of the small intestine has greatly improved the diagnosis rate of Meckel's diverticulum, particularly in those patients with the disease which can not be confirmed *via* double-balloon enteroscopy.

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INTRODUCTION

Gastrointestinal bleeding of unknown cause is mostly a result of lesions located in the small intestine, for which the diagnosis and treatment can be challenging^[1,2]. Meckel's diverticulum is a common congenital malformation of the small intestine, with gastrointestinal bleeding being the most common symptom. Preoperative diagnosis of this condition is difficult. Although the introduction of double-balloon enteroscopy has greatly improved the diagnostic rate, definite

diagnosis of Meckel's diverticulum far from the ileocecal valve is still impossible in most cases. This study describes a case of bleeding from Meckel's diverticulum diagnosed with magnetic resonance (MR) enterography of the small intestine in our department.

CASE REPORT

A 25-year-old man was first admitted to the hospital for lower abdominal pain on March 25, 2011. No abnormality was found through enhanced computed tomography (CT) scan of the whole abdomen on admission. No positive finding was reported on anal enteroscopy. Barium meal examination of the entire digestive tract showed multiple fluid levels in segments 4 and 5 of the small intestine. His condition improved with anti-inflammatory therapy, intestinal spasmolysis, and nutritional support. He was then discharged on April 3, 2011. After that, he was diagnosed as having "appendicitis" and underwent appendectomy in a local hospital. Encapsulated fluid was present in the right lower quadrant, which was improved after puncture and drainage. Later, he was again admitted to our hospital on February 17, 2012 due to melena for two days after recurrent abdominal pain for nearly a year. Examinations on admission showed a positive fecal occult blood test, stool red blood cell (RBC) +++/HP, C-reactive protein: 30.9 mg/L; abdominal CT revealed a small amount of fluid in the pelvic cavity. Routine examination of the ascites fluid collected by ultrasound-guided puncture showed red fluid, specific gravity > 1.018, positive Rivalta test, RBC ++++/HP, and nucleated cell count of 1.8×10^9 /L. Ascites smears revealed no acid-fast bacilli. Bacterial culture indicated *Streptococcus sanguis*. No bleeding lesion was found *via* colonoscopy, anal enteroscopy, or oral colonoscopy. His condition improved after hemostatic, anti-infective and symptomatic treatment, and he was discharged on March 4, 2012. His condition remained stable after discharge until three days prior to admission when he experienced lower abdominal pain after eating spicy food, and had dark red bloody stools, 2-3 times a day. He was admitted for a third time on April 24, 2012. Blood tests upon admission showed hemoglobin (HGB) of 96 g/L; MR enterography of the small intestine showed an occupying lesion of 3.0 cm in the lower segment of the ileum. He was transferred to the Department of Abdominal Surgery for surgical treatment. During surgery, a mass of 3 cm \times 2 cm was found 150 cm from the ileocecal valve, in conjunction with congestion and edema of the corresponding mesangium. Intraoperative diagnosis was small bowel diverticulum with bleeding. Postoperative pathology showed Meckel's diverticulum containing pancreatic tissues. The patient underwent partial resection of the small intestine, and was cured and discharged 7 d after operation (Figure 1).

DISCUSSION

Meckel's diverticulum is the most common congenital

malformation of the small intestine, with an incidence of 1% to 4%^[3]. It usually occurs in the ileum within 100 cm of the ileocecal valve^[4], and is a true diverticulum consisting of all layers of the bowel wall. Meckel's diverticulum harbors various ectopic tissues, with gastric mucosa being the most common (about 50%), followed by pancreatic tissues (about 5%)^[5]. The majority of people afflicted with Meckel's diverticulum are asymptomatic, and 4% to 6% of the patients are detected due to symptoms of related complications^[6-10]. Gastrointestinal bleeding is the most common symptom of Meckel's diverticulum^[11], followed by intussusception, and intestinal obstruction.

Such bleeding occurs from larger vessels invaded by erosion and ulceration of normal intestinal mucosa in the diverticulum, as a result of the secretion of gastric acid or alkaline pancreatic fluid from the ectopic mucosa^[12-15]. Consisting mainly of fundic glands, where parietal cells secrete gastric acid and chief cells secrete pepsin, the ectopic gastric mucosa is closely related to gastrointestinal bleeding, as the acid does not only directly cause corrosion of the diverticulum and intestinal mucosa, but also promotes the conversion of pepsinogen secreted by ectopic chief cells into pepsin, which in turn induces tissue digestion and leads to ulceration and bleeding.

Preoperative diagnosis is difficult when Meckel's diverticulum is complicated by small intestinal bleeding^[15]. Gastroscopy, colonoscopy, barium meal and ultrasound lack specificity, but they can be used to exclude lower gastrointestinal bleeding from other sites. Due to its high-density resolution, CT scanning can detect and accurately locate stones of low density in Meckel's diverticulum^[16,17]. CT enterography of the small intestine has been proven to be more useful than conventional CT in patients suspected of having small bowel lesions, as it not only maximizes the visibility of the mucosa and intestine structure with the aid of contrast agents, but also detects parenteral abnormalities^[18-22]. MR enterography is a radiation-free technique developed based on CT enterography, which allows evaluation of intestinal functionalities using MR fluoroscopy.

MR enterography is a novel technique that combines the advantages of both the conventional enterography and the morphological imaging of magnetic resonance imaging, thus making it an examination for both the functions and the morphologies of small intestine. Before MR enterography is done, the bowel must be completely cleansed and the intestinal canal distended. Therefore, it is critically important to select a contrast agent that can thoroughly distend the intestinal canal and clearly demonstrate the enteric cavity and intestinal wall, and does not produce artifacts or pose a hazard to human health. Although the double-contrast imaging of the small intestine is helpful for observing the early mucosal changes, it cannot visualize the lesions around the intestinal wall or in the mesentery. In contrast, MR enterography, with three-dimensional imaging capabilities and excellent soft-tissue contrast, can be used to observe the mucosa and analyze the changes around the intestinal canal^[23-25].

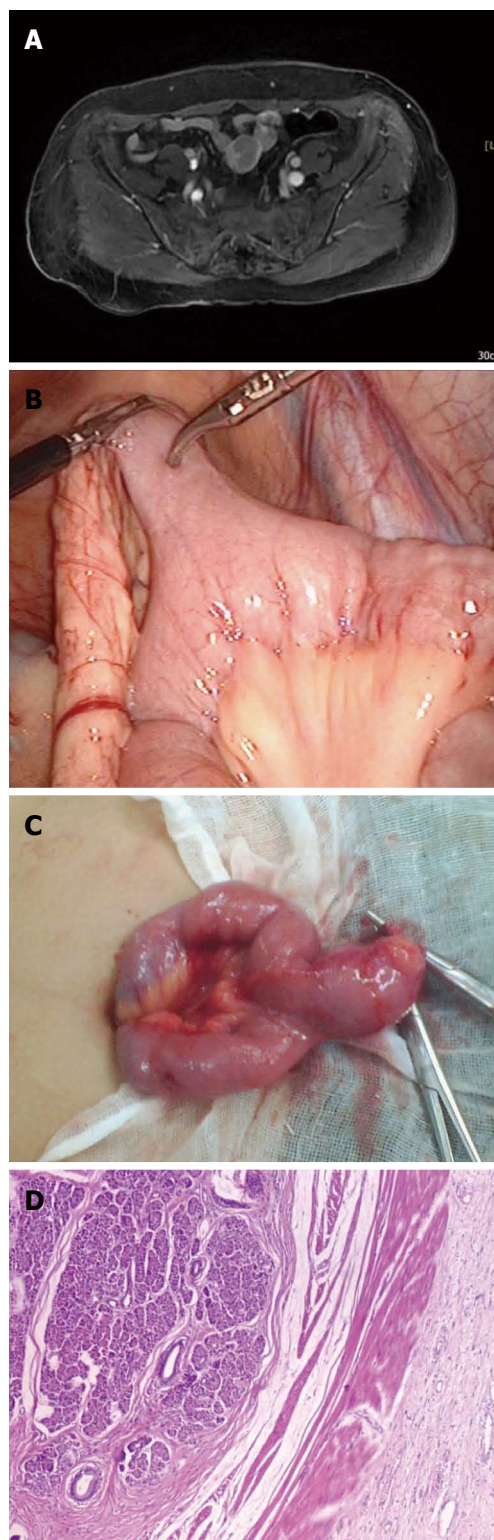


Figure 1 The patient underwent partial resection of the small intestine, and was cured and discharged 7 d after operation. A: Magnetic resonance enterography of the small intestine. An occupying lesion of 3.0 cm was found in the lower segment of the ileum; B: A mass of 3 cm × 2 cm was found 150 cm from the ileocecal valve during surgery; C: Removed lesion during surgery; D: Postoperative pathology showed Meckel's diverticulum containing pancreatic tissues.

The introduction of capsule endoscopy and double-balloon enteroscopy has made observation of the entire

small bowel mucosa possible, and the latter technique also enables biopsy for pathological diagnosis and endoscopic treatment^[26-28]. Compared with traditional push enteroscopy (90-150 cm) and retrograde terminal ileoscopy (50-80 cm), double-balloon enteroscopy has a longer average depth (240-360 cm orally and 102-140 cm anally); compared with capsule endoscopy, it enables more pathological and endoscopic applications, such as hemostasis, polypectomy, dilatation treatment, and removal of foreign substances^[29,30]. Heine *et al.*^[31] demonstrated in their study that double-balloon enteroscopy has a diagnosis rate of 63% for obscure gastrointestinal bleeding, and complete small bowel examination is achievable for about 1/3 of these cases.

Although double-balloon enteroscopy has a high diagnosis rate for unexplained gastrointestinal bleeding, since a complete small bowel examination can be done in only a few patients, the causes of bleeding still remain unknown in many other patients before surgery. In the present case, the lesion was up to 150 cm from the ileocecal valve, which was undetectable by anal colonoscopy with an average insertion depth of about 102-140 cm, therefore, no definite diagnosis could be made. In view of this, MR enterography of the small intestine was performed and an occupying lesion of 3.0 cm was found in the lower segment of the ileum, with clear boundaries, showing mixed signals. He was transferred to the Department of Abdominal Surgery for surgical treatment. During surgery, a mass of 3 cm × 2 cm was found 150 cm from the ileocecal valve, in conjunction with congestion and edema of the corresponding mesangium. Intraoperative diagnosis was small bowel diverticulum with bleeding. Postoperative pathology showed Meckel's diverticulum containing pancreatic tissues. The patient underwent partial resection of the small intestine, and was cured and discharged 7 d after operation.

In summary, Meckel's diverticulum is the most common congenital malformation of the small intestine that is easily complicated by small intestinal bleeding, for which preoperative diagnosis is difficult. The introduction of double-balloon enteroscopy has greatly improved the diagnostic rate, but definite diagnosis is still challenging in some patients, especially when the lesion is far from the ileocecal valve. MR enterography is a radiation-free technique developed based on CT enterography, which maximizes the visibility of the mucosa and intestine structure with the aid of contrast agents. MR enterography of the small intestine has greatly improved the diagnosis rate of Meckel's diverticulum, particularly in those patients with the disease which can not be confirmed *via* double-balloon enteroscopy.

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The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

- 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 Pixudiarrrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325

DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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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**, Schorfeide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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