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REVIEW

Autoimmune liver diseases in systemic rheumatic diseases

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Abstract

Systemic rheumatic diseases (SRDs) are chronic, inflammatory, autoimmune disorders with the presence of autoantibodies that may affect any organ or system. Liver dysfunction in SRDs can be associated with prescribed drugs, viral hepatitis, alternative hepatic comorbidities and coexisting autoimmune liver diseases (AILDs), requiring an exclusion of secondary conditions before considering liver involvement. The patterns of overlap diseases depend predominantly on genetic determinants with common susceptible loci widely distributing in both disorders. In AILDs, it is important to identify the overlapping SRDs at an early stage since such a coexistence may influence the disease course and prognosis. Commonly co-occurring SRDs in AILDs are Sjögren syndrome (SS), rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) in autoimmune hepatitis (AIH), and SS, RA or systemic sclerosis in primary biliary cholangitis. Owing to different disease complications and therapies, it is imperative to differentiate between SLE liver involvement and SLE-AIH overlap disease. Therapeutic options can be personalized to control coexisting conditions of liver autoimmunity and rheumatic manifestations in AILD-SRD overlap diseases. The collaboration between hepatologists and rheumatologists can lead to significant advances in managing such a complex scenario. In this review, we provide a comprehensive overview on coexisting AILDs in different SRDs and the therapeutic approach in managing these overlap diseases.

Key Words: Autoimmune liver disease; Systemic rheumatic disease; Overlap disease; Liver function test; Drug-induced liver injury; Viral hepatitis

Core Tip: Liver dysfunction in systemic rheumatic diseases (SRDs) can be associated with prescribed drugs, viral hepatitis, alternative hepatic comorbidities and coexisting autoimmune liver diseases (AILDs), requiring an exclusion of secondary conditions before considering liver involvement. In AILDs, it is imperative to identify the overlapping SRDs at an early stage since such a coexistence may influence the disease course and prognosis. Commonly co-occurring SRDs in AILDs are Sjögren syndrome (SS), rheumatoid arthritis (RA) or systemic lupus erythematosus in autoimmune hepatitis, and SS, RA or systemic sclerosis in primary biliary cholangitis. Therapeutic options can be personalized to control coexisting conditions of liver autoimmunity and rheumatic manifestations in AILD-SRD overlap diseases.

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INTRODUCTION

Systemic rheumatic diseases (SRDs) are chronic, inflammatory, autoimmune disorders with the presence of autoantibodies that may affect any organ or system; they include systemic lupus erythematosus (SLE), Sjögren syndrome (SS), systemic sclerosis (SSc), rheumatoid arthritis (RA), idiopathic inflammatory myopathies (IIM), mixed connective tissue disease (MCTD), systemic vasculitis (SV), *etc.* [1]. Although SRDs can have liver involvement, most patients only have abnormal liver enzymes without significant changes in histopathology[2,3]. Hepatic dysfunction can be a secondary phenomenon, associated with prescribed drugs, viral hepatitis (VH), alternative liver comorbidities, and coexisting autoimmune liver diseases (AILDs).

The major cause of abnormal liver function test (LFT) in patients with SRDs is associated with medications, *i.e.* drug-induced liver injury (DILI)[3]. Given that a variety of medications are used in the management of SRDs, it is frequently encountered in clinical practice. High occurrences of DILI in SRDs are due to the chronic or high-dose prescription of medications, the existence of susceptible factors that makes patients prone to hepatotoxicity, and/or the use of herbal or ayurvedic compounds[2,3]. Elevated liver enzymes with predominant cholestatic or hepatocellular damage pattern can be observed in SRDs treated with non-steroidal anti-inflammatory drugs (NSAIDs), synthetic disease modifying anti-rheumatic drugs (SDMARDs), corticosteroids (CS), immunosuppressants, biologic agents or oral small molecules[2]. Most medications only cause a mild elevation in liver enzymes, which reverses with drug cessation. On rare occasions, severely irreversible hepatic damage may occur and progress into chronic liver disease or fulminant hepatic failure. Despite the relative safety with a low-dose prescription, methotrexate, a SDMARD frequently used in SRD-related arthritis, has been reported to cause acute liver dysfunction with confounding factors like concomitant NSAIDs use, and progressive liver fibrosis and cirrhosis can occur when used chronically[4]. It usually occurs after a prolong use for no less than 2 years and with a total accumulated dose of 1.5 g[5]. Notably, there is a risk of hepatitis B virus (HBV) reactivation depending on the dose and duration of CS use and the status of hepatitis B surface antigen and hepatitis B core antibody in SRDs[6]. Furthermore, acute or progressing liver dysfunction can be related to coexisting VH, requiring screen tests for HBV and hepatitis C virus (HCV) infection to provide early antiviral treatment and avoid reactivating or worsening VH after immunosuppressive therapy[3]. Table 1 summaries the hepatic abnormalities associated with the common medications used in SRDs[2,7,8]. Although immune checkpoint inhibitors have altered the therapeutic paradigm in oncological patients, there is undesirable off-target autoimmune reaction causing adverse effects like musculoskeletal manifestations and immune hepatitis, a pan-lobular active hepatitis resembling AIH[9].

Although the liver is the largest lymphoid organ involved in the immune response against invading pathogens and in the maintenance of tolerance to self-molecules, it can also be a target of autoimmune diseases[10]. AILDs are attributed to a complex interplay of socioeconomic, environmental and genetic factors, all of which may participate in their pathogenesis[11]. Most common AILDs are autoimmune hepatitis (AIH), primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), which may occur individually or in combination[12]. These disorders are characterized by hepatic lymphocyte infiltration, elevated liver enzymes, generation of autoantibodies, and associated HLA loci. Coexisting extra-hepatic autoimmune diseases such as SRDs, have been well described in the literature[13]. AIH often goes into disease remission with first-line therapy, including CS alone or plus AZA[14]. PBC has a normal life expectancy if treated early with ursodeoxycholic acid (UDCA) in responsive patients, while no effective therapy has been found to alter the natural course of PSC, except liver transplantation (LT) [15,16]. Some patients with AILDs may eventually progress into end-stage liver disease requiring LT, and with an increased risk of recurrent activities and acute or chronic rejection[17,18]. Currently, AILD research has focused on obtaining a better understanding of the pathogenetic process for identification

Table 1 Hepatic abnormalities associated with common medications used in systemic rheumatic diseases

Medications	Hepatic abnormalities	[2]Likelihood score category in DILI
NSAIDs	LEE, cholestasis, acute liver failure, VBDS	A for diclofenac, ibuprofen, sulindac
Glucocorticoids	LEE, NAFLD, acute liver failure, HBV reactivation	A in high dosages
Immunosuppressive agents		
Azathioprine	LEE, cholestasis, NRH, peliosis hepatis, VOD	A
Mycophenolate mofetil	LEE	D
Cyclophosphamide	LEE, VOD	B
Cyclosporine	LEE, cholelithiasis	C
Tacrolimus	LEE	C
Conventional SDMARDs		
Hydroxychloroquine	LEE	C
Leflunomide	LEE, acute liver failure, HBV reactivation	B
Methotrexate	LEE, NAFLD, HBV reactivation, fibrosis, cirrhosis	A
Penicillamine	LEE, cholestasis	A
Sulfasalazine	LEE, cholestasis, DRESS	A
Biologic/targeted SDMARDs		
Abatacept	LEE, HBV reactivation	C
Anakinra	LEE	C
Apremilast	Unlikely liver injury	E
Belimumab	Unlikely liver injury	E
Mepolizumab	Unlikely liver injury	E
Rituximab	LEE, HBV reactivation	A
TNF blockers[1]	LEE, cholestasis, HBV reactivation, AIH	A for infliximab
Tocilizumab	LEE, HBV reactivation	C
Tofacitinib	Suspected liver injury, potential HBV reactivation	E′
Ustekinumab	Suspected liver injury, possible HBV reactivation	E′

[1]TNF blockers including adalimumab, certolizumab, etanercept, golimumab and infliximab.
[2]Categorization of Likelihood Score in drug-induced liver injury. A: Definite; B: Highly likely; C: Probable, D: Possible, E: Unlikely; E′: Suspected.
NSAIDs: Non-steroidal anti-inflammatory drugs; HBV: Hepatitis virus B; DILI: Drug-induced liver injury; DRESS: Drug rash with eosinophilia and systemic symptoms; LEE: Liver enzyme elevation; NAFLD: Nonalcoholic fatty liver disease; NRH: Nodular regenerative hyperplasia; SDMARDs: Synthetic disease-modifying antirheumatic drugs; SRDs: Systemic rheumatic disease; VBDS: Vanishing bile duct syndrome; VOD: Veno-occlusive disease.

of new therapeutic targets to reduce morbidity and improve survival[15]. Table 2 demonstrates the demographic, clinical, laboratory, pathological, therapeutic and prognostic characteristics of three common AILDs[11-20].

In AILDs, it is imperative to identify the co-occurring SRDs at an early stage by using autoantibody screening, since such a coexistence may influence their natural course and disease prognosis[21]. The patterns of overlap diseases depend predominantly on genetic determinants, with common susceptible loci widely distributing in both disorders[20]. The similar epidemiological links between AILDs and SRDs are further reflected in their shared pathogenesis, best exemplified by the concept of autoimmune epithelitis, *i.e.*, concomitant PBC and SS[22,23]. Furthermore, AILDs and SRDs have common serologic profiles with the presence of particular autoantibodies and hyper-gammaglobulinemia[21,24]. Progressive liver damage can be identified in overlap diseases despite rare complications with liver cirrhosis and hepatic failure[3]. Table 3 shows the reported prevalence of coexisting AILDs in different SRDs.

The therapeutic strategies in AILDs and SRDs are also overlapping, with CS as first-line treatment in most cases, followed by administration of immunosuppressants, and potential application of targeted therapy[21]. Nevertheless, therapeutic options can be personalized to control coexisting conditions of liver autoimmunity and rheumatic manifestations[24]. A collaboration between hepatologists and

Table 2 Demographic, clinical, laboratory, pathological, therapeutic and prognostic profiles in three common autoimmune liver diseases

Category	AIH	PBC	PSC
Demographic			
Sex	Predominant F, 4:1	Predominant F, 10:1	Predominant M, 2:1
Age	Any, median 45 yr	Common above 40 yr	Any, typical 30-50 yr
Prevalence	Rare, 4-25 per 100000	Rare, 2-40 per 100000	Rare, 4-16 per 100000
Laboratory			
Abnormal LFT	Majorly AST/ALT	Majorly ALP/GGT	Majorly ALP/GGT
Serum Ig	Elevated IgG	Elevated IgM	Elevated IgG, IgM
Autoantibody	I: ANA, ASMA; II: anti-LKM, -LC	ANA, AMA	ANCA
HLA-DR	DR3, DR4	DR8	DR52
Liver biopsy			
Interface HA	Typical finding	Occasional	Occasional
Portal infiltrate	Lymphoplasmacytic	Lymphocytic	Lymphocytic
Bile duct lesion	Occasional	Florid duct lesion	Obliterative duct
Granuloma	Rare	Typical finding	Rare
Diagnosis	AIH score for definite diagnosis	AMA, liver biopsy, Cholestatic LFT	Cholangiography, Cholestatic LFT
Coexistent SRD			
SLE	0.7%-2.8%	1.3%-3.7%	1.70%
SS	1.4%-35%	3.5%-38%	CR
SSc	0.80%	2.3%-12%	CR
RA	1.6%-5.4%	1.8%-13%	1.2%-3.4%
IIM	CR	0.6%-3.1%	CR
MCTD	CR	0.60%	NA
SV	1.60%	2.20%	CR
Sarcoidosis	0.60%	2.70%	0.80%
First-line Tx	CS or CS plus AZA	UDCA	No effective therapy
Prognosis	Generally responsive to IS, poor prognosis if untreated	Excellent prognosis if responsive to UDCA	Median survival without LT 12-16 yr after diagnosis

AIH: Autoimmune hepatitis; ALP: Alkaline phosphatase ALT: Alanine aminotransferase; AMA: Antimitochondrial autoantibody; ANA: Anti-nuclear antibody; ANCA: Perinuclear antineutrophil cytoplasmic antibody; APS: Antiphospholipid syndrome; ASMA: Anti-smooth muscle antibody; AST: Aspartate aminotransferase; AZA: Azathioprine; CR: Case report; CS: Corticosteroids; EHAID: Extra-hepatic autoimmune disease; HA: Hepatitis; Ig: Immunoglobulin; IIM: Idiopathic inflammatory myopathies; IS: Immunosuppressants; GGT: Gamma-glutamyl transferase; LC: Liver cytosol; LKM: Liver kidney microsomal; LFT: Liver function test; LT: Liver transplantation; MCTD: Mixed connective tissue disease; NA: Not available; PBC: Primary biliary cholangitis; PSC: Primary sclerosing cholangitis; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; SS: Sjögren syndrome; SSc: Systemic sclerosis; Tx: Treatment; UDCA: Ursodeoxycholic acid.

rheumatologists in clinical practice can lead to significant advances in managing such a complex scenario. Herein, we provide a comprehensive overview on coexisting AILDs in different SRDs and the therapeutic approach in managing these overlap diseases.

SLE

SLE is a less common SRD, occurring mostly in women of childbearing age and having heterogenous clinical manifestations affecting any organ or system as well as presenting antinuclear antibody (ANA) and a variety of autoantibodies[25]. The liver is generally not a target organ in SLE and hepatic

Table 3 Reported prevalence of concomitant autoimmune liver diseases in different systemic rheumatic diseases

Category	AIH	PBC	PSC	AIH/PBC OS
SLE	1.6%-15%	2.2%-7.5%	CR	CR
SS	0.4%-4.4%	3.4%-8.9%	CR	CR
SSc	CR	0.8%-3.3%	CR	CR
RA	1.3%	3.8%-6.3%	CR	CR
IIM	CR	0.7%	CR	CR
MCTD	1.6%	CR	NA	NA

AIH: Autoimmune hepatitis; APS: Antiphospholipid syndrome; CR: Case report; IIM: Idiopathic inflammatory myopathies; NA: Not available; OS: Overlap syndrome; PBC: Primary biliary cholangitis; PSC: Primary sclerosing cholangitis; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; SS: Sjögren syndrome; SSc: Systemic sclerosis.

involvement is not included in the classification or diagnostic criteria. Abnormal LFT is common in SLE, usually with subtle changes, in up to 60% of cases during the disease course, while elevated liver enzymes occur during disease flares in less than 20% of patients[3,26,27]. Hepatic dysfunction in SLE can be classified into primary form due to disease itself or secondary form including DILI, VH, vascular disorders, alternative liver comorbidities and coexisting AILDs[28]. Before considering the liver involvement in SLE, it is necessary to exclude other secondary conditions.

Lupus hepatitis (LH) is reactive liver damage caused by immune-complex deposition, in contrast to lupoid hepatitis, a term used in the 1950s to define what was later known as AIH[29,30]. This manifestation is usually synchronous with disease activity and affects less than 10% of patients[31-33]. It is characterized by asymptomatic transaminasemia with the presence of anti-ribosomal P antibody (commonly known as ARPA) and non-specific histopathological changes. Although CS may help to improve impaired LFT, there is a risk of flare up upon cessation of its use[34]. Figure 1 shows the liver biopsy finding from a patient with LH demonstrating non-specific histopathological changes.

The main cause of liver dysfunction in SLE was salicylate toxicity in the 1950s[35]. Later on, owing to a rare prescription, another common finding of liver biopsy was steatohepatitis, an alternative liver comorbidity. Nowadays, the known risk factors for development of non-alcoholic fatty liver disease (NAFLD) include obesity, physical inactivity and sedentary lifestyle[36], which are also shared by SLE. Furthermore, patients with SLE have been shown to have higher incidences of metabolic syndrome and insulin resistance[37], especially with the use of CS[38]. Increased frequencies of NAFLD have been found in liver biopsy specimens from patients with SLE[39].

The presence of antiphospholipid antibody (aPL) in SLE underlies an increased probability of thrombophilia, leading to antiphospholipid syndrome (APS) with vascular thrombosis[40]. APS can affect the hepatic circulation, causing hepatic arterial thrombosis, portal vein thrombosis and Budd-Chiari syndrome (BCS) as well as the rarely-observed liver infarction and hepatic veno-occlusive disease [40-42]. Notably, BCS resulting from the obstruction of hepatic venous outflow[43] can be an initial manifestation of patients with SLE-associated APS[44]. In particular, aPL has been reported to be involved in the pathogenesis of hepatic nodular regenerative hyperplasia (referred to herein as NRH), small-nodule transformation of hyperplastic hepatocytes with a later development of non-cirrhotic portal hypertension[3,45]. Although higher frequencies of aPL could be detected in AILDs, there was no definite clinical or histological correlation with their presence in such patients[3,46].

Autoimmune gastrointestinal diseases have been linked to SLE with shared pathogenic mechanisms responsible for the development of both disorders[47]. Although AIH and PBC are rare AILDs, the coexistence with either of these diseases is not uncommon among SLE patients with liver enzyme abnormalities, suggesting a causal relationship between their overlap[28,48,49]. Since SLE-PSC overlap disease rarely occurs (but has been described in case reports[28,48]), it remains to be ascertained whether they are casual associations. A review on individual AILD coexisting with SLE is depicted as follows.

AIH is a rare AILD characterized by interface hepatitis as the most specific histological change, and the presence of autoantibodies including anti-liver kidney microsomal-1 (LKM-1)/liver cytosol-1 (LC-1) in type II, a rare subgroup affecting female pediatric patients, and ANA/anti-smooth muscle antibody (ASMA) in type I[50]. Clinical manifestations vary from asymptomatic to nonspecific symptoms of varying severity, including fatigue, malaise, nausea, anorexia and abdominal pain. The criteria established by the International Autoimmune Hepatitis Group (commonly known as the IAHG) are usually used for the diagnosis of AIH[51]. Due to different disease complications and therapeutic regimens between AIH and LH, it is imperative to differentiate between two disease entities[28,34]. AIH may lead to end stage liver disease, and its immunosuppressive therapy needs to be continued for at least 2 years of hepatic biochemical remission before attempting withdrawal[50]. Liver biopsy is highly

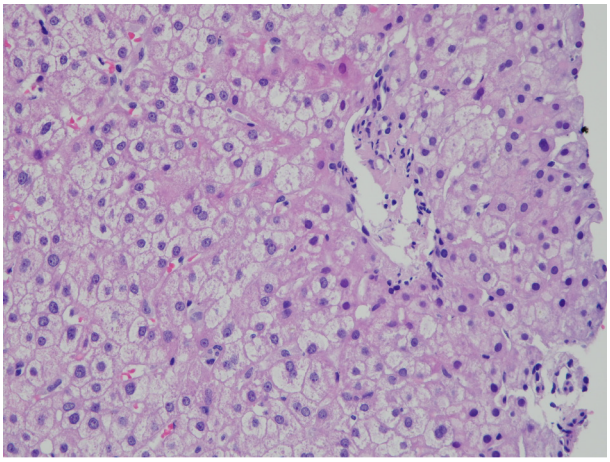

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Figure 1 Liver biopsied tissues from a patient with systemic lupus erythematosus liver involvement (lupus hepatitis). The portal area with minimal non-specific lymphocytic infiltration is shown. Hematoxylin and eosin staining, 400 × magnification.

recommended for their distinguishment[28,34]. LH usually demonstrates lobular infiltrates or occasionally mild periportal infiltrates, whereas AIH is characterized by portal mononuclear infiltrates invading nearby lobules to induce interface hepatitis and form hepatocyte rosettes, followed by confluent lytic necrosis and finally cirrhosis[30].

SLE-AIH overlap disease is defined by fulfilling American College of Rheumatology (commonly known as the ACR) criteria for the classification of SLE in patients who also meet IAIHG criteria for the diagnosis of AIH[34,51,52]. The prevalence of AIH in SLE ranges from 1.6% to 15%, lower in general cohorts and higher in patients with abnormal LFT[39,53-58]. Immunosuppressive treatment for AIH is also effective for SLE, and has been demonstrated to successfully apply to their overlap cases[28]. Most cases with coexisting SLE and AIH responded well to CS or plus immunosuppressants[48]. The long-term outcome for SLE-AIH overlap disease has been observed to be better than AIH alone[34]. Nevertheless, there are sporadic cases of acute liver failure or end-stage liver disease requiring LT[59, 60].

PBC is the most common AILD affecting women predominantly. It is characterized by destructive lymphocytic cholangitis involving small bile ducts, and leading to progressive ductopenia, hepatic cholestasis and biliary fibrosis[61]. Clinical manifestations vary from asymptomatic to non-specific symptoms with jaundice and pruritus. According to the guidance from American Association for the Study of Liver Diseases (commonly known as the AASLD), the diagnosis of PBC is established when two of three items are met, including biochemical cholestasis based on alkaline phosphatase (ALP) elevation, presence of antimitochondrial autoantibody (AMA), and histological evidences of nonsuppurative destructive cholangitis and interlobular bile ducts destruction[62]. The nomenclature of PBC has already shifted from cirrhosis to cholangitis, reflecting the dramatically improved prognosis upon first-line UDCA therapy without the development of cirrhosis[63,64].

SLE-PBC overlap is defined by fulfilling the diagnostic criteria for both diseases[34,52,62]. SLE usually affects younger females of childbearing age, whereas PBC is more common in middle-aged women. By genome-wide studies, both diseases have been reported to share the IRF5-TNPO3 gene-spanning haplotype loci[65]. The prevalence of PBC in SLE patients with liver dysfunction ranges from 2.2% to 7.5%, usually lower than that of AIH[39,53-55,57]. In a review of SLE overlapping with PBC, 69% were diagnosed first by PBC, 24% had coexisting SS, and 2 deaths were due to PBC-related hepatic failure[66]. For patients with concomitant SLE and PBC, regardless of the SLE treatment, UDCA is effective first-line therapy for PBC[49].

The diagnosis of PBC-AIH overlap is established with coexisting features of both diseases[67]. Two commonly used criteria for the diagnosis of PBC-AIH overlap syndrome are the IAIHG and Paris criteria[51,68]. Patients with overlapping PBC and AIH have been described to exhibit significantly higher rates of LC, portal hypertension and mortality as compared with those with AIH or PBC alone [69]. PBC with features of AIH should be considered for immunosuppressive therapy[49], while PBC-AIH overlap disease can benefit from combination treatment with UDCA and CS or plus AZA[69]. There is a rare association between SLE and PBC-AIH overlap disease[70]. In a large case series with 71 overlap patients, EHAIDs were identified in 31 (44%), while only 2 (3%) had concurrent SLE[71].

In contrast to western countries, AIH had been considered a rare etiology in the Asia-Pacific region, where VH is a major diagnosis in patients with chronic liver diseases[72]. A very low prevalence of AIH was found in Taiwan in earlier years, raising concerns about under-recognition in an area with a high prevalence of HBV infection and associated liver cirrhosis and hepatocellular carcinoma complications [73], where clinicians would have been more familiar with VH and might have tended to overlook AIH

[74]. Recent findings, however, have shown increasing annual incidences of AIH, indicating improved recognition of AIH in this region[72].

The clinical, laboratory, therapeutic and outcome profiles in 3 patients with SLE-AIH overlap disease diagnosed by ourselves are shown in Table 4. All met IAIHG diagnostic criteria for AIH[51], and case 2 also fulfilled AASLD diagnostic criteria for PBC[62]. Despite CS plus AZA therapy, case 1 progressed into advanced LC, and received LDLT with stabilized LFTs and low SLE activity. Case 2 had the initial diagnosis of AIH with transaminasemia, followed by the development of hepatic cholestasis and sicca symptoms, and finally full-blown manifestations of SLE. Under the diagnosis of coexistent AIH, PBC, SLE and SS, in addition to CS/AZA and UDCA, the patient received B-cell depleting therapy with anti-CD20 monoclonal antibody, with low-dose CS for maintenance, resulting in normalized LFT and low SLE activity. Figure 2 demonstrates histopathological findings in liver biopsy specimens from cases 1 and 2.

PSC is a rare cholestatic AILD characterized by persistent, progressive inflammation, fibrosis and stricture of the intrahepatic and extrahepatic bile ducts, leading to cirrhosis[75]. About half of the patients are asymptomatic. The diagnosis is made by cholestasis with ALP elevation and imaging of bile duct strictures, excluding secondary causes. Liver biopsy is indicated only when suspecting overlapping with other AILDs or small-duct PSC, a variant with normal cholangiogram. UDCA is the subject of debate with conflicting data to support its use in PSC[49], and end-stage liver disease requiring LT may develop in affected patients.

Although the association of SLE with PSC is considered to be extremely unusual[34,48], there are several published cases with SLE-PSC overlap disease[76-80]. Furthermore, a 1.7% occurrence of SLE was observed in a Swedish PSC cohort[81]. Whether such a coexistence indicates that both diseases might share common pathogenic pathways remains to be elucidated.

SS

SS is a common SRD affecting the exocrine glands with typical symptoms of dryness of eyes and mouth, histological evidence of focal lymphocytic sialadenitis and the presence of anti-Ro and -La antibodies [82]. The treatment of SS-related dry eyes and mouth is symptomatic with the use of artificial tear and saliva preparation. LFT abnormalities can be identified in nearly half of patients, either persistent or intermittent, and usually mild with cholestatic or hepatocellular pattern[3]. Liver involvement is considered as the most common extra-glandular feature, correlating with the disease activities of SS involving other organs[83]. In a large-scale investigation of 475 cases, after excluding DILI and alternative hepatic comorbidities, the main causes of liver dysfunction were VH in 50% and AILDs in around 20% of patients[84]. Several studies have confirmed a higher prevalence of AILDs among SS, mainly PBC (3.4% to 8.9%), followed by AIH (0.4% to 4.4%)[84-87].

The most frequently associated SRDs in PBC is SS with a prevalence ranging from 3.5% to 38%[88-94]. PBC can be considered a SS of the liver, whereas SS has been equally regarded as a PBC of the exocrine glands[21]. In addition to frequent clinical coexistence and comparable epidemiological features, SS and PBC have similar pathogenic mechanisms and genetic susceptibility backgrounds[95]. Pyruvate dehydrogenase complex E2 subunit, a PBC autoantigen, is also present on the surface of salivary epithelial cells in SS, while HLA-DR2 and -DR3 have been reported as the common susceptibility genes in both disorders[20].

Despite a higher frequency of ASMA than AMA in SS[96], the prevalence of coexisting AIH is lower than PBC[84,85,97]. Owing to a much higher prevalence of SS than SLE in the general population, the occurrence of concomitant SLE and SS in patients with AIH are 0.7% to 2.8% and 0.8% to 7.2%, respectively, lower in SLE than in SS[98-102].

There are published cases and case series describing SS-PSC overlap disease as well as a higher prevalence in small-scale PSC studies[84,103,104], implicating a causative association rather than sporadic occurrence. Notably, almost all reported patients with overlapping SS and PSC have chronic pancreatitis, demonstrating a triad syndrome complex[103]. A possibility of co-occurring IgG4-related disease (IgG4-RD) should be considered in SS-PSC overlap disease with the presentation of autoimmune pancreatitis (AIP)[104].

SSC

SSc is an uncommon SRD characterized by vasculopathy and fibrosis of the skin and internal organs, with the presence of anti-topoisomerase I and anti-centromere antibodies (ACA) in diffuse and limited cutaneous subsets, respectively[105]. It has a higher mortality rate than other SRDs. The gastrointestinal tract is affected in up to 90% of patients[106], and hepatic fibrosis has been identified at autopsy[107]. Since liver involvement is rarely observed in SSc[3], abnormal LFT should exclude other possibilities first before considering disease *per se*. There are diverse autoimmune diseases like AILDs co-occurring within SSc patients and their family members[108], suggesting common pathophysiological

Table 4 Clinical, laboratory, therapeutic and outcome data in 3 patients with systemic lupus erythematosus-autoimmune hepatitis overlap disease[1]

Patient number	1	2	3
Sex	Female	Female	Female
SLE Dx age	19	50	20
ACR criteria	8/11	7/11	8/11
AIH Dx age	26	37	22
IAIHG score	Definite	Definite	Definite
Clinical			
SLE	Skin, joint, renal, hematology, neurology	Skin, joint, renal, hematology, serositis	Skin, joint, renal, hematology, serositis
AILD complication	Jaundice, malaise LC with PH	Jaundice, pruritus hepatosplenomegaly	Jaundice, anorexia
Coexistent AID	Nil	PBC, SS	Nil
Laboratory			
Hemogram	HA, TP	HA, TP, leukopenia	TP, leukopenia
Proteinuria autoantibody	2 g/d	2.5 g/d	1 g/d
SLE-related	ANA, anti-dsDNA/Sm	ANA, anti-dsDNA/Sm	ANA, anti-dsDNA
AILD-related	ASMA	AMA, ASMA	ASMA
Others	ARPA, ANCA	ARPA, anti-Ro/La	ARPA
[2]IgG (mg/dL)	2130	2520	1615
[2]AST (IU/L)	1563	116	97
[2]ALT (IU/L)	1093	217	177
[2]Bil (mg/dL)	23.8	3.7	2.4
[2]ALP (IU/L)	432	621	344
HLA-DR	DR8, DR15	DR4, DR15	DR4, DR7
VH	No [3]HHV/CMV/EBV	No HHV/CMV/EBV	No HHV/CMV/EBV
Treatment	CS/AZA, LDLT and low-dose CS/FK506 after OP	CS/AZA, UDCA RTX and low-dose CS for maintenance	CS/AZA, AZA for maintenance
Outcome	Stabilized LFT and low SLEDAI	Normalized LFT and low SLEDAI	Normalized LFT and low SLEDAI

[1]Enrollment from 2018 July to 2021 June.
[2]Peak levels during autoimmune hepatitis.
[3]Human hepatitis viruses including hepatitis A virus, hepatitis B virus and hepatitis C virus.
AID: Autoimmune disease; AIH: Autoimmune hepatitis; ALP: Alkaline phosphatase ALT: Alanine aminotransferase; AMA: Antimitochondrial autoantibody; ANCA: Antineutrophil cytoplasmic antibody; ARPA: Anti-ribosomal-P antibody; ASMA: Anti-smooth muscle antibody; AST: Aspartate aminotransferase; AZA: Azathioprine; Bil: Bilirubin; CMV: Cytomegalovirus; CS: Corticosteroids; Dx: Diagnosis; EBV: Epstein-Barr virus; IAIHG: International Autoimmune Hepatitis Group; HA: Hemolytic anemia; HHV: Human hepatitis viruses; LC: Liver cirrhosis; LDLT: Living donor liver transplantation; LFT: Liver function test; OP: Operation; PBC: Primary biliary cholangitis; PH: Portal hypertension; RTX: Rituximab; SLEDAI: SLE disease activity index; SS: Sjögren syndrome; TP: Thrombocytopenia; UDCA: Ursodeoxycholic acid; VH: Viral hepatitis.

mechanisms between these disorders.

Increased prevalence of PBC has been observed in SSc, varying from 0.8% to 3.3%[108-112], and there is a 2.3% to 12.4% occurrence of SSc in PBC[90-94,111]. SSc-PBC overlap disease has the presence of both ACA and AMA[113], and tends to occur in older females with the limited cutaneous subset[114]. This overlap disorder has a slower disease progression in comparison with PBC alone; however, survival is similar due to an increase in SSc-related non-liver death. The use of UDCA has been observed to reduce skin lesions in addition to improved hepatic cholestasis in overlap patients[115].

A 0.8% prevalence of SSc has been reported from a AIH cohort[98], and patients with SSc-AIH overlap disease can be found in the literature[116,117]. In a review with 11 cases[117], all had positive ACA and a later presentation of AIH, 9 with limited cutaneous subtype and 3 with AIH-PBC overlap. Despite a risk of scleroderma renal crisis under the higher dosages of CS use, there were normalized or improved LFT without the occurrence of scleroderma renal crisis in overlap patients receiving such a

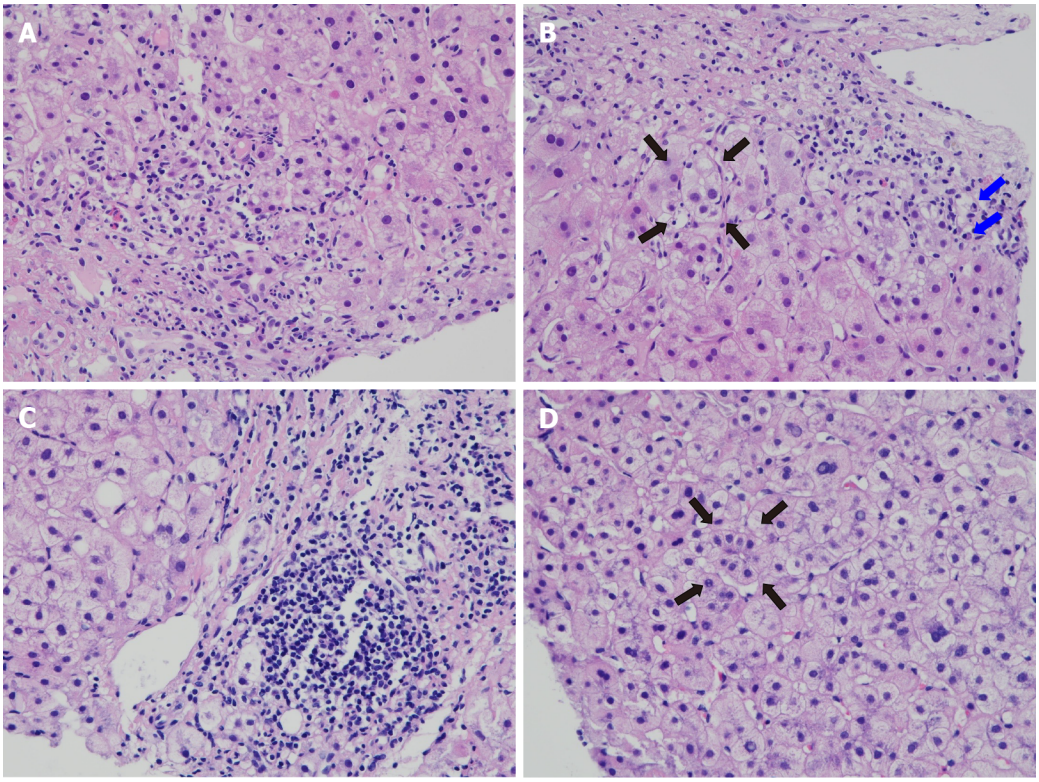

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Figure 2 Liver biopsied tissues from 2 patients with systemic lupus erythematosus-autoimmune hepatitis overlap disease. A and B: Case 1 (A) lymphoplasmacytic infiltration with interface activity and (B) lymphoplasmacytic infiltration with interface activity. Plasma cells are indicated by blue arrows and rosette formations by black arrows; C and D: Case 2 (C) lymphoplasmacytic infiltration with interface activity, and (D) rosette formation (arrows). Hematoxylin and eosin staining, 400 × magnification.

treatment[116].

Overlap condition with large- or small-duct PSC has been observed in patients with SSc[118,119], suggesting that the extensive disturbance of connective tissues in SSc can lead to abnormal collagen deposition in the bile duct epithelium of PSC[120].

RA

RA is a common SRD primarily affecting the joints and causing cartilage and bone damage, with extra-articular presentations and the presence of rheumatoid factor (commonly referred to as RF) and anti-cyclic citrullinated peptide (commonly referred to as CCP) autoantibodies[121]. Among patients with chronic inflammatory joint diseases, liver involvement has been recognized in RA, despite not showing a significant extra-articular manifestation[122]. Elevated liver enzymes have been identified in up to 50% of patients with RA[2]. DILI is not uncommonly observed in RA, especially under the treatment of NSAIDs and SDMARDs including leflunomide, methotrexate, penicillamine and sulfasalazine, all with potential hepatotoxicity[2,7,123]. Patients are at the hazard of developing NAFLD with the risk factors of chronic inflammation and CS use[2]. Prior to the widespread use of methotrexate in RA, the hepatic histopathological findings at autopsy were most commonly mild portal tract inflammation, rarely diffuse fibrosis of advanced grades[124]. Two rare extra-articular manifestations, rheumatoid vasculitis and Felty syndrome, have been reported to cause necrotizing hepatic arteritis with liver rupture and NRH with portal hypertension, respectively[125,126].

There were no differences in the prevalence of HBV and HCV infection in RA as compared with the general population[127]. Nevertheless, immunosuppressive therapy for RA may significantly worsen underlying VH, and further affect the clinical course and disease prognosis, requiring the survey of viral markers and their antibodies before its initiation[123]. Since the use of tumor necrosis factor (TNF) blockades in RA can cause inactive HBV reactivation[7,128], HBsAg-positive individuals should receive anti-viral prophylactic treatment[129]. Although the TNF pathway is involved in perpetuation of hepatic inflammation and fibrosis progression in HCV infection[130], further studies are needed to verify the safety of anti-TNF therapy in HCV-infected patients[131]. Notably, the use of TNF antagonists has been reported to be associated with the development of AIH in RA[7,132].

The most common coexisting AILDs in RA is PBC with a prevalence of 3.8% to 6.3%[53,97,123], while the occurrence of RA in PBC has been reported to be 1.8% to 13%[90-94]. Around 50% of patients with PBC were shown to be positive for RF[133]. Since RA is usually diagnosed before PBC in patients with the overlap disease, AMA should be screened in RA with elevated cholestatic liver enzymes[134]. Genetic studies have shown that RA has HLA-DQB1, STAT4, IRF5, MMEL1 and CTLA4 genes in common with PBC, predisposing to develop PBC in RA with the overlapping genetic trait[135]. Potentially hepatotoxic drugs used in RA can be avoided in patients with RA-PBC overlap disease[123].

AIH is rarely observed in RA with a 1.3% prevalence reported from patients with liver dysfunction [97]. Furthermore, in patients with AIH, there is a 1.6% to 5.4% prevalence of RA[98,100-102]. AIH can be diagnosed during the RA progression as acute or chronic hepatitis, but rarely fulminant hepatic failure[123]. In addition, in patients with AIH-PBC overlap disease, RA is accounting for an occurrence of 4.2%[71].

High circulating levels of TNF were found in AIH, while a TNF antagonist etanercept has been demonstrated to improve the AIH histological lesions in RA[136]. Nevertheless, anti-TNF therapy can induce the production of autoantibodies, including ANA and ASMA, leading to the development of distinct autoimmune diseases[137]. Notably, anti-TNF-inhibitor-associated AIH (also known as ATIAIH), a serious idiosyncratic DILI, has been well documented in a large-scale analysis of 389 cases [138]. ATIAIH has a female predominance, a period of 3-14 mo between starting therapy and AIH occurrence, and improvement upon medication stoppage and CS use. Infliximab is the most frequently administrated medication, and RA is the most commonly reported indication.

There was a 1.2% and a 3.4% prevalence of RA in two large-scale PSC cohorts[81,139]. In patients with RA-PSC overlap disease, the presence of HLA-DR4 has been reported to have unusual progression to cirrhosis, 14-48 mo after the diagnosis of PSC[140], implicating a clinical marker at a high risk of cirrhosis development.

Psoriatic arthritis (PsA) is a less common SRD with psoriasis (PsO) and inflammatory arthritis, associating with extra-articular manifestations which have an impact on their therapeutic regimens [141]. Similar to RA, liver enzyme abnormalities in PsA and PsO can be caused by comorbid NAFLD and used medications including NSAIDs and conventional or biologic/targeted SDMARDs. Despite an increased association of AIH in PsA and PsO[142], these patients might be under anti-TNF therapy, and both diseases are commonly observed complications in ATIAIH[138].

IIM

IIM including polymyositis (PM) and dermatomyositis (DM), an uncommon group of SRDs with the presence of myositis-specific/associated antibodies, have weakness due to skeletal muscle inflammation and extra-muscular involvement[143]. Since transaminases are also muscle-derived enzymes with increased levels during IIM disease activity, an increase of aspartate aminotransferase and alanine aminotransferase more than creatine kinase or an alteration of cholestatic enzymes should consider a possibility of hepatic dysfunction[3]. During the first 3 years to 5 years after the onset of DM, the risk of cancer is increased, rarely hepatocellular carcinoma. Since DM can be associated with malignancy as a paraneoplastic syndrome[144], sporadic cases had HBV-associated hepatocellular carcinoma with a concurrent or later diagnosis of DM[145,146].

Although IIM usually occur alone, these SRDs may associate with other extra-muscular autoimmune diseases including AILDs, more frequently in patients with PM than DM[147]. Positive AMA could be identified in 2.5% of patients with IIM[148], and there were sibling cases of familial clustering with PBC-PM overlap disease[149]. PBC can be identified in IIM with a prevalence of 0.7%[148], while the occurrence of PM in PBC ranges from 0.6% to 3.1%[90,92,93]. There are sporadic cases with PM coexisting with AIH, AIH-PBC overlap disease or PSC[150-152].

MCTD

In addition to the presence of anti-U1 small nuclear ribonucleoprotein (known as snRNP) antibody in high titers, MCTD has distinct features including Raynaud's phenomenon and puffy hands as well as mixed findings from PM, SLE and SSc[153]. It is a rare SRD with a strong HLA linkage, distinctly differing from ethnically matched healthy controls and other SRDs. Hepatic dysfunction occurs in MCTD usually caused by DILI and pulmonary hypertension-related liver congestion[97,153]. Coexistent AILDs are rarely observed in patients with MCTD[153]. In addition to published case reports, a 1.6% prevalence of AIH was found in MCTD[154], while a 0.6% prevalence of MCTD could be identified in PBC[90]. There was no observed association with MCTD in two PSC case series[81,141].

SV

SV is a rare SRD characterized by inflammation of vascular walls, resulting in a broad spectrum of clinical manifestations dependent on the site, type, and size of involved vessels[155]. Although the diagnosis relies on clinical presentations confirmed by histopathological findings, large/medium and small vessel involvement can be supported by angiographical examinations and laboratory tests (*e.g.*, ANCA), respectively[155,156]. Owing to hepatic vascular involvement[2,53], polyarteritis nodosa (referred to herein as PAN), a medium-vessel SV associated with HBV infection[157], may have elevated liver enzymes. A 2.2% prevalence of SV has been reported from a large-scale PBC series with 361 cases [94], while a 1.6% occurrence of SV was identified in a 122-patient AIH series[98]. There were sporadic cases of AIH coexisting with PAN[158].

Testing for ANCA can support the diagnosis of ANCA-associated vasculitis including eosinophilic granulomatosis with polyangiitis (also referred to as EGPA), granulomatosis with polyangiitis and microscopic polyangiitis (also referred to as MPA) in spite of seropositivity in only one-third of EGPA cases[159]. Notably, ANCA has a diagnostic relevance beyond SV, justifying its occurrence in suspected type I AIH which is lacking conventional autoantibodies[160]. AILDs usually develop atypical perinuclear-ANCA not targeting the classical myeloperoxidase with the positive frequencies highest in patients with PSC[21,156]. There is no clinical nor prognostic value of ANCA testing in patients with AILDs. This atypical autoantibody, referred to as peripheral anti-nuclear neutrophil antibody, can react with beta-tubulin isotype 5 and shares structural homology with the intestinal bacterial protein FtsZ [161]. Nevertheless, it is not specific for AILDs, and it is also present in VH and alcoholic liver disease [162]. Interestingly, ANCA was detected in the bile of PSC patients and correlated with the severity of bile duct stricture[163]. Sixteen cases of ANCA-associated vasculitis-AILD overlap disease have been reported, with twelve involving women, PBC in eleven, and MPA in eight[164-166].

OTHER SRDS

Adult-onset Still's disease (AOSD) is a rare SRD usually affecting young adults, with spiking fever, polyarthritis, evanescent rash and marked hyperferritinemia as well as uncommon life-threatening macrophage activation syndrome[167]. In medical practice, hyperferritinemia is a non-specific finding related to iron overload in only 10% of cases such as hereditary hemochromatosis, while underlying causes attributing to a reactive increase in the rest 90% patients such as AOSD[168]. Hepatic dysfunction is commonly observed in AOSD, mostly due to the disease itself and without any specific histological finding[97,167]. Coexisting AILDs have rarely been observed, and there are sporadic cases of AIH-AOSD overlap disease[169].

Behçet's disease (BD) is a SRD with a variable worldwide prevalence, characterized by vasculitis affecting the small/Large venous and arterial vessels, and presenting with orogenital ulcers, ocular lesions and systemic involvement[170]. The liver is rarely involved, and the commonest hepatic complication is BCS with thrombosis of the inferior vena cava and hepatic vein[171]. Elevated ALP levels of liver origin has been reported in 10% of patients, with a correlation to disease activity[172]. Case reports of Behçet's disease concomitant with AIH or PBC can be found in the literature[173,174].

IgG4-RD is a rare SRD, characterized by elevated serum IgG4 concentrations and fibroinflammation in the affected tissues, with dense lymphoplasmacytic infiltrates rich in IgG4-positive plasma cells and storiform fibrosis[175]. Cases of type I AIP and IgG4-related sclerosing cholangitis (commonly referred to as IgG4-SC), two common forms of IgG4-RD usually occurring in combination, have painless jaundice and cholestatic LFT abnormalities due to liver involvement[176,177]. Although CS has favorable therapeutic efficacy[49], AIP and IgG4-SC are associated with significant morbidity and mortality due to extra-pancreatic organ failure and malignancy[176]. AIP has been reported to be associated with PBC and PSC[178,179]. Infiltrating IgG4-positive plasma cells can be observed in the AIH liver, suggesting involvement of IgG4 in its pathogenesis[180]. Nevertheless, the disease concept of IgG4-AIH remains to be established[181].

Sarcoidosis is an uncommon SRD, characterized by the formation of noncaseating granulomas in various organs, predominantly the lungs, lymphatic system, skin, and eyes, or a different combination of these sites[182]. Abnormal LFT has been observed in one-fourth of patients with chronic sarcoidosis; among which, 15% are suspected of having liver involvement with cholestatic pattern of injury[183]. Although hepatic sarcoidosis is mainly asymptomatic, it can progress to LC, while such cases are rare [184]. AILDs coexisting with sarcoidosis have been reported, having a prevalence of 0.6% in AIH and 0.8% in PSC[81,99]. Several case reports have described the association of sarcoidosis with PBC[88]. A 2.7% prevalence of sarcoidosis was found in a PBC cohort from Greece[185], whereas an epidemiological study with 1510 patients from the United Kingdom failed to show an association between the two disorders[186].

Relapsing polychondritis is a rare SRD, characterized by cartilaginous inflammation throughout the body, especially involving the hyaline cartilage of the ears, nose and joints, and the respiratory tract [187]. Liver involvement with cholestatic hepatic dysfunction has been observed scarcely in such

patients[188]. The association of relapsing polychondritis with AILDs has been reported with PBC or PSC overlap diseases[189,190].

CONCLUSION

SRDs are chronic, inflammatory, autoimmune disorders with the presence of autoantibodies that may affect any organ or system. Liver dysfunction in SRDs can be associated with prescribed drugs, VH, alternative hepatic comorbidities and coexisting AILDs, requiring an exclusion of secondary conditions before considering liver involvement. The patterns of overlap diseases depend predominantly on genetic determinants with common susceptible loci widely distributed in both disorders. In AILDs, it is important to identify the overlapping SRDs at an early stage, since such a coexistence may influence the disease course and prognosis. Commonly co-occurring SRDs in AILDs are SS, RA or SLE in AIH, and SS, RA or SSc in PBC. Owing to different disease complications and therapies, it is imperative to differentiate between SLE liver involvement and SLE-AIH overlap disease. Therapeutic options can be personalized to control coexisting conditions of liver autoimmunity and rheumatic manifestations in AILD-SRD overlap diseases. The collaboration between hepatologists and rheumatologists in clinical practice can lead to significant advances in managing such a complex scenario.

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FOOTNOTES

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REVIEW

Fecal microbiota transplantation in the metabolic diseases: Current status and perspectives

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Abstract

With the development of microbiology and metabolomics, the relationship between the intestinal microbiome and intestinal diseases has been revealed. Fecal microbiota transplantation (FMT), as a new treatment method, can affect the course of many chronic diseases such as metabolic syndrome, malignant tumor, autoimmune disease and nervous system disease. Although the mechanism of action of FMT is now well understood, there is some controversy in metabolic diseases, so its clinical application may be limited. Microflora transplantation is recommended by clinical medical guidelines and consensus for the treatment of recurrent or refractory *Clostridium difficile* infection, and has been gradually promoted for the treatment of other intestinal and extraintestinal diseases. However, the initial results are varied, suggesting that the heterogeneity of the donor stools may affect the efficacy of FMT. The success of FMT depends on the microbial diversity and composition of donor feces. Therefore, clinical trials may fail due to the selection of ineffective donors, and not to faulty indication selection for FMT. A new understanding is that FMT not only improves insulin sensitivity, but may also alter the natural course of type 1 diabetes by modulating autoimmunity. In this review, we focus on the main mechanisms and deficiencies of FMT, and explore the optimal design of FMT research, especially in the field of cardiometabolic diseases.

Key Words: Fecal microbiota transplantation; Metabolic diseases; Inflammatory bowel disease; Type 1 diabetes; Metabolic syndrome

Core Tip: The success of fecal microbiota transplantation (FMT) depends on the microbial diversity and composition of donor feces. It is newly found that FMT may not only improve insulin sensitivity, but also alter the natural course of type I diabetes by modulating autoimmunity. In this review, we focus on the main mechanisms and deficiencies of FMT, and explore the optimal design of FMT research, especially in the field of cardiometabolic diseases.

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INTRODUCTION

Most of the research on microorganisms is confined to infectious diseases and the role of microorganisms in human health is largely ignored. The average weight of these microorganisms is about 1.5 kg, equivalent to the weight of the liver. There are 10^{12}–10^{14} microorganisms, which is 10 times the number of the human body's own cells, and they are mainly parasitic in the intestinal tract[1]. These symbiotic microorganisms include bacteria, viruses, archaea, fungi and, in some cases, protists, collectively known as the microbiome. The most important advantage of fecal microbiota transplantation (FMT) is the determination of cause and effect of disease through microbiology[2] .

During the long process of human evolution, the intestinal flora has coevolved with its host, along with social development, changes in diet, lifestyle and environment. Intestinal symbiotic bacteria can regulate a variety of metabolic activities that cannot be carried out by the human body itself[3]. They can obtain energy by decomposing polysaccharides, proteins and fats in food that cannot be fully digested by the host, and produce a series of metabolites that affect the health of the host. In this process, the intestinal microecosystem is closely related to the host metabolic capacity[4].

As early as 3000 years ago, cow dung was used in India to treat gastrointestinal diseases. As early as the Eastern Jin Dynasty (317–420 AD), a treatment similar to fecal bacterial transplantation, called "Huanglong Soup", was described in Ge Hong's "Urgent Prescription for Elbow Reserve", which was used to treat food poisoning and diarrhea. In traditional Chinese medicine, it is recorded that huanglian and rhubarb, among others, have the curative effect of "quenching thirst" (ancient term for diabetes). Berberine, a monomer component from huanglian, has been recognized internationally for its effect on improving glucose and lipid metabolism earlier. During World War II, German soldiers in North Africa treated diarrhea with camel excrement[5]. At present, FMT is mainly used for the treatment of recurrent *Clostridium difficile* infection (CDI) in clinical practice, and many clinical trials have confirmed that FMT is a feasible treatment[6].

At present, with the development of fast and accurate high-throughput sequencing technology and the improvement of bioinformatics technology methods, intestinal flora is closely related to metabolic syndrome (MS), type 1 diabetes (T1D) and type 2 diabetes (T2D), various cancers, and autoimmune diseases. Currently, it is believed that the FMT donor should be carefully selected and examined for infectious diseases[7]. However, due to the large difference in metabolism and diet of FMT donors, the effect of transplantation can be different. In this review, the mechanisms and deficiencies of FMT are discussed, and the optimal design of FMT is explored to maximize scientific research and clinical application methods.

COMPOSITION AND METHOD OF FMT

The main components of FMT are the gut flora of humans and other species. Humans have evolved to come into contact with a variety of bacteria, including those produced by food fermentation. The oral cavity is an important location of intestinal microbiota, which has an important effect on human health. Studies have shown that children who grow up on farms have a lower risk of asthma; a phenomenon that may be linked to changes in their gut microbiota[8]. In addition, babies born by cesarean section are at increased risk of developing autoimmune diseases, mainly because the initial microbes passed from the vagina to the baby at birth are replaced by skin microbes from the mother and surgical team members, which alter the baby's gut microbes[9]. An infant's gut microbiome can be reshaped in breast milk by adding small amounts of bacteria from the mother's feces, creating a pattern that more closely resembles that of babies born vaginally[10].

FMT has been processed into an odorless and tasteless preparation. In clinical practice, there are three methods of bacterial flora transplantation for patients willing to accept FMT: Upper, middle and lower digestive tract. The methods of transplanting upper digestive tract microflora mainly include oral

microflora liquid and oral microflora capsule. The middle digestive tract approach includes a nasointestinal tube, endoscopic biopsy hole, percutaneous endoscopic gastrostomy and jejunal catheterization, endoscopic catheterization such as Transendoscopic enteral tubing (TET)[11]. The lower gastrointestinal pathway includes colonoscopy, colostomy, enema, and colonic pathway TET. Colonic pathway TET is not only used for microflora transplantation, but also for whole colon administration such as mesalazine, hormones and traditional Chinese medicine. As a new endoscopic technique, TET is an important supplement for interventional treatment of inflammatory bowel disease[12].

FMT focuses on flora transplantation, but other components, such as phages, should not be ignored, which may be the reason for FMT's effectiveness in the treatment of recurrent CDI. Therefore, phage research is important, and animal studies have shown that fecal virus transplantation also plays an important role. Analysis of the feces of adults on a classic British diet found that 25% of the 100 g/d excreted was made up of bacteria and 75% of fiber, protein, fat, bile acids and short-chain fatty acids (SCFAs). In most FMT, however, feces are simply mixed with salt water and filtered to remove insoluble substances[13]. Thus, the potential effects of FMT may be partly due to the combined effects of these compounds (Figure 1).

AUTOLOGOUS FMT

Most studies have focused on fecal transplants from healthy donors (known as FMT allografts). However, autologous fecal transplants have significant advantages[14], such as reducing the risk of infection and increasing the efficiency of transplantation, especially in the treatment of recurrent CDI by freezing their own feces. Autologous fecal transplants are effective in many diseases, but not in disorders caused by intestinal flora disorders, such as inflammatory bowel disease[15]. Studies have shown that disorder of intestinal flora can aggravate the disease, and intestinal inflammation can also affect the composition of intestinal flora[16]. Therefore, it is speculated that fecal biobanks may contain probiotics, which have changed the composition of intestinal flora before the relapse of the disease. This requires further confirmation of the value of probiotics in intestinal flora, but there is insufficient evidence to confirm the value of probiotics collected in clinical remission. Therefore, autologous FMT can improve clinical symptoms by regulating intestinal flora to promote metabolism[17].

In conclusion, regulating the balance of intestinal flora is the primary goal of therapy. Autologous FMT through a duodenal tube or oral capsules can reshape the composition of small intestinal flora, which play an important role in the regulation of autoimmune diseases[18], mainly because the immune system response to antigenic stimuli occurs in the small intestine. It has also been suggested that autologous FMT *via* the duodenal tube may be valuable in a new method of preserving β-cell function for T1D diagnosis that is more effective than healthy donor FMT[19].

FMT AND METABOLIC DISEASES

MS

To date, the only reported study of FMT in the treatment of human MS was conducted by Witkowski *et al*[20]. This study examined the effects of FMT on glucose and lipid metabolism in men with MS in a double-blind randomized controlled trial in nine patients receiving fecal bacteria transplants from lean healthy donors (allograft group) and nine other patients[21]. They received their own fecal bacteria as a control (autologous transplantation group)[22]. After 6 wk of FMT treatment, insulin sensitivity and fecal microbial diversity were significantly increased in the allograft group, while no significant changes were observed in the autograft group. It should be noted that there were individual differences in the efficacy of FMT[23], and Gagliardi *et al*[24] suggested that the differences might have been more due to different donors than recipients, since the two subjects receiving fecal bacteria from the same donor showed similar benefits[24]. In a randomized, double-blind, controlled trial of fecal bacteria transplantation in patients with MS it was found that insulin sensitivity and butyric acid-producing intestinal flora significantly improved in patients receiving fecal donation[25]. FMT strengthened the intestinal barrier function and effectively reduced endotoxemia in a nutritionally obese rat model. It is also concluded that FMT can regulate the lipid content of obese rats and reduce hepatic steatosis[26]. There are currently four clinical trials of FMT for MS registered with clinicaltrials.gov. We believe that the results of these clinical trials can provide us with a better understanding of the role of intestinal flora in human metabolic disorders[27].

T1D

There is a close relationship between intestinal flora and diabetes mellitus. Some studies have found that the decrease of butyrate- and lactate-producing bacteria is related to the autoimmunity of β cells [28]. In addition, it has been found that the intestinal flora of children with β-cell-related autoimmune diseases lacks *Bifidobacterium*, and the bacteria producing butyrate and lactate are reduced while

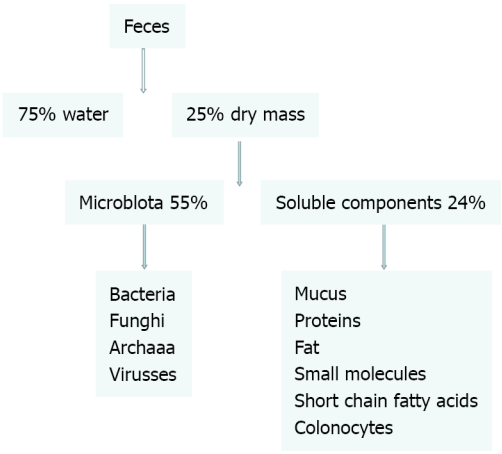

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Figure 1 Components that are transferred during fecal transplantation.

Bacteroidetes are increased[24]. Another study in Spain found similar changes in the gut flora of children with T1D, suggesting that structural changes in the gut flora may be associated with T1D[29]. A meta-genomic analysis of the intestinal flora in an included study found that, compared with the control group, patients with T1D had fewer butyrate-producing bacteria and mucin-degrading *Prevotella* and *Akkermansia*, and had more lactate-producing bacteria, and bacteria-producing SCFA other than butyrate, such as *Bacteroides* and *Riyanella*[30].All of these suggest that gut bacteria may participate in the disorder of immune function in patients with T1D[31].

Dietary fiber can be metabolized and fermented by intestinal bacteria into SCFAs, including acetic acid, propionic acid and butyric acid, which may also be involved in the pathogenesis of metabolic diseases[32]. The types and quantities of SCFAs are thought to vary with the composition of intestinal microbes. In addition to serving as an energy source for intestinal epithelial cells and liver (SCFAs are absorbed by the intestine and transported mainly through the portal vein), SCFAs are thought to have immunomodulatory effects by reducing intestinal permeability[33]. Lipopolysaccharides from intestinal translocation to the portal vein are thought to be involved in obesity-related mild inflammatory responses and insulin resistance in mice[34].

SCFAs produce a small number of microorganisms in T1D, and the incidence of T1D is significantly reduced in nonobese diabetic mice treated with *Akkermansia* or with a prebiotic diet supplemented with SCFAs[35]. It has been suggested that the restoration of intestinal flora balance through healthy donor FMT may further weaken autoimmune function and β-cell dysfunction[36]. A recent study showed that both healthy autologous and allogeneic FMT attenuates the decline of β-cell function, while donor FMT decreases at a slower rate[37]. Surprisingly, the decay rate of autologous FMT b cells was only 12 mo after three consecutive FMT treatments. Due to the significant changes in microbes from the mouth to the anus during autologous FMT[38], the immune system of the small intestine can be reshaped. Due to the lack of effective immunomodulators to treat T1D, a large number of clinical studies are needed to confirm this[39].

T2D

The association between intestinal flora and T2D was first reported by Chinese researchers led by The Shenzhen Huada Institute for Life Sciences and published in Nature in 2012[40]. This study found that the relative abundance of clostridium butyricum and its butyric acid-producing function in Chinese patients with T2D were significantly lower than those in the normal population, and lipopolysaccharide produced by conditional pathogenic Enterobacteriaceae species, hydrogen sulfide proinflammatory function and branched chain amino acid transport function levels were significantly higher than the general population[41]. These changes may be associated with impaired intestinal mucosal barrier function and increased levels of intestinal inflammation in T2D patients[42].

A randomized controlled study showed that autologous FMT can maintain normal metabolism after diet-induced weight loss[43]. It has been observed that obese donor FMT can cause rapid weight gain, so there is a link between the intestinal microbiome, obesity and insulin resistance[44]. On the contrary, non-obese donor FMT can improve insulin resistance in obese patients with MS. Another study found that donor FMT had no effect on glucose metabolism and their diets were metabolically tested. Recent studies have shown that the use of single-dose capsule FMT improves lipid metabolism and insulin resistance, mainly through continuous supplementation of low-fermenter fiber[45]. Therefore, dietary composition may affect insulin resistance of FMT, or metabolites of donor FMT may affect the enteric–brain axis[46].

NONALCOHOLIC FAATTY LIVER DISEASE

The relationship between intestinal microflora and nonalcoholic faatty liver disease (NAFLD) is increasingly close, research suggests. Intestinal flora can affect the occurrence and development of NAFLD by changing the composition of intestinal flora, increasing serum endotoxin level and intestinal permeability, producing endogenous alcohol and changing choline metabolism[47]. Animal studies have shown that FMT can improve steatohepatitis in mice induced by high-fat diet, reduce the production of lipids and proinflammatory factors in the liver, regulate the balance of intestinal flora in mice, and increase the abundance of beneficial flora[48]. After FMT treatment, the cecal butyrate concentration and intestinal tight junction protein ZO-1 increased, and the toxin release decreased, thus reducing the inflammatory response[49].

Increased intestinal permeability and metabolic endotoxin caused by changes in intestinal flora composition are involved in the progression of NAFLD in mice, and the severity of NAFLD in mice is increased when special flora are transferred to methionine- and choline-deficient diet, indicating that intestinal flora is involved in the progression of NAFLD[50]. The results of human studies also support the idea that changes in gut flora can contribute to fatty liver disease. Compared with normal subjects, NAFLD patients showed increased intestinal permeability, endotoxemia, increased numbers of g-Proteobacteria, and decreased numbers of Bacteroidetes[51].

Fatty liver often occurs in obese patients. Long-term vegans have a lower risk of NAFLD, which may be related to changes in gut flora. It is suggested that FMT treatment of long-term vegan feces can improve liver inflammation score[52]. Despite the small sample size, this study still found that the inflammatory necrotic tissue score and inflammatory gene expression were reduced after transplantation of vegan fecal flora, which may be an important indicator for predicting the progression of NAFLD to cirrhosis[53]. At the same time, an FMT study in NAFLD patients showed that healthy donor FMT reduced intestinal permeability, which is an important feature that distinguishes NAFLD from other diseases[48]. This study found that magnetic resonance imaging could not make a definitive diagnosis of hepatic adipose degeneration, which must be assessed using gold standard liver histological examination[54].

PITFALLS

Mode of delivery

Since innate and adaptive immune cell reactions occur in the small intestine, immune diseases are usually treated by oral capsules or fresh feces administered through the duodenum under strict anaerobic conditions, in order to ensure that active aerobic and anaerobic bacteria can be transplanted to the maximum extent and thus reshape the intestinal microecological balance[55]. Remodeling of the small intestinal flora is not appropriate for nonimmune diseases or diseases with distal intestinal malformations, but colonic delivery (enema or colonoscopy) may be an option[56].

Whether FMT plays an important role in other diseases besides recurrent CDI needs confirmation. Donor FMT freeze-drying capsules or frozen-solution capsules have been widely used and have gained more support due to their noninvasive administration[57], and it is also convenient for the donor and recipient to make multiple trips to the hospital for transplantation on the same day[58]. Current treatments for CDI include enemas, frozen capsules or freeze-drying formulations. However, it may not be suitable for mild intestinal microecological disorders that do not comply with GMP regulations as compared to fresh feces[59].

PREPARATION OF FMT FECAL BACTERIA LIQUID

Due to the lack of sufficient sample size and establishment of control groups in most clinical studies [55], the conclusions are not reliable, and there is controversy about the preparation of FMT fecal bacteria solution. (1) Selection of stool dilution materials[60]. It is reported that ordinary water (98.5%) has a higher disease remission rate than normal saline (86%) as a stool dilution material, but the recurrence rate of CDI with the former increased > 2 times. Other thinners, such as milk or salt water from plantain, achieved a 94% remission rate; (2) The amount of fecal bacteria liquid transplanted. When the volume of fecal bacteria liquid transplanted is > 500 mL, the remission rate of CDI is 97%, but < 200 mL, the remission rate is only 80%. However, it is difficult to compare the above conclusions because the dilution ratio of feces may vary. Currently, according to the Amsterdam protocol, 200–300 g of donor stool is dissolved in 500 mL normal saline for use (donor stool is preferably fresh within 6 h); and (3) Feasibility of frozen feces. A case report of standardized frozen stool samples used for fecal bacteria transplantation for the treatment of CDI showed that there was no statistical difference in the efficacy of standardized frozen stool compared with fresh stool. Therefore, establishment of stool donation banks and use of standardized frozen stool made fecal bacteria transplantation more feasible

in clinical practice[61]. A recent study on the treatment of CDI by oral frozen fecal bacteria capsules showed that no serious adverse reactions occurred in recurrent CDI treated by fecal bacteria transplantation *via* oral frozen fecal bacteria capsules[62]. The diarrhea relief rate of single administration was 70% (14/20), while four of six patients who did not respond to treatment achieved remission after second administration, resulting in a total remission rate of 90%. This study initially demonstrated the feasibility and safety of fecal bacteria transplantation *via* frozen fecal bacteria capsules[63].

THE PROCESSING OF FMT

Protective measures are usually taken to avoid anaerobic bacteria being killed by coming in contact with oxygen[64], but it cannot be completely avoided. Under strict anaerobic conditions, the composition of diluted or filtered microorganisms does not differ significantly before and after the entire procedure, but the activity of the preparation may have been affected[65]. Similarly, prolonged freezing at -80 °C preserved fecal components to a large extent, but whether it had an effect on fecal activity was unclear [66]. However, recent studies have shown that autologous FMT stored in glycerin at -80 °C can completely restore the intestinal lumen and mucosal microbial balance. Whether FMT regulates mild microbial disorders in these studies remains to be confirmed[67].

DIVERSITY OF THE GUT MICROBIOME

The diversity of intestinal flora increases with growth and development, and finally forms a complex and relatively stable microbial community at the age of 2–3 years, mainly including bacteria, fungi, viruses and protozoa[68]. There are > 1000 species of bacteria, most of which are obligate anaerobes, including Firmicutes, Bacteroides, Proteobacteria and Actinomycetes, among which Firmicutes and Bacteroides are dominant, accounting for > 90% of all intestinal bacteria[69].

Gut microbiome composition is temporal and spatially specific. Neonatal bacteria from the birth canal colonize the intestine within a few hours after birth. The intestinal microbial composition of early vaginally delivered babies is similar to that of the mother's vagina, while that of cesarean delivery babies is different[70]. A baby's gut microbiota can reach the level of a healthy adult at about age 1 year. Most of the microorganisms in the human intestine colonize the colon[71], and the number is 10^{12} cfu/mL. The microbial content of the jejunum, ileum and duodenum decreases successively, and there are 10^{7}, 10^{4} and 10^{3} cfu/mL, respectively. There are also differences in the types of microorganisms rich in each part. In addition, the composition of intestinal mucosa and fecal-associated microorganisms varies[72].

Individuals in the same area may have different gut microbiota. The composition and diversity of intestinal microbiota may influence the therapeutic effect of donor FMT[73], or even insulin resistance. In the past few decades, with the westernization of China's diet, intestinal microbial diversity has decreased. Preselection of donor FMT may be a feasible way to improve clinical outcomes based on the presence of a specific biological chain. Therefore, an important method to study FMT is to carefully study the baseline data of patients and the microbial composition after FMT[74]. By comparing the baseline data of the donor and the recipient and the microbial composition during a certain period of time, the number of microbes transplanted from the donor to the recipient can be calculated[75]. The most common method is fecal metagenomic sequencing, which identifies microbial species based on specific mononucleotide degeneration[76]. Sequencing techniques combined with bioinformatics analysis reveal the duration of similarity between donor and recipient strains, and how many of the transplanted microbes are likely to restore the original microbial composition[77].

DONOR-ACCEPTOR COMBINATION

FMT is thought to restore disturbed gut flora to a healthy state either by implanting a donor strain or by other donor-dependent traits, such as the amount of nonbacterial components[78]. However, not all donor gut microbiota are uniform, and comparison of gut microbiota from different donors suggests that microbial diversity and metabolites may be predictors of the success of FMT[79]. In some studies, donor microbiome and metabolomic characteristics may be associated with FMT treatment response. Therefore, the selection of appropriate donor feces is a key factor in the success of FMT[80]. However, few studies of FMT have considered the influence of the variation characteristics of the intestinal microbiome and metabolome of the donor on clinical efficacy[81].

Current studies have confirmed that after FMT treatment, the intestinal flora diversity of the recipient is significantly increased and tends to be the flora characteristics of the donor[82]. Cases that respond to FMT treatment typically show higher microbial diversity. It has been confirmed that intestinal bacterial abundance in donors that respond to FMT is significantly higher than that in donors that do not respond

[83].

To date, donor selection methods in FMT studies have included the use of a single donor or the random selection of multiple donors from a group of screened eligible donors[84]. In 2019, Zheng *et al* [85] first proposed the concept of super donors and believed that the success of FMT depends on the donor[85]. However, the definition of super donor has not been established, and the clinical efficacy of donors before FMT treatment cannot be predictedv[86]. However, the failure of randomly selected single donors may be due to the selection of ineffective donors rather than the incorrect indication selection of FMT[87]. Therefore, an alternative approach is to expose each patient to multiple donors (multidonor transplants) to reduce the risk of receiving FMT from an invalid donor[88]. However, FMT is still in the clinical research stage. Single donors can provide clearer evidence for clinical studies, while multiple donors lead to false negatives or false positives in clinical studies, thus hindering the development of the FMT field and the development of new microbiome therapies[89].

Studies have shown a surprising match between donor and recipient transplants and FMT[90]. The ability to secrete blood group antigens is associated with a reduction in gut microbial diversity, which in turn determines the likelihood of successful transplantation from nonsecreting blood group donors to secreting blood group receptors, which may also apply to human leukocyte antigens (HLAs)[91]. HLA haploidy is an important risk factor for autoimmune diseases such as T1D, and infants with HLA haploidy associated with an increased risk of T1D do form a unique microbiome[92]. What remains to be proven, however, is whether FMT can correct the high-risk microbiome associated with specific HLA haplotypes later in life[93]. In addition, it needs to be considered that intestinal immunoglobulin-secretion-binding bacteria and their components may contribute to the therapeutic effect of donor FMT [94].

CONCOMITANT MEDICATION

To facilitate colonization (also known as transplantation), the recipient's gut is usually cleaned, most commonly by enemas, laxatives, or broad-spectrum antibiotics[95]. In patients with ulcerative colitis, antibiotic administration after FMT increases the risk of transplant failure, although there is evidence that antibiotic pretreatment improves the efficacy of FMT[96]. Previous studies have shown that antibiotics, metformin, berberine and other drugs can change the intestinal flora, thus affecting the state of the body[97]. A study of Finnish children aged 2–7 years found that macrolide use was associated with subsequent long-term changes in intestinal microbiota composition and metabolism: A decrease in Actinobacteria and an increase in Bacteroidetes and Proteobacteria, decreased biliary saline hydrolyase and increased resistance to macrolides[98]. It is associated with an increased risk of asthma and weight gain. The effect of penicillin on intestinal flora was weaker than that of macrolides[99]. In addition to antibiotics, many Chinese herbal extracts can alter the intestinal flora[100]. A study in Taiwan Chang Gyeong Hospital found that ganoderma lucidum extract can reduce the body weight of high-fat-diet mice, reduce inflammatory response and insulin resistance, reduce intestinal flora Firmicutes/Bacteroidetes ratio and endotoxin levels, maintain the integrity of the intestinal barrier and reduce endotoxemia. It has been found that both berberine and metformin can reverse the changes in intestinal flora in mice induced by high-fat diet and significantly reduce the diversity of intestinal flora. Proton pump inhibitors (PPIs) are among the top 10 drugs widely used worldwide, and studies have found that PPI use is associated with intestinal infections, particularly CDI[101]. PPI users had significantly increased intestinal flora of *Enterococcus*, *Streptococcus*, *Staphylococcus* and opportunistic *Escherichia coli*[102]. In addition, cardiovascular drugs such as statins, antihypertensive drugs, antiplatelet aggregation drugs, as well as opioids and antidepressants can affect the composition of gut microbes[103]. As confounding factors such as gender and age have a great influence on these results, confounding factors should be minimized, and random number tables should be used to conduct random-grouping studies, because the reactions caused by these drugs may be caused by microbial disorders[104].

CONCOMITANT LIFESTYLE AND DIET

When a donor transplants microbes to a recipient, the difference in microbial composition between the two may be partly due to lifestyle differences between the donor and recipient[105]. If the recipient's lifestyle does not change to that of the donor after FMT, then the effect on the recipient's microbial makeup may disappear over time. People living in different continents and regions have their own unique dietary habits[106]. The diet of Europeans is rich in cheese, butter and other high-fat and high-calorie foods, while the diet of Africans is low in calories and high in dietary fiber[107]. High-throughput sequencing comparing the intestinal microbiota of European children with that of rural children from Burkina Faso, a landlocked country in Western Africa, revealed significant differences between the two[108]. *Prevotella* and Xylos bacteria, which are associated with cellulose and xylan hydrolysis, were completely absent in the intestinal flora of European children on a high-calorie and

high-fat diet, while the intestinal flora of African children on a low-calorie and high-fiber diet was rich in Bacteroidetes, especially *Prevotella* and Xylos bacteria, while Firmicutes were relatively rare[109]. In addition, African children were found to have significantly more SCFAs in their intestines than European children had, and the abundance of Enterobacteriaceae (mainly *Shigella* and *E. coli*) was found to be lower than that of European children[110]. These results suggest that the intestinal flora of African children has adapted to a diet rich in polysaccharides to ensure adequate energy intake from a fiber-rich diet and to reduce the incidence of intestinal inflammatory and infectious diseases[111]. The lack of dietary fiber in the diet of European children may be responsible for the loss of prevosiella and Xylos bacteria associated with cellulose and xylan hydrolysis[112]. Another study found that increased dietary fiber intake increased the diversity of the gut flora, as well as *Prevotella* abundance[113].

Numerous studies have shown that changes in diet determine microbial composition[114]. When autologous FMT is administered during a particular diet, the beneficial effects of the diet persist even if the diet is no longer continued[115]. Conversely, changes in an individual's microbial composition also affect diet. A large number of studies have shown that dietary response to FMT may be related to changes in microbial composition[116]. Thus, FMT transplantation using a standardized diet during clinical interventions may be more effective because an important source of microbiome variation has been eliminated, but it has been overlooked in many studies[117].

POTENTIAL MECHANISM OF ACTION

Although the causal relationship between intestinal microbiota and disease is still unclear, it is sufficient to inspire researchers to implement strategies for disease management by regulating intestinal microbiota[118]. Dietary management, antibiotics, probiotics and other interventions can directly or indirectly enable the reconstruction of intestinal flora[119]. FMT is based on microbiota treatment, in which the isolated functional bacteria are transplanted into the patient to reconstruct intestinal flora and achieve a steady state of the gut microbes so as to attain the purpose of disease treatment[120]. The specific mechanism of FMT has not been clarified yet, and its complex mechanism cannot be replaced and explained by a single strain or single signal[121]. In 2007, the "Human Microbiome Project", also known as the "Human second Genome Project", became the cornerstone of human exploration and understanding of intestinal microbes[122]. With the in-depth study of intestinal flora, the causal relationship between intestinal microbes and diseases will become more clear, and the specific action mechanism of FMT will become more clear.

FMT has made great progress in the treatment of recurrent CDI. However, due to infection and repeated use of antibiotics, the diversity of intestinal flora is low and the interactions between microorganisms are affected. Studies have shown that FMT with a healthy diversity of microbiome may increase microbial diversity levels to normal levels and enhance microbial interactions[123]. Recent studies have shown that microorganisms produced by biliary saltase may help improve the efficacy of FMT in the treatment of recurrent CDI, as the enzyme degrades taurocholic acid and effectively inhibits *C. difficile*[124]. Other studies have shown that FMT treatment can cause some subsequent problems, such as the use of antibiotics to cause bacterial dysregulation, leading to non-*C. difficile*-dependent colitis recurrence, and thus requiring new FMT corrective treatment[125].

Studies have shown that the most important source of fecal genes is prokaryotic viruses (phages)[126]. Phages are probably also the most overlooked in terms of FMT. Because diarrhea is partially relieved in patients with a small amount of microbial FMT during recurrent CDI, this suggests that phages may play an important role in maintaining host health by regulating gut microbiome composition and its phenotype[127]. Phages play an important role in gene expression of host bacteria and even determine their survival. Thus, FMT may function through donor phage regulation of the recipient flora. Currently, phage transplantation is done through aseptic filtration, which has the advantage of reducing bacterial infection. In addition, a large number of clinical studies are needed to show that phage transplantation has greater application value and potential in some diseases.

The mechanism of action of FMT therapy may be realized through multiple pathways, which may vary according to the FMT condition. However, one of the important mechanisms may be altered microbial metabolite production. This may occur during transplantation or subsequently by newly colonized microorganisms. The effect of the production of large quantities of small molecules by microorganisms on the host needs to be further clarified[128]. The most significant is SCFA butyrate, produced mainly by fibrinolytic enzyme strains, which reduces intestinal permeability and provides nutrients to intestinal cells, producing epigenetic effects[129]. In addition, fibrinolytic enzyme strains has anti-inflammatory properties and can reduce the incidence of T1D in NOD mice[130]. Therefore, FMT may modulate immune activity through autoantigens. In addition, studies have shown reduced production of butyrate strains in T1D[131]. However, when T1D patients were given high concentrations of butyrate, no significant changes were detected in immune cells, and when T1D patients were given FMT, butyrate as an active regulator of protective b cells and immune cells was not detected by metabolomics[132]. As a result, the research impact of using noninvasive biomarkers for microbial metabolism has been largely underestimated[133]. This phenomenon may partly explain some of the

differences between rodent and human studies. In addition, it should be made clear that interventional studies cannot completely exclude the potential mechanism of action of butyrate in T1D[134]. Therefore, it is a long and tortuous road to find meaningful microorganisms from clinical observational studies to improve clinical outcomes.

APPLICAATION PROSPECT OF FMT IN CLINICAL RESEARCH

FMT is a new theory and technology that has prospects in the treatment of intestinal microbiome disorders. However, the mechanism of action, ethical issues and effects of FMT are still controversial. The methodology of donor screening, the preparation and state of fecal bacteria solutions, and the approaches to transplantation are not uniform, and there are different reports on the safety and efficacy of FMT treatment[135]. In the future, more and higher-quality randomized controlled clinical trials should be carried out to address the above problems, so as to provide more adequate evidence-based medical evidence[136]. It is certain that with the deepening of scientific research, the mechanism of FMT will be gradually clarified; the intestinal microbial spectrum, microbial metabolites and their association with diseases will be more clear; and the FMT methodology will be more standardized[137]. Despite its limitations, FMT is currently one of the most important tools for studying the role of microorganisms in the pathogenesis of a range of chronic diseases. To improve the effectiveness of studies, further standardization of FMT should be carried out, such as dosage, transplantation method, and whether to use alternate pretreatment of fresh or frozen preparations[138]. In addition, accurate assessments and calculations are required to avoid type I errors in order to accurately assess efficacy. Of course, many meetings and forums are needed to reach consensus.

CONCLUSION

Donor FMT can restore intestinal microbial function and improve clinical outcomes. Therefore, the question in the future is whether the addition of specific strains of FMT to microbial-targeted therapies can help improve diet and drug therapy to improve human health. Therefore, in order to improve the clinical treatment of recurrent CDI, there is a need for more standardized FMT techniques. Rapid advances in untargeted molecules and bioinformatics have made it possible to analyze in detail the potential mechanisms of action of FMT. These results can identify important microorganisms and their metabolites, which may be used as probiotics, probiotics and epigenetic bacteria to enhance the therapeutic effect of FMT, or even replace FMT, for treatment of metabolic diseases.

FOOTNOTES

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MINIREVIEWS

Up to seven criteria in selection of systemic therapy for hepatocellular carcinoma

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Abstract

Barcelona clinic liver cancer (BCLC) intermediate stage hepatocellular carcinoma is a heterogenous disease. Transarterial chemoembolization is offered as the first line therapy in this disease stage. Recent advances in systemic therapy have markedly improved outcomes even in advanced stage disease. The use of systemic therapy in BCLC intermediate stage disease may now be of therapeutic benefit in selected patients. We will focus on "the up to seven" criteria and its utility in selecting systemic therapy.

Key Words: Chemoembolization; Hepatocellular carcinoma; Immunotherapy; Drug combinations; Review; Medical oncology

Core Tip: Barcelona clinic liver cancer intermediate stage disease that exceeds "the up to seven" criteria, especially with lesions larger than 5 cm, is less likely to respond to transarterial chemoembolization (TACE) alone and is therefore a disease that may respond better to systemic therapy. The use of "the up to seven" criteria can be a helpful guidepost for when to consider systemic therapy alone or in addition to TACE. With the recent breakthroughs in immunotherapy for advanced hepatocellular carcinoma which clearly demonstrated overall survival advantage over single agent tyrosine kinase inhibitors sorafenib, it is promising that the use of immunotherapy would likely lead to better outcome when used in intermediate disease.

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INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for 80% of primary liver cancers worldwide[1]. It is one of the cancers with the highest mortality rate, with a 5-year survival rate of only 20%[2]. Treatment of HCC depends on the staging according to the Barcelona clinic liver cancer (BCLC) staging system which is determined by tumor characteristics, liver function (assessed by Child-Pugh score) and patient performance status[3]. Using these criteria, patients may be categorized as early, intermediate or advanced stage disease.

"THE UP TO SEVEN" CRITERIA

Candidates for liver transplantation are most often assessed using the Milan Criteria which was published in 1996. It set strict guidelines to identify individuals who are most likely to benefit from transplantation in an effort to minimize cancer recurrence and maximize overall survival (OS)[4].

Recently the authors of the Milan Criteria have purposed an expansion of the guidelines termed "the up to seven" criteria. In a study of 1556 patients who underwent liver transplantation for HCC, the authors developed software that searched for combinations of tumor characteristics exceeding the Milan criteria, but resulted in an estimated 5-year OS of at least 70%. These found characteristics were termed "the up to seven" criteria. Seven being the sum of the size in centimeters and the number of tumors. Examples, as illustrated in the study, one tumor up to 6 cm in size 6 + 1 = 7, to multiple tumors with seven as the sum of the size plus number (*i.e.*, two tumors up to 5 cm in total size , three tumors up to 4 cm in total size, *etc.*)[5].

A recent retrospective study comparing OS among liver transplant patients based on their selection by the Milan or "the up to seven" criteria found no differences between the two groups[6].

TRANSARTERIAL CHEMOEMBOLIZATION THERAPY IN INTERMEDIATE STAGE DISEASE

Patients with intermediate stage disease, classified by multi-nodular disease, Child-Pugh A-B, with an ECOG performance status of 0, with no extra hepatic spread are candidates for transarterial chemoembolization (TACE).

TACE therapy preferentially targets HCC due to the tumor's disproportionally higher arterial vascular supply compared to normal liver parenchyma[3]. The success of TACE was demonstrated with two randomized control trials (RCTs) and a meta-analysis[7-9].

TACE therapy can be given in different forms including by conventional TACE (cTACE), by drug-eluting beads-TACE (DEB-TACE) and by bland embolization (TAE) which does not use chemotherapy [10].

In cTACE, a cytotoxic drug that has been emulsified in Lipidol is intra-arterially injected followed by the embolic agent. The efficacy of cTACE was recently reaffirmed with an estimated average median OS of 30 mo[3,11-13].

In DEB-TACE, the embolic agent is loaded with cytotoxic medications[14].

In TAE, embolization is performed without a cytotoxic drug[15].

The differences in outcomes between these techniques have been compared. In a phase III trial the Precision Italia Study Group compared DEB-TACE with cTACE and found no difference in response rates, median time to progression, or survival[16]. This finding was also supported by a meta-analysis of 4 RCTs and 8 observational studies which concluded there was a non-superiority of DEB-TACE *vs* cTACE[10]. Similarly a meta-analysis comparing TAE *vs* cTACE found no difference in OS, or objective response to therapy[15].

However despite similar outcomes TAE therapy has its critics who note TAE therapy results in less tumor necrosis compared to other forms of TACE therapy which may prevent its complete adoption[17,18].

Another criticism of TACE therapy in general is that as a therapy it is non-standardized[19]. This is especially true of TACE therapy with cytotoxic agents as there are several chemotherapeutic drugs which may be used[20]. Additionally the extent to which stasis of flow is achieve in the target vessel is also physician operator dependent[21]. This lack of standardization and dependency on the skill of the interventionist makes a more uniform approach *via* systemic therapy desirable.

THE POTENTIAL OF SYSTEMIC THERAPY IN INTERMEDIATE STAGE DISEASE

Predictive factors of whether to initiate TACE include: Tumor size, vascularity, arterial anatomy, infiltrative *vs* nodular growth, presence of splenomegaly, Alfa-fetoprotein changes, albumin and bilirubin levels[22]. Furthermore the decision to repeat TACE should depend on the response based on modified RECIST criteria to prior TACE therapy[23,24]. Of note as radiographic assessment is dependent on the reading physician it is important that this be carried out by a radiologist experienced in HCC[25]. Patients who have an initial complete response to TACE may undergo a second procedure if warranted as long as they are still candidates for therapy. For patients with a partial response or even stable disease repeat treatment at regular intervals may be offered but that decision should be weighed against liver toxicity from treatment[22,26]. Patients with no objective response to two TACE treatments are unlikely to benefit from further TACE and would likely benefit from alternative therapy[26,27]. Even if clinicians are hesitant to choose systemic therapies as initial treatments in intermediate stage HCC, survival maybe improved by switching to these therapies in TACE refractory disease[28,29]. The 2018 OPTIMIS trial followed 1650 patients with unresectable HCC who were to undergo TACE therapy. 31% of these patients became TACE ineligible during the study but only 9% received sorafenib when deemed ineligible for TACE with the remainder having systemic therapy delayed or not receiving it at all[30]. It is therefore critical to determine which patients would be unlikely to benefit from TACE early as to not delay appropriate care (Table 1).

Although current guidelines recommend TACE as first line treatment in intermediate stage HCC, this disease is characterized by high heterogeneity and its real world management may be as equally diverse [27,31].

HCC exceeding "the up to seven" criteria is less likely to respond to TACE due to higher tumor burden[32,33]. In fact, patients beyond "the up to seven" criteria who undergo TACE had higher rates of liver function deterioration post procedure[34]. This is particularly concerning considering poor liver function may preclude patient's from promising systemic therapies[35,36].

In a retrospective propensity matched study by Kudo *et al*[37], patients with BCLC intermediate stage HCC beyond "the up to seven" criteria were treated with lenvatinib systemic therapy or TACE. Whereas TACE treatment led to a decline in liver function, lenvatinib treatment did not result in such a decline. OS was significantly longer in the lenvatinib group 37.9 mo *vs* 21.3 mo; hazard ratio: 0.48, $P < 0.01$. In the study protocol, after progression on lenvatinib, second line treatment including TACE, hepatic arterial infusion chemotherapy, sorafenib, regorafenib, or investigational therapies were allowed. Of note, about 70% of the patients who received lenvatinib underwent subsequent TACE. Patients who received TACE as initial treatment where allowed to undergo repeat TACE. After becoming TACE refractory, second line treatments were identical to the ones in the levantinib group [37].

Recently results from the phase III IMbrave-150 trail have changed management of locally advanced or metastatic/unresectable HCC who are either not TACE candidates or became refractory to TACE. In this trial, the immunotherapy and vascular endothelial growth factor inhibitor combination atezolizumab + bevacizumab was compared against sorafenib, the old standard of care. Median OS was 19.2 mo with the combination therapy *vs* 13.4 mo with sorafenib [HR, 0.66 (95%CI: 0.52-0.85); $P = 0.0009$][38,39]. This combination was the first to show clinical benefit over sorafenib since 2007 and is now first line therapy in the treatment of advanced stage liver cancer[40]. Immunotherapy doublet combination treatments have also shown promise. In the Checkmate-40 trial, nivolumab plus ipilimumab in the second line setting (after sorafenib) showed median OS of 22.8 mo with an overall response rate (ORR) of 32%[41]. A similar combination in a phase II study using the anti-programmed death-ligand 1 antibody durvalumab plus tremelimumab (CTLA-4 antibody) for patients who progressed on, were intolerant to, or refused sorafenib showed a median OS of 18.7 mo and an ORR of 22.7%. A trial of this combination in the first line is being tested in the phase III HIMALAYA study[42]. A press releases from the trial stated that the combination significantly improved OS compared to sorafenib with an HR of

Table 1 Considerations in initiating systemic therapy over transarterial chemoembolization[26,44,53-55]

No.	Considerations
1	Tumor exceeds "the up to seven" criteria
2	Tumor(s) larger than 5cm
3	Contiguous multinodular tumors
4	Poorly differentiated or undifferentiated HCC
5	No objective response to 2 consecvutive TACE treatments

HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization.

Table 2 Combination therapy trials

Trial therapies	Study name	Phase	Patient number or estimation	ORR	Median PFS	Median OS
Lenvatinib + TACE *vs* Lenvatinib[47]	LAUNCH	Phase 3	338	54.1% *vs* 25%	10.6 mo *vs* 6.4 mo	17.8 mo *vs* 11.5 mo
(cTACE or DEB-TACE) + durvalumab followed by durvalumab + placebo *vs* (DEB-TACE or cTACE) + durvalumab followed by durvalumab + bevacizumab *vs* (DEB-TACE or cTACE)[48]	EMERLD	Phase 3	600	In progress	In progress	In progress
Lenvatinib + Pembrolizumab + TACE *vs* Placebo + TACE[49]	LEAP-012	Phase 3	950	In progress	In progress	In progress
Nivolumab + Ipilimumab + TACE *vs* Nivolumab + Placebo + TACE *vs* Placebo + Placebo + TACE[50]	Checkmate-74W	Phase 3	765	In progress	In progress	In progress
Brivanib + TACE *vs* Placebo + TACE[51]	BRISK-TA	Phase 3	502	48% *vs* 42%	8.4 mo *vs* 4.9 mo[1]	26.4 mo *vs* 26.1 mo
Oranitib + TACE *vs* Placebo + TACE[52]	ORIENTAL	Phase 3	889	Not reported	2.9 mo *vs* 2.5 mo[1]	31.1 mo *vs* 32.3 mo
Tremelimumab + TACE[53]		Phase 2	11	18%	7.4 mo[1]	13.6 mo

[1]Reported as time to radiographic progression.
ORR: Overall response rate; PFS: Progression free survival; OS: Overall survival; TACE: Transarterial chemoembolization.

0.78[43,44].

In select BCLC intermediate stage disease systemic therapy should be considered in the frontline setting, especially for patients who have been refractory to TACE or in whom TACE is unlikely to be effective. Patient's unlikely to respond well to TACE include patients who exceed "the up to seven" criteria, as well as those who have tumors without a clear boundary, multifocal tumors, or poorly differentiated HCC[33,34,36,45,46].

As a heterogenous disease BCLC intermediate stage HCC maybe best treated with combination therapy. In fact, the success of combination therapy in advanced disease is now being tested in BCLC intermediate stage disease. Current investigations that combine TACE with systemic therapy include the phase III LAUNCH study in which patients with BCLC stage C disease was treated with lenvatinib + TACE *vs* lenvatinib alone. The combination group saw an improved OS from 11.5 to 17.8 mo. Additional the combination had higher ORR, 54.1% *vs* 25%, and higher disease control rate (DCR), 94.1% *vs* 73.2%, as well as a longer progression free survival, 10.6 mo *vs* 6.4 mo[47]. Other upcoming TACE and systemic therapy combination treatments include the studies EMERLD-1, LEAP-012, and Checkmate-74W. EMERLD-1 will assess efficacy and safety for durvalumab monotherapy with DEB-TACE or cTACE followed by durvalumab with or without bevacizumab therapy in patients with HCC not amenable to curative therapy. LEAP-012 will test lenvatinib plus pembrolizumab *vs* placebo in combination with TACE in patients with intermediate HCC. Checkmate-74W will analyze the combination of dual immune checkpoint blockade and TACE *vs* mono-therapy immune checkpoint blockade and TACE for patients with HCC exceeding the up to seven criteria[48-50].

Although these ongoing trials are exciting, it is worth noting that several studies which combined TACE and systemic therapy have failed to show desired efficacy. These include BRISK-TA and ORIENTAL which both compared targeted therapy and TACE to TACE alone. In both trials there was no improvement in OS compared to TACE alone[51,52]. Finally in a 2017 study by Duffy *et al*[53] the

addition of anti CTLA-4 immunotherapy in 11 patients previously treated with TACE showed a OS of 13.6 mo which is comparable to systemic therapy alone[53] (Table 2).

CONCLUSION

BCLC intermediate stage disease that exceeds "the up to seven" criteria, especially with lesions larger than 5 cm, is less likely to respond to TACE alone and is therefore a disease that may respond better to systemic therapy[32,33,37,54]. The use of "the up to seven" criteria can be a helpful guidepost for when to consider systemic therapy alone or in addition to TACE. With the recent breakthroughs in immunotherapy for advanced HCC which clearly demonstrated OS advantage over single agent tyrosine kinase inhibitors sorafenib, it is promising that the use of immunotherapy would likely lead to better outcome when used in intermediate disease. However, this conjecture requires validation from prospective phase III studies.

Improvements in the treatment of liver cancer have the ability to change the lives of the nearly 800000 patients diagnosed with liver cancer annually. The use of TACE therapy rightfully remains a cornerstone of treatment. However for patients who are unlikely to benefit from TACE therapy alone such as patients exceeding "the up to seven" criteria, alternative treatments including systemic therapies warrant consideration especially with recent advancements in the field.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Family with sequence similarity 134 member B-mediated reticulophagy ameliorates hepatocyte apoptosis induced by dithiothreitol

Yi-Xin Guo, Bing Han, Ting Yang, Yu-Si Chen, Yi Yang, Jia-Yao Li, Qin Yang, Ru-Jia Xie

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Abstract

BACKGROUND

Endoplasmic reticulum (ER) stress-related hepatocyte apoptosis is responsible for multiple hepatic diseases. Previous studies have revealed that endoplasmic reticulophagy (ER-phagy) promotes the selective clearance of damaged ER fragments during ER stress, playing a crucial role in maintaining ER homeostasis and inhibiting apoptosis. Family with sequence similarity 134 member B (FAM134B) is a receptor involved in ER-phagy that can form a complex with calnexin (CNX) and microtubule-associated protein 1 light chain 3 (LC3). The complex can mediate the selective isolation of ER fragments to attenuate hepatocyte apoptosis. However, the precise regulatory mechanisms remain unclear.

AIM

To elucidate the effect of FAM134B-mediated ER-phagy on ER stress-induced apoptosis in buffalo rat liver 3A (BRL-3A) rat hepatocytes and the potential regulatory mechanisms.

METHODS

ER stress-related hepatocyte apoptosis was induced using dithiothreitol (DTT). Proteins related to ER stress and autophagy were measured with western blotting. Protein complex interactions with FAM134B were isolated by co-immunoprecipitation. ER-phagy was evaluated in immunofluorescence experiments. Cell cycle distribution and apoptosis were measured by flow cytometry. Mitochondrial Ca^{2+} levels were evaluated by the co-localization of intracellular Ca^{2+}-tracker and Mito-

tracker. The small interfering RNA against *FAM134B* was used to knockdown *FAM134B* in BRL-3A cells.

RESULTS

ER stress-related and autophagy-related proteins in BRL-3A cells were elevated by both short and long-term DTT treatment. Furthermore, co-immunoprecipitation confirmed an interaction between FAM134B, CNX, FAM134B, and LC3 in BRL-3A cells. Immunofluorescence assays revealed that autolysosomes significantly decreased following short-term DTT treatment, but increased after long-term treatment. Mitochondrial Ca^{2+} levels and apoptotic rates were dramatically elevated, and more cells were arrested in the G1 stage after short-term DTT treatment; however, these decreased 48 h later. Moreover, FAM134B downregulation accelerated mitochondrial apoptotic pathway activation and aggravated hepatocyte apoptosis under ER stress.

CONCLUSION

FAM134B-mediated ER-phagy attenuates hepatocyte apoptosis by suppressing the mitochondrial apoptotic pathway. Our findings provide new evidence highlighting the importance of FAM134B-mediated ER-phagy in attenuating hepatocyte apoptosis.

Key Words: Hepatocytes; Reticulophagy; Family with sequence similarity 134 member B; Apoptosis; Endoplasmic reticulum stress; Endoplasmic reticulum homeostasis

Core Tip: We show that family with sequence similarity 134 member B (FAM134B)-mediated reticulophagy maintains the endoplasmic reticulum (ER) homeostasis in ER-stressed hepatocytes *via* the clearance of damaged ER fragments. Thereby FAM134B-mediated reticulophagy ameliorates dithiothreitol-induced hepatocyte apoptosis. Our findings provide emerging evidence of the prominence of ER-phagy in ER stress-related hepatocyte apoptosis. FAM134B may represent a potential therapeutic target for liver disease treatment.

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INTRODUCTION

Endoplasmic reticulum (ER) stress-related hepatocyte apoptosis participates in multiple hepatic diseases, including viral hepatitis[1], hepatic fibrosis[2], fatty liver[3,4] and cirrhosis[5]. Therefore, the alleviation of ER stress-mediated hepatocyte apoptosis is crucial in the treatment of hepatic diseases. Recent findings have indicated that endoplasmic reticulophagy (ER-phagy) promotes degradation of damaged ER fragments during ER stress. Although ER-phagy has a vital role in maintaining ER homeostasis and inhibiting cell apoptosis[6-8], the exact regulatory mechanisms behind this are largely unknown.

Glucose-regulated protein 78 (GRP78) is a prominent ER molecular chaperone, while calnexin (CNX) is a membrane-bound lectin protein in the ER that can increase the protein folding capacity[9,10]. Even though the excessive build-up of misfolded or unfolded proteins can be alleviated *via* ER stress, previous studies reported that a selective autophagic mechanism, defined as ER-phagy, can also be activated by ER stress to restore ER homeostasis[11,12]. Family with sequence similarity 134 member B (FAM134B), an ER-resident protein, may interact with CNX in the cytosol or the ER membrane[13]. Since FAM134B is not predicted to have an ER lumenal domain, there is an indirect interaction between FAM134B and lumenal proteins through the lumen-resident segment, which has a chaperone activity attributed to CNX. CNX forms transient but relatively stable complexes with unfolded ER proteins until they either become folded or are degraded. Moreover, it has been reported that as with other cargo receptor molecules, FAM134B can interact directly with microtubule-associated protein 1 light chain 3 (LC3) when its LIR motif is exposed. The CNX-FAM134B-LC3 complex can mediate the selective isolation of ER fragments containing misfolded proteins, which are subsequently transported to lysosomes for degradation[14-16]. Thus, FAM134B-mediated ER-phagy may play an essential role in

maintaining ER homeostasis and promoting cell survival. However, it is unclear whether FAM134B-mediated ER-phagy is involved in the regulation of hepatocyte apoptosis induced by ER stress. In this study, dithiothreitol (DTT) was used to induce ER stress in buffalo rat liver 3A (BRL-3A) hepatocytes, and the expression of ER stress-related and autophagy-related proteins was assessed. In addition, small interfering RNA (siRNA) was used to knockdown the expression of *FAM134B* in hepatocytes and an apoptosis analysis followed. Our study reveals an emerging role of FAM134B-mediated ER-phagy in ER stress-mediated hepatocyte apoptosis, which may provide a novel target for the treatment of hepatic diseases.

MATERIALS AND METHODS

Antibodies and reagents

Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, United States). Trypsin-EDTA solution, trypsin solution without EDTA, and penicillin-streptomycin were purchased from Biological Industries (BioInd, Israel). Bicinchoninic acid (BCA) protein assay kit, DTT, RIPA lysis buffer, and protease inhibitor were obtained from Solarbio (Beijing, China). Annexin V-FITC/PI Apoptosis Detection Kit and Cell Cycle Detection Kit were purchased from KeyGEN BioTECH (Nanjing, China). PVDF membranes were obtained from Merck Millipore. Rabbit polyclonal antibody against FAM134B was purchased from Proteintech (Wuhan, China). Rabbit polyclonal antibodies against ATG12, cytochrome c (cyt c), and cleaved caspase-3 were obtained from Cell Signaling Technology (Danvers, MA, United States). Rabbit polyclonal antibodies against β-actin, LC3, CNX, CHOP and GRP78, and the Ca^{2+} indicator (Rhod-2 AM) were purchased from Abcam (Cambridge, United Kingdom). Dynabeads protein G immunoprecipitation kit and lipofectamine 3000 reagent were purchased from Thermo Fisher Scientific, Inc. HRP-labeled Goat Anti-Rabbit IgG (H + L), Mito-Tracker Green, Lyso-Tracker Green, ER-Tracker Red, and immunofluorescence-related reagents were purchased from Beyotime Institute of Biotechnology (Nanjing, China).

Cell culture and experiment protocol

BRL-3A cells, bought from Cell Bank of the Chinese Academy of Sciences (Shanghai, China), were cultivated and maintained in DMEM culture media supplemented with 1% penicillin-streptomycin and 10% FBS. BRL-3A cells were seeded at 37 °C and 5% CO_2 in a constant temperature and humid atmosphere, pre-cultured every 3 d, and further passaged until the density reached approximately 80%. To induce the ER stress, BRL-3A cells were treated with DTT (2.0 mmol/L based on previous studies[17]) for 0, 3, 6, 12, 24, or 48 h.

Apoptosis assessment

Cells were cultured to 80% confluency and treated with 2.0 mmol/L DTT for the specified point-in-time intervals. To determine the efficacy of the different DTT treatments, a cell apoptosis analysis was evaluated with flow cytometry. Each group of cells was trypsinized without EDTA and rinsed thrice with PBS. After centrifugation at 2000 rpm for 5 min, cells were loaded with 500 μL binding buffer and labeled with 5 μL of Annexin V-FITC/PI, according to the manufacturer's instructions. Labeled cells were detected and analyzed with flow cytometry and NovoExpress® software 1.4.1. The experiments were performed in triplicate.

Cell cycle analysis

To determine the effect of DTT's 0, 3, 6, 12, 24, and 48 h incubation on the cell cycle progression of BRL-3A, the harvested cells were trypsinized without EDTA and rinsed three times with cold PBS, followed by fixation with 70% ethanol in cold storage. After 24 h incubation at 4 °C, 500 μL PI/RNase was added to each group and maintained at 37 °C for 60 min in a dark place. Stained cells were processed using flow cytometry and further measured *via* the NovoExpress® software 1.4.1. The experiments were performed in triplicate.

Western blot analysis

BRL-3A cells were grown on 10 cm diameter dishes and treated with 2.0 mmol/L DTT for different times. Cells were rinsed three times with pre-cooled PBS after experimentation and collected with cell scrapers in 100 μL RIPA buffer containing 1 mmol/L PMSF. After centrifugation at 12000 rpm for 25 min at 4 °C, the concentrations of total cellular protein extracts were determined using the BCA kit (Solarbio Science, Beijing, China), and known concentrations of BSA were used as standard. The total cellular protein extracts were denatured by boiling at 100 °C using dry bath incubator (Hangzhou Miu Instruments Co., Ltd, Zhejiang, China). Protein samples (30–40 mg) were loaded onto SDS-PAGE and transferred onto PVDF membranes for immunostaining. After blocking with 5% defatted milk for 90 min, membranes were stained overnight with primary antibodies, including β-actin (1:1000), GRP78 (1:1000), CNX (1:3000), ATG12 (1:1000), LC3 (1:1000), FAM134B (1:1000), CHOP (1:1000), cleaved

caspase-3 (1:1000), cyt c (1:1000) in cold storage, followed by incubation with secondary antibodies (1:4000). The density of protein bands on membranes was exposed and quantified *via* fluorography using Image J software. The images shown are representative of experiments carried out at least three times.

Co-immunoprecipitation analysis

BRL-3A cells, treated with DTT (2.0 mmol/L for 0 h and 24 h), were lysed in RIPA lysis buffer and the lysates were centrifuged at 12000 rpm for 15 min at 4 °C. The supernatant was resuspended in ice-cold PBS to a total volume of 500 μL, and 5 μL of the designated antibody was added overnight at 4 °C. The next day, the Ab-Ag complexes were bound to Dynabeads magnetic beads on a rotary shaker for 10 min. The magnetic bead-Ab-Ag complex was washed and eluted by adding a washing buffer and elution buffer, respectively, according to the manufacturer's protocol. Immunocomplexes were heated for 5 min at 100 °C and prepared for analysis by western blot. The images shown are representative of experiments carried out at least three times.

Calcium imaging and mitochondrial labeling

To observe the effects of DTT treatment at 2.0 mmol/L for specified time points, mitochondrial Ca^{2+} levels were determined using Rhod-2 AM, a specific detection dye for calcium. The treated cells were rinsed with HBSS three times and stained with a mixture of 5 μM Rhod-2 AM and 20 nM Mito-Tracker Green at 37 °C for 30 min in the dark. Finally, live cells were extensively rinsed thrice by adding HBSS without calcium, and images were visualized with Zeiss LSM Image Browser using a Zeiss LSM 900 confocal microscope. The images shown are representative of experiments carried out at least three times.

Live imaging of ER and lysosome

To observe the intracellular localization of the ER and lysosomes, after treatment with 2.0 mmol/L DTT for 0, 3, 6, 12, 24, and 48 h, ER and lysosomes were stained with ER-tracker and Lyso-tracker. Prior to staining, trackers were diluted appropriately in DMEM, on the basis of the manufacturer's instructions. Following dilution, cells were simultaneously incubated with the two trackers listed above, maintained for 30 min at 37 °C, and finally rinsed thrice with HBSS. Stained cells were visualized under the Zeiss LSM 900 confocal microscope. Images shown are representative of experiments carried out at least three times.

SiRNA transfections

Specific siRNA against buffalo rat *FAM134B* was designed and synthesized by OriGene. Product number and targeting sequence: SR510501A-rGrGrArArGrUrGrGrUrUrUrArUrCrArArArUr-UrCrUrGrATA; SR510501B-rArArArUrUrUrGrArCrUrUrArCrArGrUrGrGrArArArCrCAA; SR510501C-rArArGrUrGrGrUrUrUrArUrCrArArArUrUrCrUrGrArUrAGA. Cells were cultured in six-well dishes until the density of cell fusion reached 60%. Briefly, 75 pmol of *FAM134B* siRNA were added to Lipofectamine 3000 Transfection Reagent and gently mixed for 15 min, then administered to BRL-3A cells, which were resuspended in DMEM. After transfection for 6 h, cells were washed, and then supplemented with fresh medium. Finally, cells were treated with DTT (2.0 mmol/L) for a further 24 h and subjected to western blot assay and apoptosis assessment.

Statistical analysis

GraphPad Prism 7 software was used to perform all the statistical analyses and prepare experimental graphs. Data are expressed as the mean ± SD. Shapiro-Wilk normality test was used to test the normal distribution of the data and all the data were fit to a normal followed by Tukey's post hoc test was performed, and a significant difference was considered as $P < 0.05$.

RESULTS

DTT-mediated ER stress upregulates ER-phagy-related FAM134B in BRL-3A cells

To assess whether the drug treatments could alter the protein expression of CNX and GRP78, BRL-3A cells were subjected to short-term (3, 6, 12, 24 h) or long-term (48 h) treatment with DTT, and the protein extracts from BRL-3A cells were analyzed by western blot. We found that treatment of BRL-3A cells with 2.0 mmol/L DTT resulted in a prominent increase in CNX and GRP78 levels, both in a time-dependent manner (Figure 1A and B). Moreover, CHOP is a specific and stress-responsive transcription factor during ER stress and its protein expression was significantly increased in the 12, 24, and 48 h groups (Figure 1A and B). However, the expression of CHOP in BRL-3A cells treated with DTT for 48 h was lower than that after DTT treatment for 24 h. These alterations in CNX, GRP78, and CHOP confirm that ER stress in BRL-3A was activated.

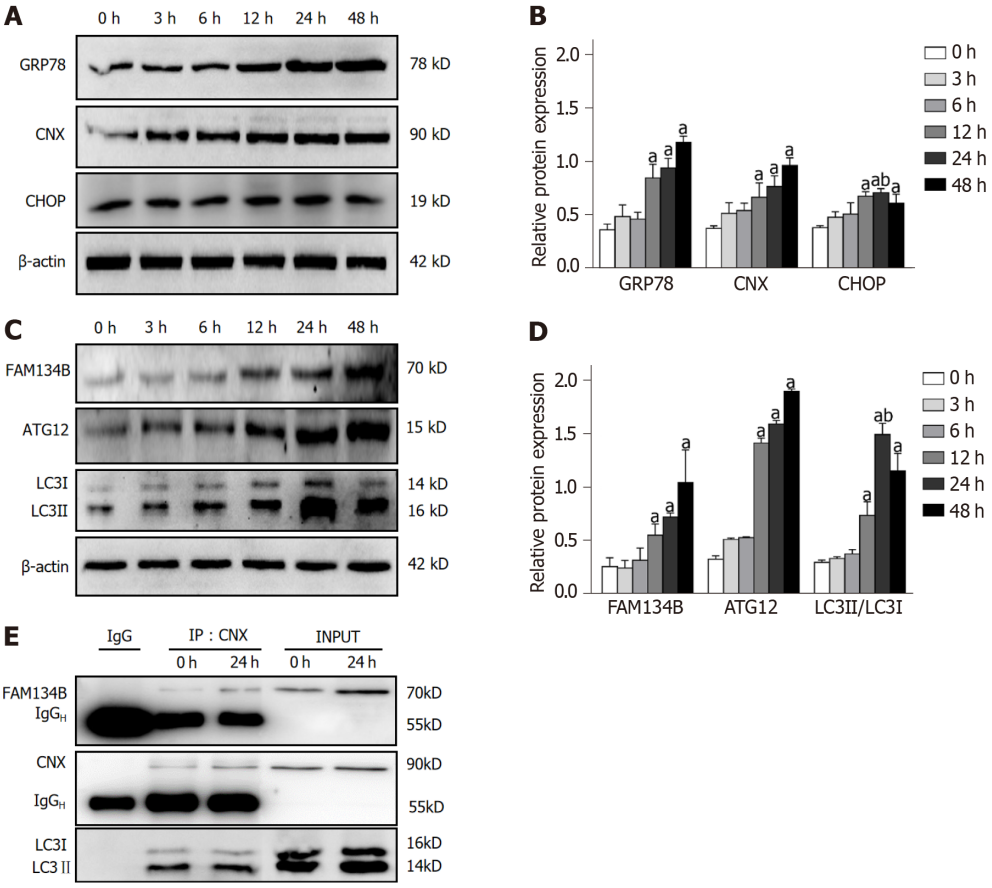

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Figure 1 Impact of the endoplasmic reticulum stressor, dithiothreitol, on endoplasmic reticulophagy mediated by family with sequence similarity 134 member B in buffalo rat liver 3A cells. A and B: Buffalo rat liver 3A (BRL-3A) cells were treated with 2.0 mmol/L dithiothreitol (DTT) for the time intervals (0, 3, 6, 12, 24, 48 h); Western blot showed the effect of endoplasmic reticulum (ER) stressor, DTT, on expression of the ER stress-related proteins glucose-regulated protein 78 (GRP78), calnexin (CNX), and C/EBP homologous protein (CHOP); β-actin was used as a control for normalization; C and D: Analysis of autophagy related gene 12 (ATG12), family with sequence similarity 134 member B (FAM134B), and microtubule-associated protein 1 light chain 3 (LC3) protein expression by western blot. Protein levels were normalized to β-actin; E: BRL-3A cells were treated with 2.0 mmol/L DTT for 0 and 24 h; co-immunoprecipitation analysis detected the presence of CNX-FAM134B-LC3 complex in BRL-3A cells. Values are represented as mean ± SD (n = 3), $^aP < 0.05$ *vs* 0 h group; $^bP < 0.05$ *vs* 48 h group.

To determine the effects of ER stress on FAM134B-mediated ER-phagy, alterations in FAM134B, ATG12, and LC3 expression were detected by western blot. As expected, DTT treatment for 3, 6, 12, 24, and 48 h increased the conversion ratio of LC3-I to LC3-II and the FAM134B and ATG12 expression levels compared to those in the 0 h group (Figure 1C and D). Thus, our results revealed that the expression of FAM134B is induced in response to ER stress.

Furthermore, we used an anti-CNX antibody to immunoprecipitate the CNX-FAM134B-LC3 complex, confirming the hypothesis that FAM134B forms a complex with CNX and LC3, exerting a positive influence on ER-phagy (Figure 1E).

Long-term DTT treatment relieved the gradually blocked ER autolysosome delivery in BRL-3A cells

Typically, ER is delivered to lysosomes and finally degraded. To analyze whether ER autolysosomes are formed, we examined the subcellular location of the ER and lysosomes using cell organelle markers. As shown in Figure 2, the treatment groups of 3, 6, 12, 24, and 48 h DTT incubation significantly alleviated the co-localization of the ER with lysosomes, compared to that in the 0 h group. Notably, the colocalization of ER and lysosomes in BRL-3A cells treated with DTT for 48 h was increased compared to those treated for 24 h (Figure 2).

Short-term DTT treatment induces mitochondrial calcium uptake while prolonged DTT treatment reduces it

Calcium in the ER can be released and transferred to the mitochondria owing to an imbalance of ER homeostasis. To explore the altered localization of calcium, collected cells were co-loaded with Rhod-2 AM and Mito-Tracker Green. In response to DTT treatment for 3, 6, 12, 24, and 48 h, the co-localized fluorescence increased considerably (Figure 3). However, the distribution of the co-localized signal was

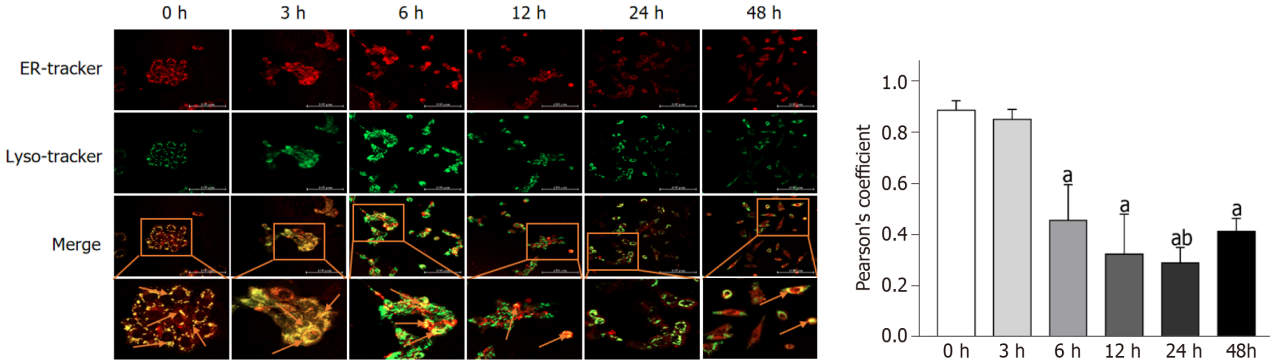

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Figure 2 Impact of dithiothreitol treatment on the formation of autolysosomes in buffalo rat liver 3A cells. After dithiothreitol treatment for 0, 3, 6, 12, 24, and 48 h, the buffalo rat liver 3A cells labeled with endoplasmic reticulum (ER)-Tracker Red and Lyso-Tracker Green were observed and captured under confocal fluorescence microscopy (200 ×) in a live cell imaging experiment. Insets show the magnification of the pictures. Scale bars indicate 100 μm. Arrows head to indicate ER-localized lysosomes. Values are represented as mean ± SD (n = 3), $^{a}P < 0.05$ *vs* 0 h group; $^{b}P < 0.05$ *vs* 48 h group.

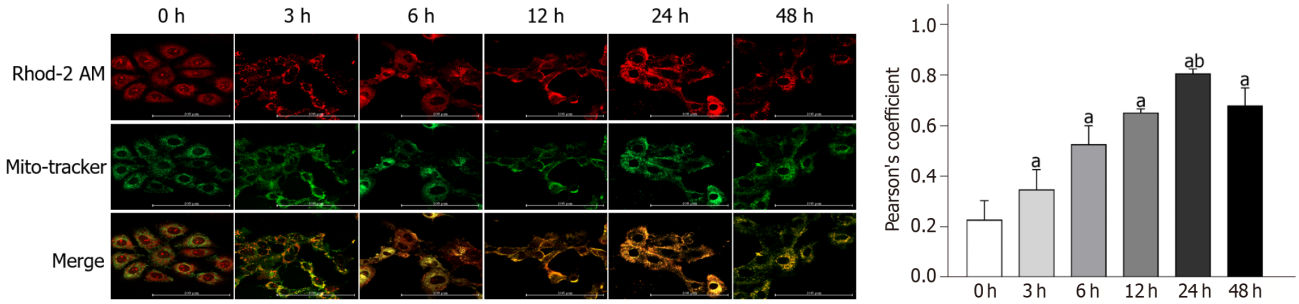

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Figure 3 Impact of dithiothreitol treatment on mitochondrial calcium uptake in buffalo rat liver 3A cells. Buffalo rat liver 3A cells were treated for 0, 3, 6, 24, and 48 h with 2.0 mM dithiothreitol, followed by co-incubating with Mitochondria-Tracker Green and Rhod-2 AM, and visualized by confocal microscopy (400 ×). Scale bars indicate 100 μm. Values are represented as mean ± SD (n = 3), $^{a}P < 0.05$ *vs* 0 h group; $^{b}P < 0.05$ *vs* 48 h group.

weaker in the 48 h group, compared to that in the 24 h group (Figure 3). These results strongly suggest that mitochondrial calcium accumulation is related to DTT treatment.

DTT treatment induces cell cycle arrest and apoptosis in BRL-3A cells, which is relieved at 48 h

To further validate that DTT treatment leads to apoptosis in BRL-3A cells, we quantitatively measured the number of apoptotic cells using the Annexin V-FITC/PI double staining assay. As shown in Figure 4A and B, the ratio of apoptotic cells treated with DTT for 0, 3, 6, 12, and 24 h exhibited a time-dependent increase. Interestingly, the apoptotic percentage in the 48 h group was significantly lower than that in the 24 h group (Figure 4A and B). Subsequently, we sought to use flow cytometry to determine the impact of DTT treatment on the cell cycle progression, and the data suggests that the proportion of BRL-3A cells in G1 phase after DTT treatment was noticeably higher than that of the 0 h group (Figure 4C and D and Table 1). Moreover, the number of cells in G1 phase in the 48 h group was smaller than that of the 24 h group.

BRL-3A cells undergo apoptosis upon FAM134B knockdown

We further verified whether *FAM134B* knockdown could alter DTT-induced apoptosis. We first investigated the transfection efficiency of siRNA with three different siRNAs targeting *FAM134B* (siRNA 1, 2, and 3) and found that the *FAM134B* siRNA2 was the most effective (Figure 5A and B). Next, we investigated FAM134B protein levels by performing a western blot on already transfected samples, which were treated with DTT for 24 h. As shown in Figure 5C and D, FAM134B and β-actin expression levels were determined, and it was found that FAM134B protein levels were down-regulated compared with the control and control siRNA groups.

It has been reported that cyt c and cleaved caspase-3 are apoptosis-related proteins and important hallmarks of apoptosis activation involved in mitochondrial dysfunction. Consequently, siRNA-mediated silencing of *FAM134B* caused a high level of cleaved caspase-3 and cyt c in BRL-3A cells treated with DTT for 24 h (Figure 5E and F). We examined the rates of apoptotic cells using Annexin-V-FITC/PI staining assays, which revealed that the apoptotic rates also increased in the *FAM134B* siRNA

Table 1 The cell cycle distribution of buffalo rat liver 3A cells treated with dithiothreitol for different times was detected by flow cytometry

Group	G_0/G_1	S	G_2/M
0 h	17.08 ± 0.13	58.48 ± 3.82	23.05 ± 4.46
3 h	24.28 ± 2.03[a]	42.12 ± 3.98[a]	33.6 ± 4.72[a]
6 h	33.91 ± 1.39[a]	25.11 ± 0.11[a]	41.71 ± 2.45[a]
12 h	41.57 ± 1.08[a]	24.81 ± 5.45[a]	33.62 ± 4.73[a]
24 h	51.83 ± 1.14[a,b]	38.1 ± 3.00[a]	10.08 ± 3.28[a,b]
48 h	38.72 ± 1.18[a]	37.12 ± 8.06[a]	24.16 ± 8.38

[a]$P < 0.05$ *vs* 0 h group.
[b]$P < 0.05$ *vs* 48 h group.

group, compared with those in the control and control siRNA groups (Figure 5G and H). These results suggest that ER-phagy mediated by FAM134B is likely to serve a cytoprotective function in response to DTT treatment in BRL-3A cells.

DISCUSSION

Hepatic injury caused by multiple harmful factors is closely associated with ER stress-induced hepatocyte apoptosis[18-20]. The ER is responsible for proper protein folding, intracellular calcium storage, and lipid biosynthesis[21,22]. Various stressors, including unfolded protein aggregation in the ER, intracellular Ca^{2+} disturbance, and pharmacological inducers, such as DTT, can disrupt ER homeostasis and lead to ER stress in hepatocytes. If the ER stress cannot be alleviated, aberrant ER stress can trigger cell apoptosis[23]. In the present study, we found that the protein levels of GRP78 and CNX, which are ER stress biomarkers, were upregulated in BRL-3A cells during ER stress. GRP78 and CNX are ER chaperone proteins and accelerate the proper folding of the accumulated unfolded proteins in the ER, which engages effector mechanisms to rebalance ER homeostasis[24,25]. A series of studies have revealed that ER-phagy is an ER selective autophagy mechanism that can promote the clearance of damaged ER lumens containing the unfolded proteins, and helps restore ER homeostasis[26-28]. ER-phagy is a critical quality control mechanism for the ER in multiple cell types. Defects in ER-phagy pathways are associated with multiple human pathologies, including infectious and neurodegenerative diseases, aging and cancer. However, whether ER-phagy is involved in the regulation of ER homeostasis in hepatocytes under ER stress remains elusive. In this study, we assessed the levels of reticulophagy-related proteins in BRL-3A cells treated with DTT. We found that the levels of FAM134B and ATG12 were markedly elevated, and the ratio of LC3II/LC3I also increased. These data indicate that DTT-induced ER stress increases the level of reticulophagy-associated proteins.

Recent findings have indicated that receptor proteins of ER-phagy play crucial roles in driving the sequestration of isolated ER fragments into autophagosomes[29]. FAM134B, an ER-anchored protein, was recently proposed as a major mammalian receptor for reticulophagy[30,31]. FAM134B contains an LC3-interacting region that can interact with LC3 protein to form autophagosomal membranes, leading to efficient ER sequestration into an autophagosomal lumen[32-34]. In a previous report, the authors found that CNX serves as a co-receptor that recognizes misfolded proteins within the ER lumen and interacts with FAM134B[35,36]. In turn, the CNX-FAM134B complex binds with LC3, the autophagosome membrane-related protein, which delivers ER lumens containing misfolded proteins to the lysosome for degradation. To investigate how FAM134B modulates ER-phagy in BRL-3A cells, immunoprecipitation was performed to detect the interaction between CNX, FAM134B, and LC3. The results confirmed that CNX interacted with FAM134B, and FAM134B interacted with LC3 after DTT treatment. Thus, the formation of the CNX-FAM134B-LC3 complex allows for the selective delivery of ER lumens containing misfolded proteins to the lysosome for eventual degradation. Complete ER-phagy indicates that autophagosomes fuse to form autolysosomes[37,38], hence, we detected the number of autolysosomes in BRL-3A cells treated with DTT. We found that the formation of autolysosomes decreased in the early stages of ER stress, whereas autolysosomes were elevated in later stages. As it has been reported that CHOP can suppress autolysosome formation[39], we speculated that decreased autolysosomes in the early stages of ER stress were associated with increased CHOP expression.

The ER is the main pool for Ca^{2+} storage, and ER dysfunction leads to Ca^{2+} efflux from the ER[40,41]. In the early stages of ER stress, the suppression of the autophagosomes' fusion with lysosomes may lead to calcium release and subsequent Ca^{2+} overload in mitochondria[42-44]. As expected, we found that

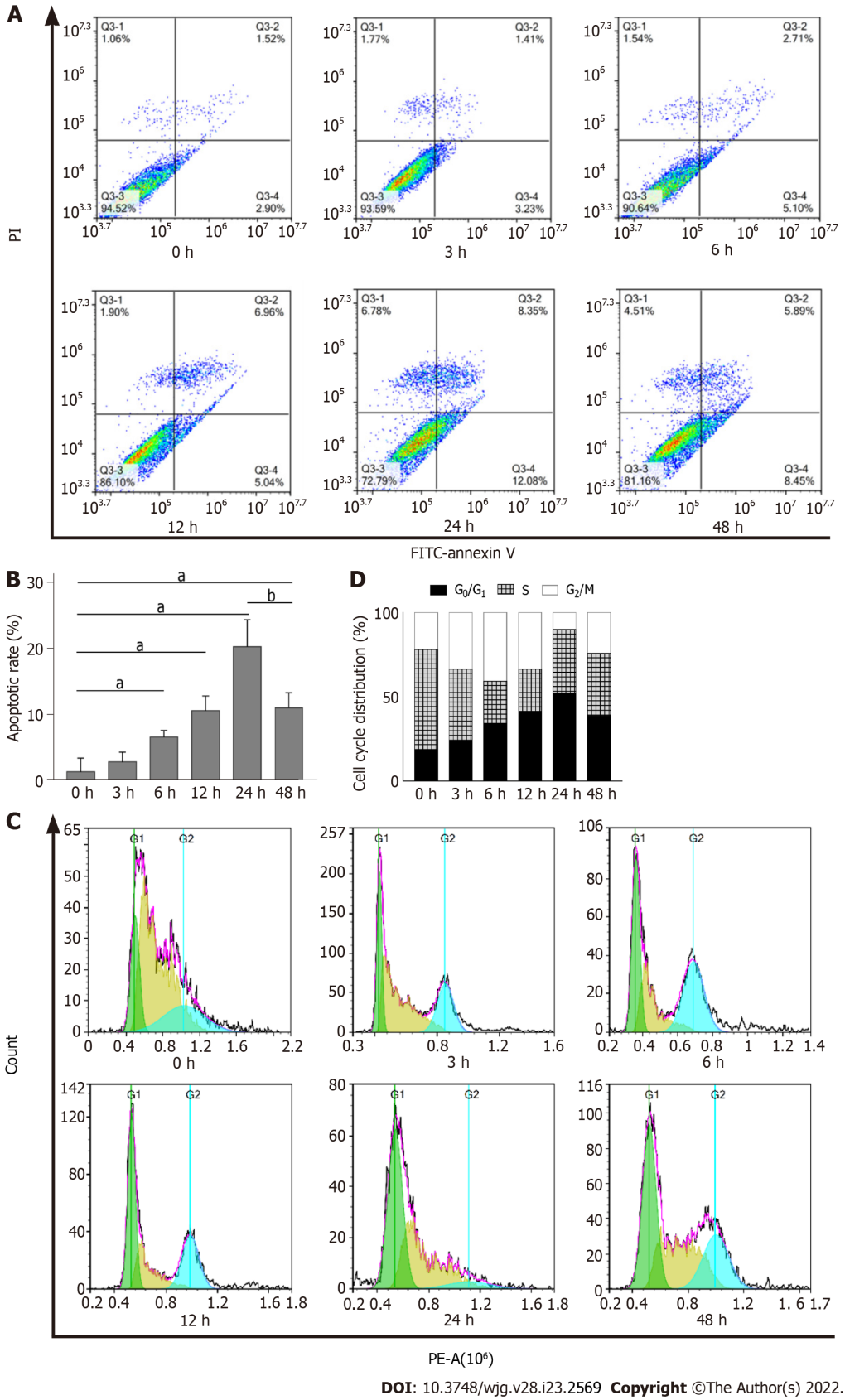

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Figure 4 Impact of dithiothreitol treatment on cell cycle and apoptosis of buffalo rat liver 3A cells. A and B: Buffalo rat liver 3A (BRL-3A) cells were treated with 2.0 mmol/L dithiothreitol (DTT) for 0, 3, 6, 12, 24 and 48 h. The population of apoptotic cells was detected by flow cytometry. The lower right quadrant represents the early apoptotic cells, and the upper right quadrant represents the late apoptotic cells; C and D: BRL-3A cells were treated with 2.0 mmol/L DTT for 0, 3, 6, 12, 24 and 48 h. The analysis of the cell cycle was assessed by flow cytometry. $^{a}P < 0.05$ *vs* 0 h group; $^{b}P < 0.05$ *vs* 48 h group.

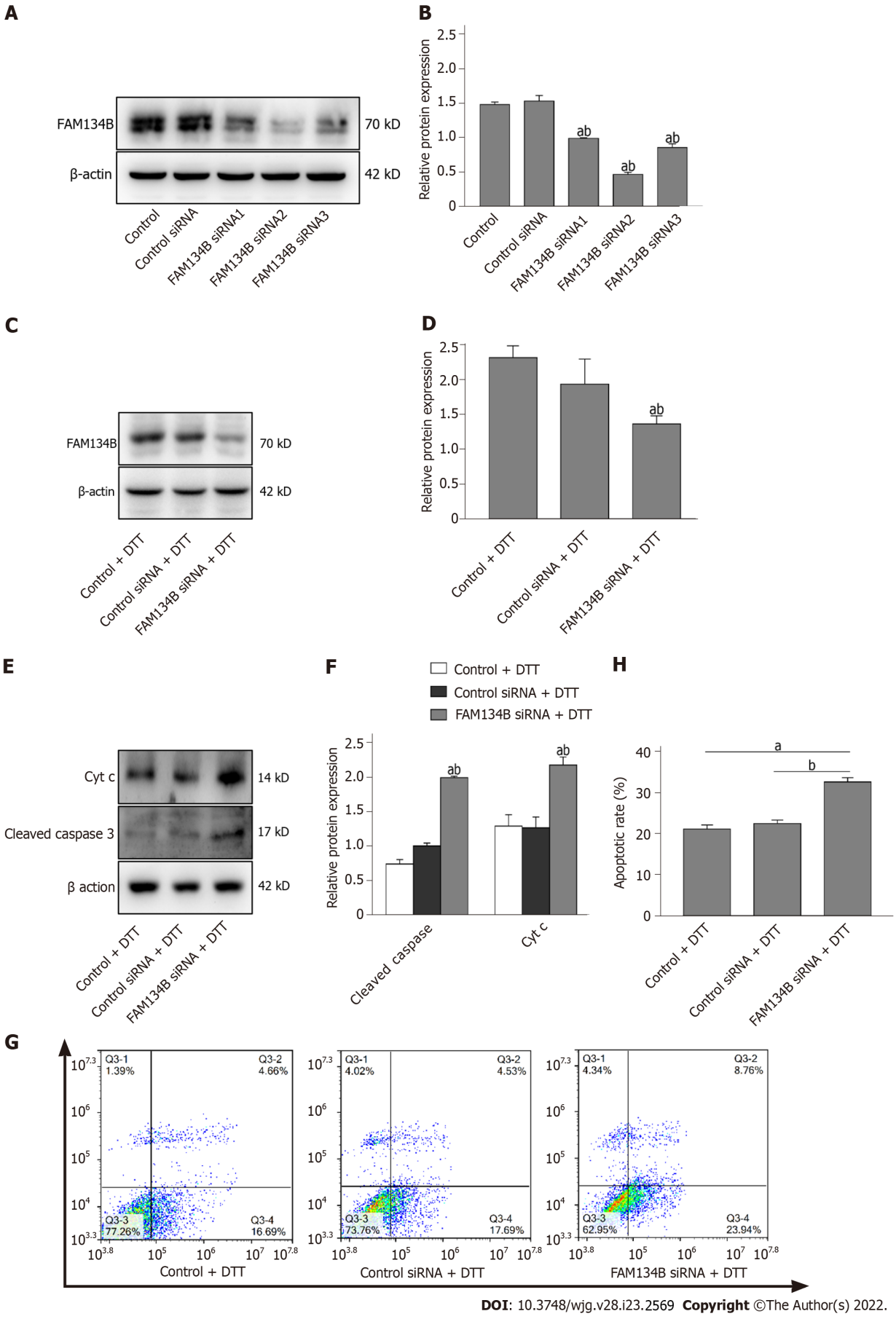

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Figure 5 Impact of dithiothreitol treatment on apoptosis of buffalo rat liver 3A cells lacking *FAM134B*. A and B: Buffalo rat liver 3A (BRL-3A) cells were transfected with *FAM134B* small interfering RNAs (siRNAs) 1, 2, and 3; immunoblot was used to detect the transfection efficiency of siRNA. Protein levels were

normalized to β-actin; C and D: BRL-3A cells were transfected with *FAM134B* siRNA, followed by treatment with 2.0 mmol/L dithiothreitol (DTT) for 24 h. Immunoblot was used to detect the expression of *FAM134B* in BRL-3A cells. Protein levels were normalized to β-actin; E and F: BRL-3A cells were transfected with *FAM134B* siRNA, followed by treatment with 2.0 mmol/L DTT for 24 h. Immunoblot showed the expression of cleaved caspase-3 and cyt c. Protein levels were normalized to β-actin; G and H: BRL-3A cells were transfected with *FAM134B* siRNA, followed by treatment with 2.0 mmol/L DTT for 24 h. Representative results showed the apoptotic rate in BRL-3A cells. Untransfected cells served as controls. Cells transfected with control siRNA served as transfection controls. The lower right quadrant represents the early apoptotic cells and the upper right quadrant represents the late apoptotic cells. Values are mean ± SD (n = 3), $^{a}P < 0.05$ *vs* control group; $^{b}P < 0.05$ *vs* transfection control siRNA group.

DTT treatment dramatically elevated the levels of mitochondrial Ca^{2+}, the apoptotic rate, and G1 arrest in BRL-3A cells. Nevertheless, these trends were relieved after treatment with DTT for 48 h. Our results reveal that hepatocytes initiate adaptive mechanisms in response to DTT-induced ER stress; consequently, apoptosis in BRL-3A cells treated with DTT for 48 h was lower than that in cells treated with DTT for 24 h.

To clarify whether FAM134B is involved in the regulation of cellular homeostasis during ER stress, we used a small interference RNA technique to knockdown *FAM134B* expression in hepatocytes. We found that *FAM134B* silencing not only significantly attenuated the DTT-upregulated FAM134B expression, but also accelerated the activation of the mitochondrial apoptotic pathway and aggravated DTT-triggered hepatocyte apoptosis.

CONCLUSION

In conclusion, DTT treatment significantly upregulated the protein levels of GRP78, CNX, FAM134B, and ATG12, and also increased the ratio of LC3II/LC3I in BRL-3A cells. Moreover, FAM134B-mediated reticulophagy ameliorates DTT-induced hepatocyte apoptosis *via* selective clearance of damaged ER lumens. Accordingly, knockdown of FAM134B enhanced ER stress-mediated apoptosis in BRL-3A cells. Our data show that FAM134B-mediated reticulophagy plays a key role in rebalancing ER homeostasis in hepatocytes undergoing ER stress. Therefore, FAM134B-mediated reticulophagy may be a novel therapeutic target, and our findings may provide emerging evidence to demonstrate the prominence of ER-phagy in ER stress-related hepatocyte apoptosis. Alleviation of ER stress-mediated hepatocyte apoptosis *via* restoring ER homeostasis is critical in the treatment of liver diseases.

ARTICLE HIGHLIGHTS

Research background

Hepatocyte apoptosis induced by endoplasmic reticulum (ER) stress has a strong association with the development of fibrosis, cirrhosis, and hepatocellular carcinoma. Previous studies have revealed that endoplasmic reticulophagy (ER-phagy) promotes the selective clearance of damaged ER fragments during ER stress, playing a crucial role in maintaining ER homeostasis and inhibiting apoptosis. However, the precise regulatory mechanisms remain unclear.

Research motivation

Defects in ER-phagy pathways are associated with multiple human pathologies, including infectious and neurodegenerative diseases, aging and cancer. However, whether ER-phagy is involved in the regulation of ER homeostasis in hepatocytes under ER stress remains elusive.

Research objectives

To elucidate the effect of family with sequence similarity 134 member B (FAM134B)-mediated ER-phagy on normal buffalo rat hepatocytes apoptosis induced by dithiothreitol (DTT) and explore the potential regulatory mechanism.

Research methods

A model of ER stress was established by DTT. The levels of proteins related to ER stress and ER-phagy were determined by western blot. An interaction between FAM134B, calnexin (CNX), and microtubule-associated protein 1 light chain 3 (LC3) was investigated by co-immunoprecipitation. ER-Tracker Red probe and Lyso-Tracker Green probe were used to detect the colocalization of ER with lysosome in cells. Mito-Tracker Green and Rhod-2 AM probes were used to detect the level of mitochondrial Ca^{2+} under the confocal microscopy. Flow cytometry was conducted to analyze the effect of DTT treatment on cell cycle distribution and apoptosis. The small interfering RNA against *FAM134B* was used to knockdown *FAM134B* in buffalo rat liver 3A (BRL-3A) cells.

Research results

DTT treatment upregulated glucose-regulated protein 78 (GRP78), CNX, FAM134B, and autophagy related gene 12 (ATG12) protein levels and increased the ratio of LC3II/LC3I in BRL-3A cells. FAM134B-mediated reticulophagy maintains ER homeostasis in ER-stressed hepatocytes *via* the clearance of damaged ER fragments. FAM134B-mediated reticulophagy ameliorates DTT-induced hepatocyte apoptosis. Knockdown of *FAM134B* enhanced ER stress-mediated apoptosis in BRL-3A cells.

Research conclusions

FAM134B-mediated ER-phagy attenuates hepatocyte apoptosis by suppressing the mitochondrial apoptotic pathway.

Research perspectives

FAM134B-mediated reticulophagy may be a novel therapeutic target, and our findings provide emerging evidence demonstrating the prominence of ER-phagy in ER stress-related hepatocyte apoptosis. Alleviation of the ER stress-mediated hepatocyte apoptosis *via* restoring ER homeostasis is critical in the treatment of liver diseases.

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FOOTNOTES

Author contributions: Yang Q and Xie RJ designed and coordinated the study; Guo YX, Han B and Yang T performed the experiments and acquired data; Chen YS, Yang Y and Li JY analyzed and interpreted data; Guo YX and Xie RJ drafted the manuscript; all authors approved the final version of the article.

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Institutional animal care and use committee statement: This study did not involve human subjects or living animals.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Data sharing statement: The data used to support the findings of this study are available from the corresponding author at 592153968@qq.com upon request.

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ORIGINAL ARTICLE

Retrospective Study

Infliximab trough level combined with inflammatory biomarkers predict long-term endoscopic outcomes in Crohn's disease under infliximab therapy

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Abstract

BACKGROUND

Infliximab trough level (ITL) severely affects therapeutic outcomes of Crohn's disease (CD) patients under infliximab (IFX). Recently, frontier research has focused on identifying ITL based on different therapeutic targets. Although previous studies have elaborated clinical value of ITL monitoring on short-term outcomes in CD patients during therapy, studies contraposing the predictive value of ITL on long-term endoscopic outcomes in CD patients are still scarce domestically and overseas.

AIM

To explore the predictive value of ITL in combination with inflammatory biomarkers on long-term endoscopic outcomes in CD with clinical remission during IFX maintenance therapy.

METHODS

CD patients with endoscopic remission under long-term IFX maintenance therapy in the First Affiliated Hospital of Zhejiang Chinese Medicine University from January 2012 to December 2020 were collected. ITL and inflammatory biomarkers were continuously monitored during the therapy. The Step I study was conducted from weeks 14 to 54 of IFX treatment. The Step II study was conducted from weeks 54 to 108 of IFX treatment. Endoscopic outcomes were defined as endoscopic activity (Crohn's disease endoscopic index of severity score > 2 points or Rutgeerts score > i1) and endoscopic remission (Crohn's disease endoscopic index of severity score ≤ 2 points or Rutgeerts ≤ i1). Endoscopic relapse free survival was defined as endoscopic remission at the beginning of the study stage and maintaining endoscopic remission during the study stage.

RESULTS

At week 14, low ITL [odds ratio (OR) = 0.666, 95% confidence interval (CI): 0.514-0.862, $P < 0.01$] and high fecal calprotectin (FCP) level (OR = 1.002, 95%CI: 1.001-1.004, $P < 0.01$) increased the risk of endoscopic activity at week 54. At week 54, low ITL (OR = 0.466, 95%CI: 0.247-0.877, $P < 0.01$) and high C-reactive protein (CRP) level (OR = 1.590, 95%CI: 1.007-2.510, $P < 0.01$) increased the risk of endoscopic activity at week 108. At week 14, ITL ≤ 5.60 μg/mL [area under the curve (AUC) = 0.83, 95%CI: 0.73-0.90, $P < 0.001$] and FCP > 238 μg/g (AUC = 0.82, 95%CI: 0.72-0.89, $P < 0.001$) moderately predicted endoscopic activity at week 54. ITL ≤ 5.60 μg/mL in combination with FCP > 238 μg/g indicated 82.0% possibility of endoscopic activity. At week 54, ITL ≤ 2.10 μg/mL (AUC = 0.85, 95%CI: 0.72-0.93, $P < 0.001$) and CRP > 3.00 mg/L (AUC = 0.73, 95%CI: 0.60-0.84, $P = 0.012$) moderately predicted moderate endoscopic activity at week 108. ITL ≤ 2.10 μg/mL in combination with CRP > 3.00 mg/L indicated 100.0% possibility of endoscopic activity. From weeks 14 to 54 of IFX treatment, patients with ITL > 5.60 μg/mL had higher rate of endoscopic relapse free survival than those with ITL ≤ 5.60 μg/mL (95.83% *vs* 46.67%). From weeks 54 to 108 of IFX treatment, patients with ITL > 2.10 μg/mL had higher rate of endoscopic survival free relapsed rate than those with ITL ≤ 2.10 μg/mL (92.68% *vs* 30.77%).

CONCLUSION

Combination of ITL, CRP, and FCP contribute to long-term endoscopic prognosis monitoring. During IFX maintenance treatment, low ITL, high CRP level, and high FCP level were independent risk factors of CD patients with clinical remission in adverse endoscopy outcomes within 1-year follow-up.

Key Words: Infliximab trough level; C-reactive protein; Fecal calprotectin; Crohn's disease; Clinical remission; Long-term endoscopic outcomes

Core Tip: Previous investigations, contraposing Crohn's disease patients under infliximab (IFX) maintenance therapy, have indicated that higher IFX trough levels (ITLs) were associated with sustained drug response and clinical remission in inflammatory bowel disease patients, while lower ITLs were linked to secondary unresponsiveness of IFX. Currently, endoscopic remission or mucosal healing has been considered the main goal of biological therapy. Our study manifested that Crohn's disease patients with higher levels of IFX blood concentration and lower levels of inflammatory biomarkers tended to have a better long-term endoscopic prognosis. Combining ITL, fecal calprotectin and C-reactive protein monitoring was helpful for the timely adjustment of IFX treatment strategy.

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INTRODUCTION

Crohn's disease (CD) is a persistently progressive disease with nonspecific inflammation characterized by disease scope involving the whole digestive tract and disease depth involving the whole intestinal wall. The accumulation damage of intestinal walls contributes to the occurrence of stenosis, fistula and even abscess, reducing the life quality. Therefore, recent clinical studies have consistently concluded

that therapeutic strategies and targets play key roles in controlling CD progression. Setting different therapeutic targets will have different disease outcomes. Clinical response, focusing only on the improvement of clinical symptoms, can improve the quality of daily life but not affect long-term treatment outcomes. CD patients who do not achieve deep remission may be aggravated persistently, while achieving deep remission could reduce long-term hospitalization and surgery rates. Deep remission is mainly defined in previous studies as endoscopic remission or mucosal healing. Biologics, as an important step in the therapeutic strategy of CD, can effectively control the disease progression if conducted early and completely.

In consideration of the wide use of infliximab (IFX), precisely predicting the long-term endoscopic outcomes is stressed by more and more inflammatory bowel disease (IBD) physicians. Although the IFX trough level (ITL) has been proven to be closely related to the outcome of CD, ITL alone may be biased in predicting the outcome of CD. Monitoring inflammation biomarkers is one of the important links of IFX therapy, including C-reaction protein (CRP), fecal calprotectin (FCP), *etc*. High inflammatory load affects the pharmacokinetics of IFX, inducing secondary nonresponse by decreasing blood drug concentration. Currently, it is believed that inflammatory biomarkers are good predictors of disease activity, but there is still a lack of reliable evidence for predicting disease remission. Therefore, this study intends to evaluate long-term endoscopic outcomes of CD patients receiving IFX treatment by combining the blood drug concentration and inflammatory biomarkers.

MATERIALS AND METHODS

Study subjects design

A single-center retrospective research was implemented at the First Affiliated Hospital of Zhejiang Chinese Medical University. CD patients under IFX therapy from January 2012 to December 2020 were collected. One hundred and eighty-one CD patients underwent IFX treatment. One hundred and fifty-one CD patients underwent endoscopy as well as serum concentration monitoring at week 14 after the third dose of IFX induction therapy. Inclusion criteria: (1) Endoscopic remission at week 14 [Crohn's disease endoscopic index of severity (CDEIS) score ≤ 2 points or Rutgeerts ≤ i1]; (2) Clinical remission after IFX induction therapy without corticosteroids more than 6 mo; and (3) Therapeutic strategy during maintenance stage was designed as IFX 5 mg/kg every 8 wk combined with azathioprine (AZA) 50 mg every day. Therapeutic strategic was modulated if CD patients were confronted with clinical relapse or secondary loss of response (LOR), and data analysis focused on the treatment course when patients received IFX 5 mg/kg and AZA therapy regularly. Secondary LOR was defined as a recurrence of the disease during IFX maintenance therapy. Two criteria were met to determine LOR: The recurrence of symptoms of IBD in clinical remission after induction therapy and symptoms caused by the inflammatory activity of IBD itself. Clinical relapse means Crohn's disease activity index > 150 points. Blood drug concentration monitoring and clinical, laboratory, endoscopic and imaging evaluation were implemented every 2 mo since the third dose of IFX induction therapy in all patients. The study was divided into two stages, step I study period defined as IFX maintenance therapy during week 14 to week 54 and step II study period defined as IFX maintenance therapy during week 54 to week 108.

Data collection

General data included age, sex, course, disease location, disease behavior, medication history and history of intestinal surgery. Laboratory indicators include white blood cell count, blood platelet count, CRP, erythrocyte sedimentation rate, serum albumin, FCP, ITL and anti-IFX antibody. Evaluation indicators of disease severity included Crohn's disease activity index score on clinical severity, CDEIS score on endoscopic severity in CD patients without intestinal surgery and Rutgeerts score on endoscopic severity in CD patients with intestinal surgery.

Outcome definition

Endoscopic outcomes at week 54 and week 108 after IFX initial therapy were evaluated by specialist physicians on IBD under electronic colonoscopy. Endoscopic remission was defined as CDEIS score ≤ 2 or Rutgeerts score ≤ i1, and endoscopic activity was defined as CDEIS score > 2 or Rutgeerts score > i1. Survival outcomes during IFX maintenance therapy were concentrated on endoscopic relapse-free survival, defined as sustained endoscopic remission during step I study period or step II study period.

Statistical analysis

Descriptive statistical analysis was used to describe characteristics of CD patients. Number of cases (percentage) was used to describe categorial variable. mean ± SD was used to describe continuous variable. Nonparametric Mann-Whitney test was used to compare two groups in enumeration data or measurement data without normal distribution. Two-sample t test was used to compare two groups in measurement data with normal distribution. One-way analysis of variance was used to compare multi-group if data satisfied homogeneity of variance. Nonparametric Kruskal-Wallis test was used to

compare multi-group if data not satisfied homogeneity of variance. SPSS 23.0 (Armonk, NY, United States) was used to analyze differences between groups. A P value < 0.05 was considered significant.

Receiver-operating characteristic (ROC) analysis was used to identify the best cut off level of ITL on predicting endoscopic remission as well as sensitivity, specificity, positive predictive value, negative predictive value, area under the curve and Youden Index. Univariate logistic regression analysis was used to identify the association between endoscopic activity and predictors. Log-rank test was used to identify the association between endoscopic relapse and predictors. GraphPad Prism9.0 (San Diego, CA, United States) was used to draw histograms and survival analysis curves and implement log-rank test. MedCalc19.0 was used to draw ROC curve and analyze the predictive value of indicators on endoscopic outcomes. A P value < 0.05 was considered significant.

RESULTS

Characteristics of study subjects

In total, the study cohort collected 112 CD patients achieving clinical remission after IFX induction therapy. In step I study, 19 CD patients were excluded due to data absence (n = 1, 5.26%) and endoscopic activity at week 14 (n = 18, 94.74%), while 93 CD patients with endoscopic remission at week 14 were included. In step II study, 58 CD patients were excluded due to course of therapy shorter than 2 years (n = 10, 17.24%), secondary non-response of IFX (n = 12, 20.69%), suspension of IFX therapy within 2 years for disease remission (n = 10, 17.24%) and endoscopic activity at week 54 (n = 26, 44.83%), while 54 CD patients with endoscopic remission at week 54 were included. These 12 patients did not satisfy indications of operation and received hormonotherapy as the primary choice to alleviate disease, for our center lacked other biological agents at that time. All CD patients under IFX maintenance therapy were combined with AZA (Figure 1). The dose of IFX was 5 mg/kg every 8 wk, and the dose of AZA was 50 mg every day. Characteristics of CD patients included in study are shown in Table 1.

Correlation between ITL, inflammatory biomarkers and endoscopic outcomes

In step I study, 67/93 CD patients (72.04%) sustained endoscopic remission at week 54 among. Multivariable regression analysis revealed that only ITL (OR = 0.666, 95%CI: 0.514-0.862, P = 0.002) and FCP (OR = 1.002, 95%CI: 1.001-1.004, P = 0.002) were independent risk of endoscopic activity at week 54 (Table 2). Based on incremental gain analysis, an ITL range of 5.0-7.4 μg/mL was correlated with sustained endoscopic remission rate of more than 85% (Figure 2).

In step II study, 42/54 CD patients (77.78%) sustained endoscopic remission at week 108. Multivariable regression analysis revealed that only ITL (OR = 0.466, 95%CI: 0.247-0.877, P = 0.018) and CRP (OR = 1.590, 95%CI: 1.007-2.510, P = 0.047) were independent risks of endoscopic activity at week 108 (Table 2). Based on incremental gain analysis, an ITL range of 2.0-3.9 μg/mL was correlated with sustained endoscopic remission rate of more than 85% (Figure 2).

Predictive value of ITL and inflammatory biomarkers on endoscopic outcomes

In step I study, the ROC analysis demonstrated that the best cut off level of ITL and FCP at week 14 on predicting endoscopic relapse at week 54 was 5.60 μg/ml (AUC = 0.83, 95%CI: 0.73-0.90, P < 0.001) and 238 μg/g (AUC = 0.82, 95%CI: 0.72-0.89, P < 0.001) (Table 3 and Figure 3). CD patients with ITL ≤ 5.60 μg/ml and FCP > 238 μg/g at week 14 had 82% probability of endoscopic relapse at week 54. However, CD patients with ITL > 5.60 μg/ml and FCP ≤ 238 μg/g at week 14 had 98% probability of sustained endoscopic remission at week 54.

In step II study, the ROC analysis demonstrated that the best cut off level of ITL and CRP at week 54 on predicting endoscopic relapse at week 108 was 2.10 μg/mL (AUC = 0.85, 95%CI: 0.72-0.93, P < 0.001) and 3.00 mg/L (AUC = 0.73, 95%CI: 0.60-0.84, P = 0.012) (Table 3 and Figure 3). CD patients with ITL ≤ 2.10 μg/mL and CRP > 3.00 mg/L at week 54 had 100% probability of endoscopic relapse at week 108. However, CD patients with ITL > 2.10 μg/mL and CRP ≤ 3.00 mg/L at week 54 had 97% probability of sustained endoscopic remission at week 108.

Correlation between ITL, inflammatory biomarkers and endoscopic relapse-free survival

In step I study, 26/93 (27.96%) CD patients had experienced endoscopic relapse from week 14 to week 54 of IFX maintenance therapy. The estimated endoscopic relapse-free rate was 46/48 (95.83%) in CD patients with ITL > 5.6 μg/ml and 21/45 (46.67%) in CD patients with ITL ≤ 5.6 μg/ml. The median time to endoscopic relapse of CD patients with ITL ≤ 5.6 μg/ml was 32.00 wk shorter than those with ITL > 5.6 μg/ml [hazard ratio (HR) = 16.19, 95%CI: 7.44-35.22, P < 0.0001] (Figure 4A). The estimated endoscopic relapse-free rate was 6/25 (24.00%) in CD patients with FCP > 238 μg/g and 61/68 (89.71%) in CD patients with FCP ≤ 238 μg/g. The median time to endoscopic relapse of CD patients with FCP > 238 μg/g was 21.00 wk shorter than those with FCP ≤ 238 μg/g (HR = 11.25, 95%CI: 4.26-29.73, P < 0.0001) (Figure 4B).

Table 1 Clinical characteristics of Crohn's disease patients with endoscopic remission

Variable	Week 14 (after initial IFX therapy)			Week 54 (after initial IFX therapy) *n* = 54		
	Total, *n* = 93	ER at week 54, *n* = 67	EA at week 54, *n* = 26	Total, *n* = 54	ER at week 108, *n* = 42	EA at week 108, *n* = 12
Median age in yr, mean ± SD	28.96 ± 9.37	29.03 ± 10.09	28.77 ± 7.39	27.57 ± 10.13	26.93 ± 9.87	29.83 ± 11.13
Course in yr, median (IQR)	3.0 (1.0, 6.0)	2.0 (1.0, 6.0)	3.5 (2.0, 8.3)	2.0 (1.0, 5.0)	2.0 (1.0, 5.0)	2.0 (1.3, 8.5)
Male sex, *n* (%)	55 (59.1)	43 (64.2)	12 (46.2)	35 (64.8)	28 (66.7)	7 (58.3)
Disease location, *n* (%)						
L1 (terminal ileum)	18 (19.4)	14 (20.9)	4 (15.4)	10 (18.5)	9 (21.4)	1 (8.3)
L2 (colon)	11 (11.8)	6 (9.0)	5 (19.2)	5 (9.3)	4 (9.5)	1 (8.3)
L3 (ileocolon)	64 (68.8)	47 (70.1)	17 (65.4)	39 (72.2)	29 (69.0)	10 (83.3)
L4 (upper digestive tract)	22 (23.7)	15 (22.4)	7 (26.9)	14 (25.9)	11(26.2)	3 (25.0)
Disease behavior, *n* (%)						
B1 (no)	19 (20.4)	15 (22.4)	4 (15.4)	13 (24.1)	9 (21.4)	4 (33.3)
B2 (stenosis)	11 (11.8)	9 (13.4)	2 (7.7)	6 (11.1)	5 (11.9)	1 (8.3)
B3 (penetration)	40 (43.0)	27 (40.3)	13 (50.0)	23 (42.6)	18 (42.9)	5 (41.7)
B2 (stenosis) + B3 (penetration)	23 (24.7)	16 (23.9)	7 (26.9)	12 (22.2)	10 (23.8)	2 (16.7)
Perianal diseases, *n* (%)	55 (59.1)	38 (56.7)	17 (65.4)	32 (59.3)	25 (59.5)	7 (58.3)
Previous medical therapy, *n* (%)	64 (68.8)	46 (68.7)	18 (69.2)	34 (63.0)	25 (59.5)	9 (75.0)
Previous surgical therapy, *n* (%)	15 (16.1)	11 (16.4)	4 (15.4)	8 (14.8)	8 (19.0)	0 (0.0)
Laboratory indicators, mean ± SD						
Fecal calprotectin, μg/g	399.96 ± 562.47	178.62 ± 242.38	970.35 ± 734.49	353.17 ± 557.71	178.57 ± 276.56	964.25 ± 830.56
IFX trough level, μg/ml	6.12 ± 3.72	7.23 ± 3.48	3.25 ± 2.67	3.80 ± 2.25	4.37 ± 2.02	1.80 ± 1.90
White blood count, × 10^9/L	5.32 ± 1.87	5.16 ± 1.47	5.73 ± 2.65	5.27 ± 1.41	5.27 ± 1.43	5.28 ± 1.41
Hematoglobin, g/L	128.37 ± 20.42	130.96 ± 20.03	121.69 ± 20.27	136.59 ± 16.06	136.79 ± 16.94	135.92 ± 13.13
Platelet, × 10^9/L	210.66 ± 69.77	202.36 ± 58.34	232.04 ± 90.94	205.02 ± 46.19	204.62 ± 45.82	206.42 ± 49.54
Erythrocyte sedimentation rate, mm/h	9.88 ± 12.25	6.89 ± 7.96	17.46 ± 17.28	7.31 ± 10.35	6.33 ± 6.89	10.75 ± 17.97
Albumin, g/L	42.13 ± 4.17	42.65 ± 3.93	40.77 ± 4.54	44.51 ± 3.40	44.99 ± 3.38	42.85 ± 3.02
C-reactive protein, mg/dl	2.98 ± 5.43	1.82 ± 2.77	5.96 ± 8.69	1.99 ± 3.18	1.23 ± 1.79	4.66 ± 5.19

IFX: Infliximab; IQR: Interquartile range; SD: standard deviation; EA: Experimental adhesive; ER: Endoplasmic reticulum.

In step II study, 12/54 (22.22%) CD patients had experienced endoscopic relapse from week 54 to week 108 of IFX maintenance therapy. The estimated endoscopic relapse-free rate was 38/41 (92.68%) in CD patients with ITL > 2.1 μg/mL and 4/13 (30.77%) in CD patients with ITL ≤ 2.1 μg/mL. The median time to endoscopic relapse of CD patients with ITL ≤ 2.1 μg/mL was 40.00 w, shorter than those with ITL > 2.1 μg/mL (HR = 13.14, 95%CI: 3.07-56.27, $P < 0.0001$) (Figure 4D). The estimated endoscopic relapse-free rate was 4/8 (50.00%) in CD patients with CRP > 3.00 mg/L and 40/46 (86.96%) in CD patients with CRP ≤ 3.00 mg/L. The median time to endoscopic relapse of CD patients with CRP > 3.00 mg/L was 50.00 wk shorter than those with CRP ≤ 3.00 mg/L (HR = 7.85, 95%CI: 1.31-46.85, $P < 0.0001$) (Figure 4C).

DISCUSSION

Several studies have confirmed that different ITLs brought about different outcomes of CD under IFX therapy (Table 4). Tang *et al*[1] discovered that CD patients achieving mucosal healing at week 14 of IFX

Table 2 Predictive indicators of endoscopic relapse in Crohn's disease patients with endoscopic remission

Variable	Week 14 (after initial IFX therapy), *n* = 93				Week 14 (after initial IFX therapy), *n* = 54			
	Predict endoscopic relapse at week 54				Predict endoscopic relapse at week 108			
	Univariate analysis		Multivariable analysis		Univariate analysis		Multivariable analysis	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Median age in year, median (IQR)	0.997 (0.950-1.047)	0.904	–	–	1.028 (0.967-1.093)	0.381	–	–
Course in year, median (IQR)	1.054 (0.945-1.176)	0.346	–	–	1.065 (0.906-1.253)	0.444	–	–
Male sex, *n* (%)	0.478 (0.191-1.199)	0.116	–	–	0.700 (0.188-2.607)	0.595	–	–
Disease location, *n* (%)								
L1 (terminal ileum)	0.413 (0.114-1.495)	0.178	–	–	1.158 0.117-11.454)	0.900	–	–
L2 (colon)	1.453 (0.430-4.908)	0.548	–	–	3.000 (0.341-26.427)	0.322	–	–
L3 (ileocolon)	–	–	–	–	–	–	–	–
L4 (upper digestive tract)	1.277 (0.452-3.612)	0.645	–	–	0.939 (0.215-4.113)	0.934	–	–
Disease behavior, *n* (%)								
B1 (no)	–	–	–	–	–	–	–	–
B2 (stenosis)	0.889 (0.345-2.294)	0.808	–	–	0.600 (0.141-2.561)	0.490	–	–
B3 (penetration)	1.860 (0.658-5.264)	0.242	–	–	0.700 (0.188-2.607)	0.595	–	–
B2 (stenosis) + B3 (penetration)	–	–	–	–	–	–	–	–
Perianal diseases, *n* (%)	1.442 (0.562-3.696)	0.446	–	–	0.952 (0.259-3.502)	0.941	–	–
Previous medical therapy, *n* (%)	1.027 (0.386-2.736)	0.957			2.040 (0.481-8.650)	0.333		
Previous surgical therapy, *n* (%)	0.926 (0.266-3.218)	0.903	–	–	–	–	–	–
Laboratory indicators, median (IQR)								
Fecal calprotectin, μg/g	1.003 (1.002-1.005)	0.000	1.002 (1.001-1.004)	0.002	1.002 (1.001-1.004)	0.001	NS	NS
IFX trough level, μg/mL	0.650 (0.532-0.796)	0.000	0.666 (0.514-0.862)	0.002	0.470 (0.289-0.766)	0.002	0.466 (0.247-0.877)	0.018
White blood count, x 10^9/L	1.167 (0.921-1.478)	0.201	–	–	1.004 (0.636-1.586)	0.986	–	–
Hematoglobin, g/L	0.977 (0.954-1.000)	0.053	–	–	0.997 (0.957-1.038)	0.867	–	–
Platelet, x 10^9/L	1.006 (0.999-1.013)	0.081	–	–	1.001 (0.987-1.015)	0.904	–	–
Erythrocyte sedimentation rate, mm/h	1.073 (1.028-1.120)	0.001	NS	NS	1.035 (0.978-1.096)	0.239	–	–
Albumin, g/L	0.895 (0.800-1.002)	0.054	–	–	0.821 (0.667-1.010)	0.062	–	–
C-reactive protein, mg/dL	1.245 (1.080-1.435)	0.003	NS	NS	1.389 (1.070-1.804)	0.014	1.590 (1.007-2.510)	0.047

IFX: Infliximab; NS: Not significant; CI: Confidence interval; IQR: Interquartile range; OR: Odds ratio.

Table 3 Predictive value of indicators on endoscopic relapse

	Youden index	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Area under the ROC curve	*P* value
Predictors at week 14 of Endoscopic relapse at week 54							
ITL ≤ 5.6 μg/mL	0.61 (0.41-0.72)	0.92 (0.75-0.99)	0.69 (0.56-0.79)	0.53 (0.44-0.62)	0.96 (0.86-0.99)	0.83 (0.73-0.90)	< 0.001
FCP > 238 μg/g	0.64 (0.38-0.78)	0.73 (0.52-0.88)	0.91 (0.82-0.97)	0.76 (0.59-0.88)	0.90 (0.82-0.94)	0.82 (0.72-0.89)	< 0.001
ITL ≤ 5.6 μg/mL and FCP > 238 μg/g	0.63 (0.40-0.80)	0.69 (0.48-0.86)	0.94 (0.85-0.98)	0.82 (0.63-0.92)	0.89 (0.82-0.93)	0.82 (0.72-0.89)	< 0.001
ITL ≤ 5.6 μg/mL or FCP > 238 μg/g	0.62 (0.47-0.74)	0.96 (0.80-1.00)	0.66 (0.53-0.77)	0.52 (0.44-0.60)	0.98 (0.87-1.00)	0.81 (0.71-0.88)	< 0.001
Predictors at week 54 of Endoscopic relapse at week 108							
ITL ≤ 2.1 μg/mL	0.68 (0.40-0.87)	0.75 (0.43-0.95)	0.93 (0.81-0.99)	0.75 (0.49-0.90)	0.93 (0.83-0.97)	0.85 (0.72-0.93)	< 0.001
CRP > 3.0 mg/dL	0.45 (0.20-0.68)	0.50 (0.21-0.79)	0.95 (0.84-0.99)	0.75 (0.41-0.93)	0.87 (0.79-0.92)	0.73 (0.60-0.84)	0.012
ITL ≤ 2.1 μg/mL and CRP > 3.0 mg/dL	0.33 (0.08-0.58)	0.33 (0.10-0.65)	1.00 (0.92-1.00)	1.00 (1.00-1.00)	0.84 (0.78-0.89)	0.67 (0.53-0.79)	0.019
ITL ≤ 2.1 μg/mL or CRP > 3.0 mg/dL	0.80 (0.50-0.93)	0.92 (0.62-1.00)	0.88 (0.74-0.96)	0.69 (0.49-0.84)	0.97 (0.85-1.00)	0.90 (0.79-0.96)	□0.001

ITL: Infliximab trough level; FCP: Fecal calprotectin; CRP: C-reactive protein; ROC: Receiver operating characteristic.

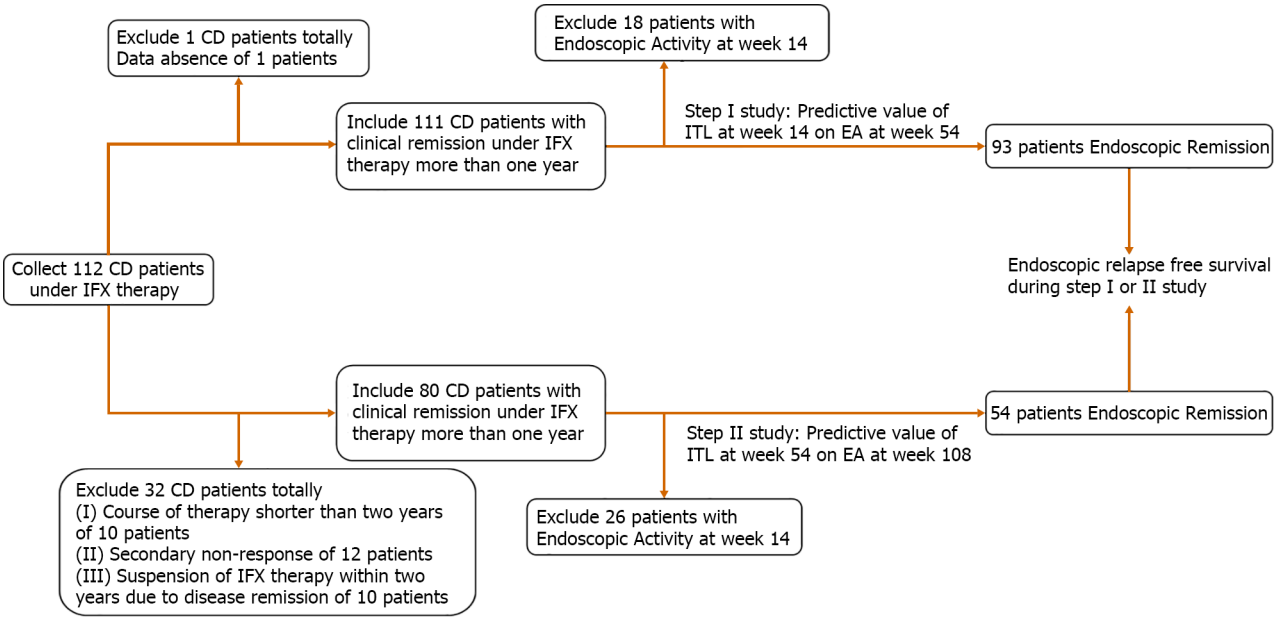

Figure 1 Patients selected and outcome of infliximab therapy. CD: Crohn's disease; EA: Endoscopic activity; IFX: Infliximab.

therapy with ITL > 2.5 μg/mL at week 14 had 71% chance of mucosal healing at week 54, while patients with ITL < 2.5 μg/mL had only 33% chance. ITL ≥ 3 μg/mL at the beginning of IFX maintenance therapy was confirmed as a predictor of sustained response to IFX in CD patients[2]. Recently a prospective study in Japan verified that CD patients with ITL ≥ 3 μg/mL at week 14 after IFX initial therapy had much better long-term clinical outcomes than patients with ITL < 3 μg/mL, of which survival analysis indicated 100% probability of clinical remission at week 108 in the former and 33.3% probability in the latter[3]. A meta-analysis determined that ITL > 2.0 μg/mL of IBD patients under IFX maintenance therapy contributes to better prognosis such as clinical remission or mucosal healing[4]. Similarly, this study found that CD patients with ITL > 5.6 μg/mL at week 14 had a large chance of

Table 4 Previous research of infliximab trough level on deep remission in inflammatory bowel disease

Study design	Optimal cut-off value	Predictive content	Yes/No, *n*	SE	SP	PPV	NPV	AUC
A retrospective observational single-center study in China	4.85 μg/mL at week 14	Mucosal healing (complete absence of any sign of ulceration)	82/59	67%	80%			0.80
	4.85 μg/mL at week 14	Mucosal healing (CDEIS of < 3)	84/57	68%	83%			0.79
	2.85 μg/mL at week 30	Mucosal healing (complete absence of any sign of ulceration)	59/50	73%	84%			0.78
	2.85 μg/mL at week 30	Mucosal healing (CDEIS of < 3)	62/47	68%	81%			0.73
A retrospective observational single-center study in China	2.50 μg/mL at week 14	Mucosal healing (SES-CD/Rutgeerts of 0 or 1) at week 52	31/42	87%	60%			0.70
	2.50 μg/mL at week 14	Sustained remission (no treatment failure, no need for surgery or intensification of IFX nor new introduction during IFX therapy) at week 52	70/38	64%	63%			0.70
A prospective multicenter study in Spanish	3.40 μg/mL	(1) SES-CD<3 for CD patients; (2) Rutgeerts score < i2 for CD patients in the postoperative setting; and (3) Mayo endoscopic score < 2 for UC patients	58/30	60%	60%	73%	42%	0.63
A multicenter, randomized, double-blind, controlled trial in Europe	23.10 mg/L at week 2	Endoscopic remission (CDEIS < 3) at week 12	54/52	56%	80%	72%	65%	0.67
	10.00 mg/L at week 6	Endoscopic remission (CDEIS < 3) at week 12	54/52	37%	89%	76%	59%	0.64
	10.60 mg/L (dose escalation to 10 mg/kg)	The absence of ulcers at week 54	85/51	94%	42%	49%	92%	0.71
A retrospective multicenter study in United States	9.70 μg/mL	Endoscopic remission (absence of any mucosal break (ulceration or erosion)/Rutgeerts score of ≤ i1)	62/34	57%	73%	80%	48%	0.65
	9.80 μg/mL	Histologic remissions (absence of active inflammation)	43/44	63%	66%	64%	64%	0.62
	2.20 μg/mL	Biochemical remission (CRP ≤ 5 mg/dL)	48/23	92%	35%	75%	67%	0.64
A retrospective observational single-center study in Japan	4.00 μg/mL	Mucosal healing (modified Rutgeerts scoring system: 0 or 1) after 30 days	20/58	71%	70%			0.63
	0.60 μg/mL	CRP normalization (≤ 0.3 mg/dL)	28/22	73%	62%			0.67
	1.00 μg/mL	Serum albumin normalization (≥ 4.0 mg/dL)	17/33	67%	71%			0.72
	1.10 μg/mL	Fecal calprotectin (≥ 300 μg/g)	13/25	72%	56%			0.63
A retrospective cross-sectional multicenter study in South Korea	4.20 μg/mL	Mucosal healing (SES-CD = 0)	51/54	65%	70%	67%	68%	0.68
	3.71 μg/mL	Partial mucosal healing (SES-CD < 3)	63/42	70%	71%	79%	61%	0.73
	3.26 μg/mL	Clinical remission (PCDAI < 10)	95/10	71%	100%	100%	73%	0.90
	2.52 μg/mL	Biochemical remission (CRP < 0.3 mg/dL)	87/18	86%	56%	90%	46%	0.71
A prospective cohort multicenter study in Canada	8.02 μg/mL	Histologic remission (an absence of active chronic inflammation)	56/48	79%	68%			0.72
	8.27 μg/mL	Sustained histologic remission (histologic remission documented at both the baseline and follow-up colonoscopies)	36/16	88%	72%			0.77
A retrospective cross-sectional study in United Kingdom	7.10 μg/mL	Fistula healing (no spontaneous discharge or no discharge on palpation in the absence of seton drainage)	18/11	78%	100%			0.93
	7.10 μg/mL	Fistula closure (the absence of an external skin opening)	13/16	64%	100%			0.97

SE: Sensitivity; SP: Specificity; PPV: Positive predictive value; NPV:Negative predictive value; AUC: Areas under the curve; CDEIS: Crohn's disease endoscopic index of severity; SES-CD: Simplified endoscopic score for Crohn's disease; CD: Crohn's disease; UC: Ulcerative colitis; CRP: C-reactive protein; PCDAI: Pediatric Crohn's disease activity index.

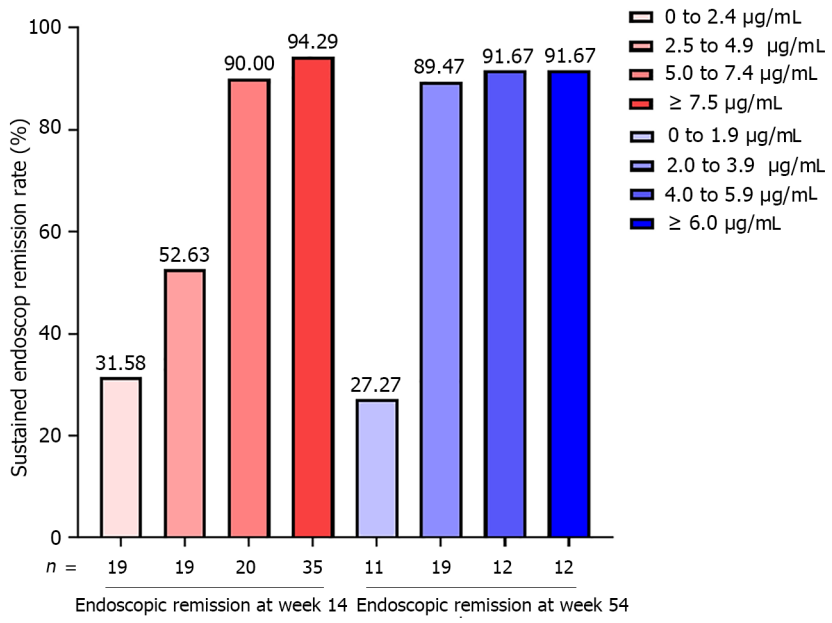

Figure 2 Incremental gain analysis of sustained endoscopic remission rate in relation to infliximab trough level at week 14 and week 54.

achieving sustained endoscopic remission during IFX maintenance therapy as well as CD patients with ITL > 2.1 μg/mL at week 54. Borren *et al*[5] implemented a multi-center retrospective study and concluded that low ITL in IBD patients during IFX maintenance therapy could not be a good predictor of clinical relapse in the next 2 years, suggesting that proactive therapeutic drug monitoring was not suitable in this group. However, this study discovered that CD patients with ITL ≤ 5.6 μg/mL at week 14 or ITL ≤ 2.1 μg/mL at week 54 were more likely to experience endoscopic relapse during the 1-year follow-up period.

According to previous studies, the challenge for IBD physicians is to frame the more suitable blood trough level of IFX to achieve better disease prognosis in clinical therapy. The elements associated with the blood trough level of IFX can be classified into three areas. Above all, the better the therapeutic goal desired by IBD physicians or patients, the higher the blood trough level of IFX is required. An observational study contraposing to CD patients with a history of intestinal surgery by Imaeda *et al*[6] verified that mucosal healing required higher ITLs as more than 4.0 μg/mL compared to those to achieve normalization of routine clinical markers. Papamichael *et al*[7] considered that ITL surpassing 9.7 μg/mL indicated 80% probability of endoscopic remission in CD patients under IFX maintenance therapy, while ITL surpassing 2.2 μg/mL was associated only with biochemical remission. Recently, a prospective study verified that ITL > 8.0 μg/mL was highly correlated with histological emission and sustained histological remission in IBD patients[8]. Perianal fistula, the most universal complications of CD patients, is another therapeutic goal. A retrospective cross-sectional study by Plevris *et al*[9] manifested that perianal fistula healing or closure is associated with higher ITLs as more than 7.1 μg/mL.

Secondly, each clinical study had different stages of IFX drug monitoring, especially during maintenance therapy. ITL continues to decrease as time passed during IFX maintenance therapy[10]. A cross-sectional study of IBD patients under IFX therapy with a fixed dose more than 6 mo found that IBD patients with ITL ≥ 3.4 μg/mL had a 73% chance of endoscopic mucosal healing[11]. Kang *et al*[12] showed that ITL ≥ 5 μg/mL during IFX maintenance therapy could identify mucosal healing in pediatric CD patients with 80% specificity. Feng *et al*[13] innovatively integrated ITL levels in different time stages to identify endoscopic mucosal healing in CD patients, indicating that patients with ITL > 4.85 μg/mL at week 14 and ITL > 2.85 μg/mL at week 30 had an 80% chance of achieving endoscopic mucosal healing. Based on incremental gain analysis in our study, sustained endoscopic remission rate at week 54 reached only 54.63% at an ITL range of 2.5 to 4.9 μg/mL at week 14 while corresponding numbers at week 108 was 89.47% at an ITL range of 2.0 to 3.9 μg/mL at week 54. Therefore, the study held the view that CD patients with endoscopic remission need higher ITL at the beginning of IFX maintenance therapy (≥ 5.6 μg/mL at week 14) than after IFX maintenance therapy over a half year (≥ 2.1 μg/mL at week 54). In addition, CD patients achieving endoscopic remission after IFX induction therapy and sustained endoscopic remission more than a half year may not need high ITL to maintain endoscopic remission.

The third element is therapeutic optimization of IFX in IBD. Adverse IFX response as high ATI level or low ITL may occur in a few CD patients during IFX maintenance therapy. Several clinical studies held the view that severe inflammatory activity of CD patients could change pharmacokinetics of anti-

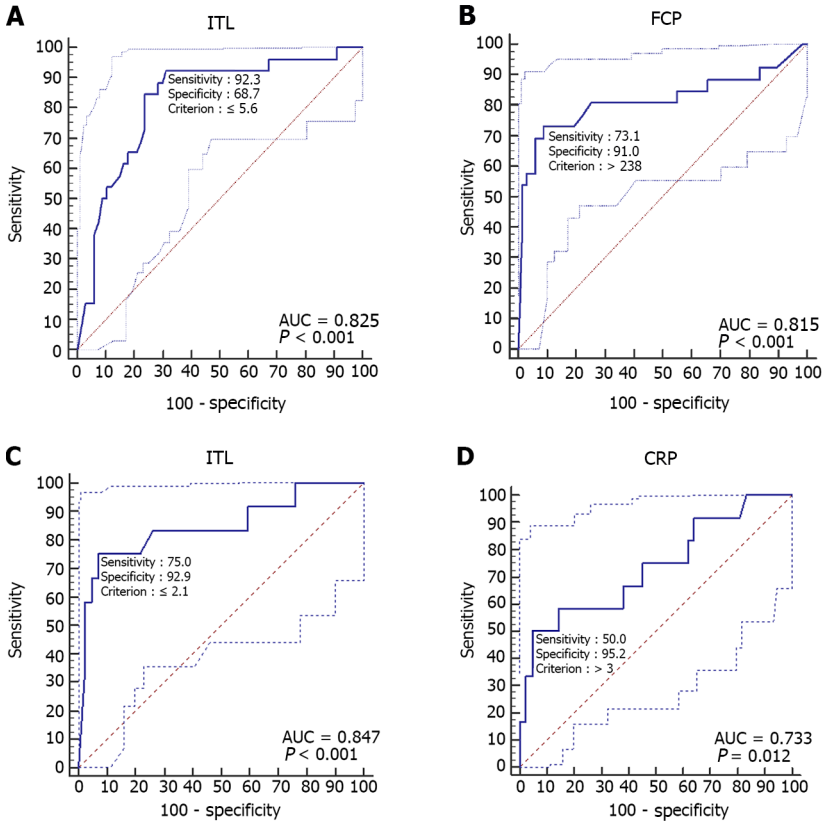

Figure 3 Receiver operator characteristic curve of infliximab trough level and inflammatory biomarkers in predicting endoscopic outcomes. A: Receiver operator characteristic (ROC) curve of infliximab trough level at week 14 in predicting endoscopic remission of Crohn's disease (CD) at week 54; B: ROC curve of fecal calprotectin at week 14 in predicting endoscopic remission of CD at week 54; C: ROC curve of infliximab trough level at week 54 in predicting endoscopic remission of CD at week 108; D: ROC of C-reactive protein at week 54 in predicting endoscopic remission of CD at week 108. ITL: Infliximab trough level; CRP: C-reactive protein; FCP: Fecal calprotectin; AUC: Area under the curve.

tumor necrosis factor α biology[14-16]. Therapeutic optimization as increasing fixed dose from 5 mg/kg to 10 mg/kg or shortening injection interval form every 8 wk to 4-6 wk contributes to increase ITL and decrease ATI level. A study from Greece demonstrated that, for the initial measurement after therapeutic adjustment, ITL increased from 1.47 μg/mL to 8.50 μg/mL in patients with therapeutic optimization while ITL decreased from 5.65 μg/mL to 3.8μg/ml in patients without therapeutic optimization[10]. A multi-center randomized clinical trial conducted by Dreesen *et al*[17] showed that CD patients under IFX maintenance therapy as 5 mg/kg had high probability of no mucosal ulcer under endoscopy at week 54 with ITL more than 7.3mg/L, and CD patients under intensified dose IFX therapy as 10 mg/kg had 94% probability of no mucosal ulcer under endoscopy with ITL rising to more than 10.6 mg/L. Therefore, intensified therapy may contribute to mucosal healing in CD patients with ulceration if IFX injection dose is less than 10 mg/kg and ITL is less than 10.6 μg/mL. However, a few CD patients will accept combination therapy of IFX and immunosuppressant to boost the efficacy, especially AZA and mercaptopurine. AZA is a precursor of mercaptopurine, and two components ultimately produce thioguanine to exert clinical effect during metabolism. A study verified that thioguanine concentration more than 125 pmol/8 × 10^8 red blood cells could enhance ITL to 8.3 μg/mL or more and decrease positive rate of ATI[18,19]. Hence, this study mainly included CD patients with sustained clinical remission more than 6 mo under fixed therapeutic strategy of IFX 5 mg/kg every 8 wk combined with AZA 50 mg every day. Retrospective records of CD patients included would suspend if therapy strategy changed, such as intensive therapy of IFX, conversion therapy of other biologics and combination therapy of surgery or other medications. The study design eliminated the influence of therapeutic adjustment on ITL.

The greatest strength of inflammatory biomarkers compared with blood trough level is that they are unaffected by time during different monitoring stages of biological therapy in IBD patients. This study showed that ITL > 5.6 μg/mL combined with FCP ≤ 238 μg/g at week 14 moderately predicted sustained endoscopic remission during the 1-year follow-up period on CD patients with positive predictive value more than 95% as well as ITL > 2.1 μg/mL combined with CRP ≤ 3.00 mg/L at week 54, superior to use ITL as the only predictor. FCP and CRP are considered as the most universal and typical biomarkers of inflammatory evaluation in IBD, also verified to be the independent risk factors of adverse endoscopic outcomes. The study confirmed that combining blood trough level with inflam-

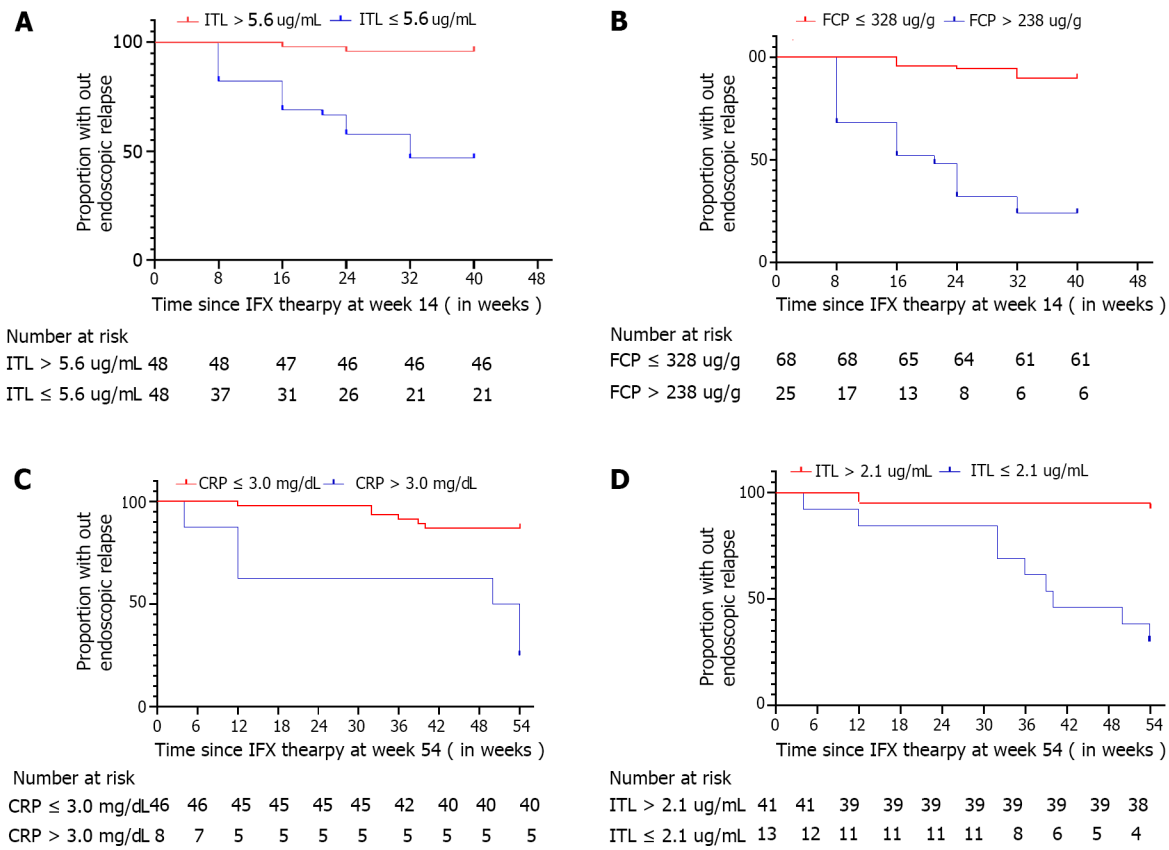

Figure 4 Proportion without endoscopic relapse. A. Time since infliximab (IFX) therapy at week 14 [IFX trough level (ITL) > 5.6 μg/mL *vs* ITL ≤ 5.6 μg/mL]; B: Time to IFX therapy at week 14 (fecal calprotectin ≤ 238 μg/g *vs* fecal calprotectin > 238 μg/g]; C: Time to IFX therapy at week 54 (C-reactive protein ≤ 3.0mg/L *vs* C-reactive protein > 3.0 mg/L); D: Time to IFX therapy at week 54 (ITL > 2.1 μg/mL *vs* ITL ≤ 2.1 μg/mL). ITL: Infliximab trough level; CRP: C-reactive protein; FCP: Fecal calprotectin; IFX: Infliximab.

matory biomarkers contributed to improving the accuracy of the prediction on endoscopic outcomes. A post hoc analysis from the CALM study manifested that CD patients with FCP < 250 μg/g mostly achieved CDEIS < 4 without deep ulceration, regardless of whether CRP < 5 mg/L. However, among patients with CRP < 5 mg/L but FCP ≥ 250 μg/g, only 16.7% achieved CDEIS < 4 without deep ulceration[20]. The result indicated that the correlation between FCP normalization and endoscopic mucosal healing in CD patients was stronger than that of CRP normalization. A previous study verified that FCP is suitable for distinguishing mild endoscopic activity from endoscopic remission, while it is difficult to distinguish partial endoscopic remission from complete endoscopic remission[21]. Similar to blood trough level, the optimal cut off value of FCP for distinguishing endoscopic activity from endoscopic remission ranges from 71 μg/g to 250 μg/g with moderate diagnostic performance[22-27]. The study identified that FCP > 276 μg/g predicted endoscopic activity at week 54 of CD patients with clinical remission at week 14 moderately with 84.6% sensitivity and 92.1% specificity. Unlike FCP, the sensitivity of CRP to mild intestinal inflammation was low and the level of CRP increased much more dramatically in CD patients with moderate to severe inflammation. Therefore, the previous study preferred to utilize CRP to distinguish moderate to severe endoscopic activity from mild to moderate endoscopic activity rather than distinguish mild endoscopic activity from endoscopic remission. A Spanish study showed that FCP > 155 μg/g in combination with CRP > 6.7 mg/L could identify endoscopic activity with 82% specificity[27].

However, the study has shortcomings in some areas. Firstly, the retrospective single-center study with small sample, inferior to prospective multi-center with greater sample, comprised some confounding factors. More real-world studies and randomized controlled trials on guidance significance of ITL to therapeutic outcomes in IBD need to be conducted. Secondly, the study primarily concentrated on mucosal inflammation located in large intestine, ignoring small intestine due to the high cost and the incomplete scoring system of small intestinal evaluation accompanied by the poor compliance of patients and the laborious operation of endoscopists. Correlation between ITL and various small intestinal examinations including endoscopy or imageology may be the focus of the future study. Last but not least, definition of deep remission on CD has been tightened. Considering transmural inflammation of CD, endoscopy is confined to mucosal inflammation and macroscopical evaluation while imageology can accurately evaluate complete volume of intestinal wall and histopathological examination, which contributes to microscopical examination. Notwithstanding endoscopic remission

considered as the main targets and histological remission considered as a novel target, the new concept of 'disease clearance', which includes clinical, endoscopic and microscopic remission, has drawn more and more attention from IBD physicians and may bring about a new upsurge of studies on IFX monitoring and new therapeutic targets[28].

CONCLUSION

In conclusion, during IFX maintenance treatment, low ITL, high CRP level and high FCP level were independent risk factors of long-term adverse endoscopy outcomes in CD patients with clinical remission. Combination of ITL, CRP and FCP contribute to long-term endoscopic prognosis monitoring. The best cut off values of ITL for predicting endoscopic activity within 1-year follow up were 5.60 μg/mL at week 14 and 2.10 μg/mL at week 54. In addition, ITL ≤ 5.60 μg/mL in combination with FCP > 238 μg/g at week 14 as well as ITL ≤ 2.10 μg/mL in combination with CRP > 3.00 mg/L at week 54 increased the precision of prediction on endoscopic outcomes at week 54 and week 108, respectively. Therapeutic optimization is still recommended in CD patients achieving endoscopic remission, provided that low ITLs or high levels of inflammatory biomarkers, such as CRP or FCP, arise to prevent endoscopic recurrence as soon as possible.

ARTICLE HIGHLIGHTS

Research background

Existing studies have confirmed that infliximab (IFX) blood concentration is closely related to remission and recurrence of Crohn's disease (CD) patients under IFX therapy. In addition, monitoring inflammatory biomarkers regularly is another important tool for prognosis assessment of CD patients. Current studies have confirmed that C-reactive protein (CRP) and fecal calprotectin (FCP) are good predictors of disease activity, but there is still a lack of reliable evidence for predicting disease remission. Therefore, in the early stage of IFX treatment, combination of IFX blood concentration and inflammatory biomarkers may contribute to predict the change of CD outcomes.

Research motivation

The best therapeutic goal of CD was initially defined as clinical remission, and then the definition was converted to endoscopic remission with precise therapy. Nowadays, some clinicians even pursue whole-wall healing with individualized therapy. However, long-term clinical prognosis rather than long-term endoscopic prognosis is still a research priority of clinical studies contrapose to CD patients under IFX therapy. Therefore, prediction on long-term endoscopic prognosis of CD patients under IFX therapy has been based solely on models because of a lack of available data.

Research objectives

To explore the predictive value of blood drug concentration on long-term endoscopic outcomes of IFX therapy for CD and establish a comprehensive outcome prediction model combining IFX blood drug concentration, CRP and FCP, so as to provide a basis for clinical decision making.

Research methods

A single-center retrospective research has been implemented in the First Affiliated Hospital of Zhejiang Chinese Medical University. CD patients under IFX therapy from January 2012 to December 2020 were collected. One hundred and eighty-one CD patients underwent IFX treatment. One hundred and fifty-three CD patients underwent endoscopy as well as serum concentration monitoring at week 14 after the third dose of IFX induction therapy. Inclusion criteria: (1) Endoscopic remission at week 14 [Crohn's disease endoscopic index of severity (CDEIS) score ≤ 2 points or Rutgeerts ≤ i1]; (2) Clinical remission after IFX induction therapy without corticosteroids more than 6 mo; and (3) Therapeutic strategy during maintenance stage was designed as IFX 5 mg/kg every 8 wk combined with azathioprine 50 mg every day. The study was divided into two stages, the Step I study was conducted from week 14 to 54 of IFX treatment, and the Step II study was conducted from week 54 to 108 of IFX treatment. Endoscopic outcomes were defined as endoscopic activity (CDEIS score > 2 points or Rutgeerts score > i1) and endoscopic remission (CDEIS score ≤ 2 points or Rutgeerts ≤ i1). Endoscopic relapse free survival was defined as endoscopic remission at the beginning of the study stage and maintaining endoscopic remission during the study stage.

Research results

In step I study, 67/93 CD patients (72.04%) sustained endoscopic remission at week 54. Multivariable regression analysis demonstrated that only ITL [odds ratio (OR) = 0.666, 95% confidence interval (CI):

0.514-0.862, P = 0.002] and FCP (OR = 1.002, 95%CI: 1.001-1.004, P = 0.002) were independent risk of endoscopic activity at week 54. The receiver-operating characteristic analysis demonstrated that the best cut off level of ITL and FCP at week 14 on predicting endoscopic relapse at week 54 was 5.60 μg/mL [area under the curve (AUC) = 0.83, 95%CI: 0.73-0.90, P < 0.001] and 238 μg/g (AUC = 0.82, 95%CI: 0.72-0.89, P < 0.001). The median time to endoscopic relapse of CD patients with ITL ≤ 5.6 μg/ml was 32.00 wk shorter than those with ITL > 5.6 μg/mL [hazard ratio (HR) = 16.19, 95%CI: 7.44-35.22, P < 0.0001]. The median time to endoscopic relapse of CD patients with FCP > 238 μg/g was 21.00 wk shorter than those with FCP ≤ 238 μg/g (HR = 11.25, 95%CI: 4.26-29.73, P < 0.0001). (II) In step II study, 42/54 CD patients (77.78%) sustained endoscopic remission at week 108. Multivariable regression analysis found that only ITL (OR = 0.466, 95%CI: 0.247-0.877, P = 0.018) and CRP (OR = 1.590, 95%CI: 1.007-2.510, P = 0.047) were independent risks of endoscopic activity at week 108. The receiver-operating characteristic analysis demonstrated that the best cut off level of ITL and CRP at week 54 on predicting endoscopic relapse at week 108 was 2.10 μg/ml (AUC = 0.85, 95%CI: 0.72-0.93, P < 0.001) and 3.00 mg/L (AUC = 0.73, 95%CI: 0.60-0.84, P = 0.012). The median time to endoscopic relapse of CD patients with ITL ≤ 2.1 μg/mL was 40.00 w shorter than those with ITL > 2.1 μg/mL (HR = 13.14, 95%CI: 3.07-56.27, P < 0.0001). The median time to endoscopic relapse of CD patients with CRP > 3.00 mg/L was 50.00 wk shorter than those with CRP ≤ 3.00 mg/L (HR = 7.85, 95%CI: 1.31-46.85, P< 0.0001).

Research conclusions

The best cut off values of ITL for predicting endoscopic activity within 1-year follow up was 5.60 μg/mL at week 14 and 2.10 μg/mL at week 54. In addition, ITL ≤ 5.60 μg/mL in combination with FCP > 238 μg/g at week 14 as well as ITL ≤ 2.10 μg/mL in combination with CRP > 3.00 mg/L at week 54 increased the precision of prediction on endoscopic outcomes at week 54 and week 108, respectively.

Research perspectives

In view of the fact that conduction of intensive monitoring for biological management plays a vital role in precise treatment for CD patients, much larger and more stringent prospective studies are warranted to provide the best predictive models as acknowledged globally in allusion to long-term endoscopic outcomes of CD patients under IFX therapy.

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FOOTNOTES

Author contributions: Fan YH designed the research; Cao WT performed the research; Cao WT and Liu S analyzed the data; Cao WT, Huang R, Ni H and Xu MS wrote the paper; Xu Y supervised the paper; All authors have read and approve the final manuscript.

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ORIGINAL ARTICLE

Retrospective Study

Higher infliximab and adalimumab trough levels are associated with fistula healing in patients with fistulising perianal Crohn's disease

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Abstract

BACKGROUND

Tumor necrosis factor-alpha inhibitors, including infliximab and adalimumab, are

effective medical treatments for perianal fistulising Crohn's disease (CD), but not all patients achieve fistula healing.

AIM

To determine the correlation between perianal fistula healing and closure with infliximab and adalimumab trough levels.

METHODS

In this multicentre retrospective study conducted across four tertiary inflammatory bowel disease centres in Australia, we identified CD patients with perianal fistulae on maintenance infliximab or adalimumab who had a trough level within twelve weeks of clinical assessment. Data collected included demographics, serum infliximab and adalimumab trough levels (mg/L) within 12 wk before or after their most recent clinical assessment and concomitant medical or surgical therapy. The primary outcome was fistula healing, defined as cessation in fistula drainage. The secondary outcome was fistula closure, defined as healing and closure of all external fistula openings. Differences between patients who did or did not achieve fistula healing were compared using the chi-square test, *t* test or Mann-Whitney *U* test.

RESULTS

One hundred and fourteen patients (66 infliximab, 48 adalimumab) were included. Forty-eight (72.7%) patients on maintenance infliximab achieved fistula healing and 18 (27.3%) achieved fistula closure. Thirty-seven (77%) patients on maintenance adalimumab achieved fistula healing and 17 (35.4%) achieved fistula closure. Patients who achieved fistula healing had significantly higher infliximab and adalimumab trough levels than patients who did not [infliximab: 6.4 (3.8-9.5) *vs* 3.0 (0.3-6.2) mg/L, $P = 0.003$; adalimumab: 9.2 (6.5-12.0) *vs* 5.4 (2.5-8.3) mg/L, $P = 0.004$]. For patients on infliximab, fistula healing was associated with lower rates of detectable anti-infliximab antibodies and younger age. For patients on adalimumab, fistula healing was associated with higher rates of combination therapy with an immunomodulator. Serum trough levels for patients with and without fistula closure were not significantly different for infliximab [6.9 (4.3-10.2) *vs* 5.5 (2.5-8.3) mg/L, $P = 0.105$] or adalimumab [10.0 (6.6-12.0) *vs* 7.8 (4.2-10.0) mg/L, $P = 0.083$].

CONCLUSION

Higher maintenance infliximab and adalimumab trough levels are associated with perianal fistula healing in CD.

Key Words: Crohn's disease; Perianal disorders; Biologics; Inflammatory bowel disease

Core Tip: This multicentre retrospective study demonstrated a significant association between both infliximab and adalimumab trough levels with fistula healing, with higher levels associated with increased healing rates. Higher tertiles of both infliximab and adalimumab levels were associated with a higher proportion of patients achieving fistula healing. Fistula healing, defined as cessation of fistula drainage, is a clinically relevant endpoint that impacts on patient quality of life. Our results support dose-escalation of both infliximab and adalimumab in non-responders, targeting higher levels to achieve fistula healing prior to changing biologic therapy. Importantly, this study is the largest study to date assessing the relationship between adalimumab trough levels and clinical fistula healing.

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INTRODUCTION

Perianal fistulising disease is a common manifestation occurring in up to 30% of patients with Crohn's disease (CD). The development of abnormal tracts between the bowel and perineum can cause perianal drainage, pain, bleeding, abscess formation, sepsis and faecal incontinence[1,2]. Perianal CD is

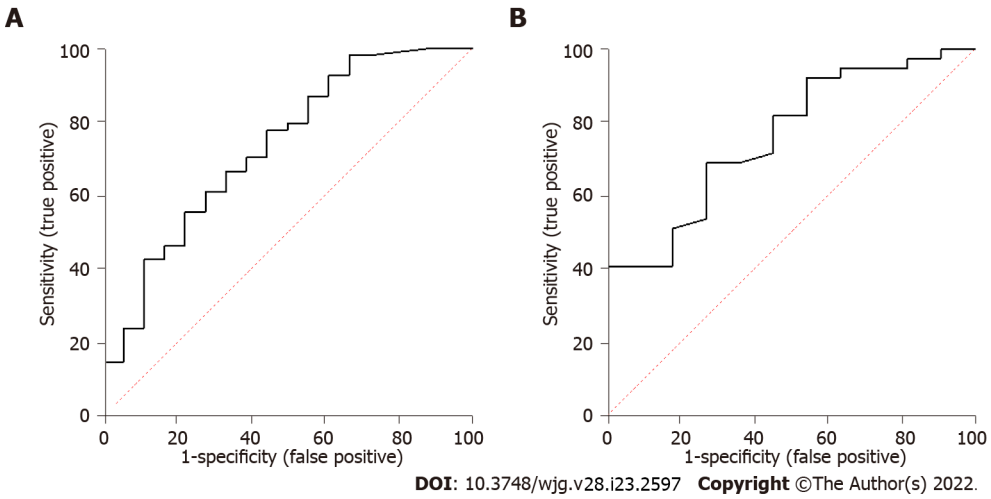

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Figure 1 Correlation between serum trough level of infliximab, adalimumab and fistula healing. A: Infliximab; B: Adalimumab.

associated with significant morbidity and decreased quality of life, negatively impacting physical, emotional, sexual and social wellbeing[1-3] and is an independent predictor for decreased productivity in patients with CD[4,5]. Given that the incidence of perianal fistulising CD is highest in the third and fourth decades of life, this places significant burden on patients, society, the economy and the health care system[6].

Treatment for perianal fistulising CD requires a multidisciplinary approach involving medical management with immunosuppressants and antibiotics, as well as surgical management with sepsis control, seton insertion and sometimes diversion or resection. Anti-tumor necrosis factor (anti-TNF) alpha agents, including infliximab[7,8] and adalimumab[9,10], are the most effective medical therapies available for inducing and maintaining remission of fistulas. Unfortunately, up to 60% of patients treated with maintenance infliximab lose response within one year[7,8]. Accumulating evidence suggests that this loss of response is partly due to subtherapeutic anti-TNF trough levels. Retrospective studies and post-hoc analyses of prospective data have identified that higher infliximab trough levels are associated with fistula healing and closure compared to what is observed for mucosal healing in luminal disease, with emerging data suggesting similar results for adalimumab[11-14]. Quantitative assays for therapeutic drug monitoring (TDM) permit individualisation of infliximab and adalimumab dosing[15,16], however there are very few studies on perianal fistulising CD and the optimal target levels for perianal fistulising CD remain unclear. Our study aims to assess the association between serum trough infliximab and adalimumab levels and perianal fistula healing and closure and identify optimal target levels.

MATERIALS AND METHODS

Study design and patient population

This was a multicentre retrospective cross-sectional study of patients with perianal fistulising CD at four tertiary inflammatory bowel disease centres across Australia between January 2014 and June 2020. All patients qualified for infliximab or adalimumab under the Australian Pharmaceutical Benefits Scheme criteria[17] which constitutes the following: (1) A confirmed diagnosis of CD using clinical, radiological, histological and/or endoscopic criteria; and (2) At least one active externally draining complex perianal fistula. We included patients on maintenance infliximab or adalimumab with a documented perianal examination who had a serum infliximab or adalimumab trough level collected within 12 wk before or after their most recent clinical assessment. Infliximab and adalimumab trough levels as well as antibodies to infliximab and adalimumab were measured using a drug sensitive enzyme-linked immunosorbent assay (Grifols Promonitor for adalimumab; LISA-Tracker and Grifols Promonitor for infliximab). Infliximab and adalimumab trough levels were measured both in a proactive manner and reactive manner in patients failing treatment across the study sites. Patients who had been changed from infliximab to adalimumab or vice versa and had relevant data were included in both the infliximab and adalimumab groups.

All patients had received standard infliximab or adalimumab induction dosing (infliximab 5 mg/kg intravenously at weeks 0, 2, and 6; adalimumab subcutaneously 160 mg at week 0, 80 mg at week 2) followed by maintenance therapy. The current dose of anti-TNF therapy was recorded and patients with or without dose-escalated maintenance therapy were included. Patients who had a diversion ostomy, rectovaginal fistula or no documented perianal examination were excluded.

Demographic data

Data was retrospectively collected from a clinical database that was updated prospectively during routine clinical practice. Patient demographics collected included age, gender, weight, body mass index, smoking status and CD phenotype classified according to the Montreal Classification[18]. The location of CD was identified as ileal, ileocolonic, colonic, upper gastrointestinal involvement or no luminal disease. The presence or absence of fistulising and stricturing disease was noted, in particular the presence of anal strictures. Biochemical markers of disease activity including C-reactive protein (CRP) and albumin were also recorded.

Current management

Prior history of surgical management of perianal disease or fistula was recorded and categorised as examination under anaesthesia and curettage, examination under anaesthesia and seton insertion or fistulotomy. The duration from the last surgical procedure to the follow up visit was recorded. Concomitant medical therapy at the time of follow up was assessed, including corticosteroid use, 5-aminosalicylates and immunomodulators. The doses of infliximab and adalimumab were recorded and stratified according to dose and interval between doses. For patients on dose-escalated anti-TNF therapy, the duration between last dose escalation and follow up was recorded.

Primary and secondary outcomes

The primary outcome was fistula healing, which was defined as cessation of fistula drainage, with or without a seton *in situ*[7]. The secondary outcome was fistula closure, which was defined as healing and closure of all external fistula openings[7].

Statistical analysis

Statistical review of this study was performed by a biostatistician from the Ingham Institute for Applied Medical Research. Descriptive statistics were used to assess the baseline characteristics of both the infliximab and adalimumab cohorts. Categorical variables were expressed as percentages and compared using the chi-square test. Continuous variables were expressed using mean ± SD for normally distributed variables and median and interquartile range (IQR) for non-normally distributed variables. The means were compared using the *t* test for normally distributed variables and the mean ranks compared using the Mann-Whitney *U* test for non-normally distributed variables. A receiver operating characteristic (ROC) curve analysis was used to assess the sensitivity and specificity of infliximab and adalimumab levels at different cut-off points for predicting fistula healing. All reported *P* values were 2-sided, with $P < 0.05$ considered statistically significant. Multivariate analysis using logistic regression with forwards selection was used to analyse variables that predicted fistula healing. Variables which were statistically significant in the univariate analysis were included in the multivariate analysis model. Ethics approval was obtained from the South Western Sydney Local Health District (Human Research Ethics Committee LNR/18/LPOOL/404; Local Project Number: HE18/261).

RESULTS

Out of 454 patients screened, 114 patients (66 infliximab, 48 adalimumab) on maintenance infliximab or adalimumab for perianal CD had a trough level collected within 12 wk of clinical assessment. Five patients had been changed from infliximab to adalimumab or vice versa and were included in both the infliximab and adalimumab groups. Seventy-five (66%) patients were on combination therapy (43 azathioprine, 16 6-mercaptopurine, 16 methotrexate). Nineteen patients (28.8%) on maintenance infliximab were on dose escalated infliximab therapy (5, 7.5, 10, 15 or 20 mg/kg every 6 or 8 wk). For these patients, the median duration between last infliximab dose adjustment and follow up was 60.0 wk (IQR = 44.5-81.0). Eleven (22.9%) patients on maintenance adalimumab were on dose escalated adalimumab therapy (40 mg weekly). For these patients, the median duration between last adalimumab dose adjustment and follow up was 39.0 wk (IQR = 24.0-86.0). Fifty-nine (89.3%) patients on infliximab had prior surgical management of their fistula, with a median duration of 93.0 wk (IQR = 45.5-284.5) between their last surgical procedure and their most recent follow up visit. Thirty-seven (77.1%) patients on adalimumab had prior surgical management of their fistula, with a median duration of 83.0 wk (IQR = 28.75-223.0) between their last surgical procedure and their most recent follow up visit. Patient demographics and disease characteristics of the population are summarised in Table 1.

Association between fistula healing and closure with infliximab trough levels

Forty-eight (72.7%) patients on maintenance infliximab achieved fistula healing. Table 2 summarises the differences between patients on infliximab with and without fistula healing. Patients who achieved fistula healing had higher infliximab trough levels [6.4 (3.8-9.5) *vs* 3.0 (0.3-6.2) mg/L, $P = 0.003$], lower rates of detectable anti-infliximab antibodies (4.3% *vs* 33.3%, $P = 0.004$) and a younger age (33.0 *vs* 43.5 years old; $P = 0.003$) compared to patients who did not achieve fistula healing. The presence of

Table 1 Patient demographics and disease characteristics

	Infliximab (*n* = 66)	Adalimumab (*n* = 48)
Median age, yr (IQR)	36.0 (28.8-43.3)	34.5 (29.0-51.8)
A1, *n* (%)	2 (3.0)	7 (14.6)
A2, *n* (%)	52 (78.8)	26 (54.2)
A3, *n* (%)	9 (13.6)	13 (27.1)
Female gender, *n* (%)	28 (42.4)	14 (29.2)
Mean weight, kg (SD)	80.9 (18.7)	82.1 (21.4)
Mean BMI, kg/m^2 (SD)	28.1 (4.8)	28.5 (5.5)
Median age at diagnosis of Crohn's disease (IQR)	26.0 (21.0-34.0)	24.0 (19.0-41.3)
Current smoker, *n* (%)	12 (18.2)	5 (10.4)
Disease location		
Ileal, *n* (%)	16 (24.2)	17 (35.4)
Colonic, *n* (%)	26 (39.4)	7 (14.6)
Ileocolonic, *n* (%)	15 (22.7)	18 (37.5)
No luminal disease, *n* (%)	4 (6.1)	2 (4.2)
Upper gastrointestinal involvement, *n* (%)	4 (6.1)	2 (4.2)
Stricturing, *n* (%)	10 (15.2)	11 (22.9)
Penetrating, *n* (%)	7 (10.6)	17 (35.4)
Median duration on anti-TNF agent, wk (IQR)	144.0 (80.0-280.0)	180.0 (107.3-309.8)
Anti-TNF dosing, *n* (%)		
IFX, 5 mg/kg/8 wk	47	-
IFX, 7.5 mg/kg/8 wk	1	-
IFX, 10 mg/kg/8 wk	12	-
IFX, 15 mg/kg/8 wk	1	-
IFX, 20 mg/kg/8 wk	1	-
IFX, 5 mg/kg/6 wk	3	-
IFX, 10 mg/kg/6 wk	1	-
ADA, 40 mg fortnightly	-	37
ADA, 40 mg weekly	-	11
Concurrent steroids, *n* (%)	0 (0.0)	1 (2.1)
Concurrent aminosalicylates, *n* (%)	4 (6.1)	5 (10.4)
Combination with immunomodulator, *n* (%)	46 (69.7)	29 (60.4)
Methotrexate, *n* (%)	8 (12.1)	8 (16.7)
6-mercaptopurine, *n* (%)	9 (13.6)	7 (14.6)
Azathioprine, *n* (%)	29 (43.9)	14 (29.2)
Concurrent allopurinol, *n* (%)	10 (15.2)	4 (8.3)
Mean albumin, g/L (SD)	39.9 (4.9)	40.2 (4.7)
Median CRP, mg/L (IQR)	1.4 (0.7-5.5)	2.3 (1.2-5.2)

ADA: Adalimumab; BMI: Body mass index; CRP: C-reactive protein; IFX: Infliximab; IQR: Interquartile range; SD: Standard deviation; TNF: Tumor necrosis factor.

Table 2 Differences between patients on infliximab with and without fistula healing

	Patients with fistula healing (*n* = 48)	Patients without fistula healing (*n* = 18)	*P* value
Median age, yr (IQR)	33.0 (28.0-38.0)	43.5 (34.3-57.3)	0.005
Female gender, *n* (%)	20 (41.7)	8 (44.4)	0.839
Mean weight, kg (SD)	82.2 (19.4)	76.5 (15.7)	0.378
Mean BMI, kg/m^2 (SD)	28.5 (5.0)	26.7 (3.8)	0.318
Median age at diagnosis of Crohn's disease (IQR)	26.0 (20.75-30.5)	30.0 (24.0-43.0)	0.121
A1, *n* (%)	1 (2.1)	1 (5.6)	-
A2, *n* (%)	41 (85.4)	11 (61.1)	-
A3, *n* (%)	4 (8.3)	5 (27.8)	-
Current smoker, *n* (%)	8 (16.7)	4 (22.2)	0.696
Location			
Ileal, *n* (%)	14 (29.2)	2 (11.1)	-
Colonic, *n* (%)	19 (39.6)	7 (38.9)	-
Ileocolonic, *n* (%)	11 (22.9)	4 (22.2)	-
No luminal disease, *n* (%)	2 (4.2)	2 (11.1)	-
Upper gastrointestinal involvement, *n* (%)	3 (6.3)	1 (5.6)	-
Stricturing, *n* (%)	7 (14.6)	3 (16.7)	0.822
Penetrating, *n* (%)	5 (10.4)	2 (11.1)	0.927
Median duration on anti-TNF agent, wk (IQR)	153.0 (86.0-285.0)	95.5 (40.25-322.75)	0.387
Dose escalated anti-TNF therapy, *n* (%)	15 (31.3)	4 (22.2)	-
Concurrent steroids, *n* (%)	0 (0.0)	1 (5.6)	-
Concurrent aminosalicylates, *n* (%)	4 (8.3)	1 (5.6)	0.219
Combination with immunomodulator, *n* (%)	35 (72.9)	11 (61.1)	0.522
Methotrexate, *n* (%)	6 (12.5)	2 (11.1)	0.937
6-mercaptopurine, *n* (%)	6 (12.5)	3 (16.7)	0.597
Azathioprine, *n* (%)	23 (47.9)	6 (33.3)	0.368
Concurrent allopurinol, *n* (%)	9 (18.8)	1 (5.6)	0.197
Mean albumin, g/L (SD)	40.3 (4.7)	40.7 (4.6)	0.590
Median CRP, mg/L (IQR)	2.1 (1.0-4.5)	5.5 (1.1-8.7)	0.094
Median trough level, mg/L (IQR)	6.4 (3.8-9.5)	3.0 (0.3-6.2)	0.003
Detectable antibody, *n* (%)	3 (4.3)	6 (33.3)	0.004

ADA: Adalimumab; BMI: Body mass index; CRP: C-reactive protein; IFX: Infliximab; IQR: Interquartile range; SD: Standard deviation; TNF: Tumor necrosis factor.

detectable anti-infliximab antibodies was associated with lower infliximab trough levels (P = 0.02). The CRP and albumin levels were not significantly different between patients with and without fistula healing. The rates of combination therapy with an immunomodulator were not significantly different between patients who achieved fistula healing and those who did not (P = 0.522).

ROC curve analysis identified a positive correlation between infliximab trough levels and healing [area under the curve (AUC) = 0.74, 95% confidence interval (CI): 0.60-0.88, P = 0.003; Figure 1A] with an infliximab trough level of 6.10 mg/L that maximised the sensitivity and specificity of predicting fistula healing [sensitivity 58%, specificity 78%, odds ratio (OR) = 4.9, P = 0.013]. Upon tertile analysis, higher tertiles of infliximab levels were associated with a higher proportion of patients achieving fistula healing with 54.5% healing rate for tertile 1 compared to 90.1% for tertile 3 (Figure 2A; P = 0.026). Out of the patients who achieved fistula healing on infliximab, 90% and 95% of the patients who achieved fistula healing were healed with an infliximab trough level of 12.7 and 14.4 mg/L respectively. Given

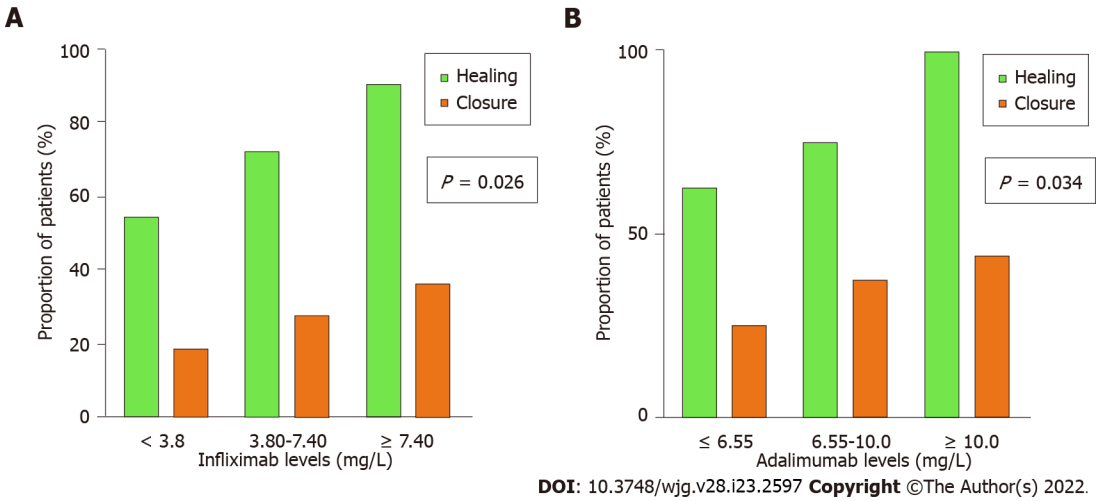

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Figure 2 Tertile analysis of infliximab and adalimumab trough levels for patients with fistula healing and fistula closure. A: Infliximab; B: Adalimumab.

that a drug-sensitive infliximab assay was used where anti-infliximab antibody titres were only performed if infliximab concentrations were < 2.0 mg/L, anti-infliximab antibodies were not included in the multivariate analysis. On multivariate logistic regression analysis, age was associated with healing ($P = 0.026$) but adequate infliximab levels ≥ 6.10 mg/L were not ($P = 0.097$). Within our cohort, 18 (27.3%) of patients on infliximab achieved fistula closure. The infliximab trough level for patients with and without fistula closure was not significantly different [6.9 (4.3-10.2) *vs* 5.5 (2.5-8.3) mg/L, $P = 0.105$].

Association between fistula healing and closure with adalimumab trough levels

Thirty-seven (77%) patients on maintenance adalimumab achieved fistula healing. Table 3 summarises the differences in patients on adalimumab with and without fistula healing. Patients who achieved fistula healing had higher adalimumab trough levels compared to those who did not [9.2 (6.5-12.0) *vs* 5.4 (2.5-8.3) mg/L, $P = 0.004$]. Patients who achieved fistula healing had higher rates of combination therapy with an immunomodulator than those who did not ($P = 0.048$). The CRP and albumin levels were not significantly different in patients with and without fistula healing. ROC curve analysis identified a positive correlation between adalimumab trough levels and healing (AUC = 0.79, 95%CI: 0.66-0.93, $P = 0.004$) with an adalimumab trough level of 7.05 mg/L that maximised the sensitivity and specificity of infliximab levels in predicting fistula healing (sensitivity 70%; specificity 73%; OR = 6.3; $P = 0.016$; Figure 1B). Upon tertile analysis, higher tertiles of adalimumab levels were associated with a higher proportion of patients achieving fistula healing, with 62.5% healing rate for tertile 1 compared to 100% for tertile 3 (Figure 2B; $P = 0.034$). Out of the patients who achieved fistula healing on adalimumab, 90% and 95% of the patients who achieved fistula healing were healed with an adalimumab trough level of 12.0 and 18.0 mg/L respectively. On multivariate logistic regression analysis, adequate adalimumab trough levels ≥ 7.05 mg/L ($P = 0.008$) and concurrent immunomodulator therapy ($P = 0.026$) both remained associated with healing. Within our cohort, 17 (35.4%) of patients on adalimumab achieved fistula closure. The adalimumab trough level for patients with and without fistula closure was not significantly different [10.0 (6.6-12.0) *vs* 7.8 (4.2-10.0) mg/L, $P = 0.083$].

DISCUSSION

Fistulising perianal CD is a highly morbid condition for which treatment outcomes remain suboptimal in many patients. While there is limited data on the role of newer biologic agents such as ustekinumab in perianal CD[19], anti-TNF agents remain the treatment of choice. Our study showed a significant association between both infliximab and adalimumab trough levels and fistula healing, with higher levels associated with increased healing rates. We demonstrated that higher tertiles of both infliximab and adalimumab levels were associated with a higher proportion of patients achieving fistula healing. Notably, when plotting the cumulative percentage of healed patients against infliximab level, we found that 50% of the patients who achieve healing will heal with a level of 6.4 mg/L, 90% of the patients who achieve healing will heal with a level of 12.7 mg/L and 95% of the patients who achieve healing will heal with a level of 14.4 mg/L. Similarly, for patients on adalimumab, 50% of the patients who achieve healing will heal with a level of 9.2 mg/L, and 90% and 95% of patients who achieved fistula healing were healed with levels of 12.0 and 18.0 mg/L respectively. Our results support dose-escalation of both infliximab and adalimumab in non-responders, targeting higher levels to achieve fistula healing prior to

Table 3 Differences in patients on adalimumab with and without fistula healing

	Patients with fistula healing (*n* = 37)	Patients without fistula healing (*n* = 11)	*P* value
Median age, yr (IQR)	33.0 (28.5-52.0)	44.9 (33.0-52.0)	0.254
Female gender, *n* (%)	10 (27.0)	4 (36.4)	0.550
Mean weight, kg (SD)	82.5 (22.3)	79.7 (16.7)	0.812
Mean BMI, kg/m^2 (SD)	29.2 (5.9)	25.5 (2.7)	0.241
Median age at diagnosis of Crohn's disease (IQR)	24.0 (18.0-42.0)	30.0 (19.0-41.0)	0.570
A1, *n* (%)	6 (16.2)	1 (9.1)	-
A2, *n* (%)	19 (51.4)	7 (63.6)	-
A3, *n* (%)	10 (27.0)	3 (27.3)	-
Current smoker, *n* (%)	5 (13.5)	0 (0.0)	0.198
Location			
Ileal, *n* (%)	12 (32.4)	5 (45.5)	-
Colonic, *n* (%)	5 (13.5)	2 (18.2)	-
Ileocolonic, *n* (%)	15 (40.5)	3 (27.3)	-
No luminal disease, *n* (%)	2 (5.4)	0 (0.0)	-
Upper gastrointestinal involvement, *n* (%)	2 (5.4)	1 (9.1)	-
Stricturing, *n* (%)	9 (24.3)	2 (18.2)	0.644
Penetrating, *n* (%)	14 (37.8)	3 (27.3)	0.481
Median duration on anti-TNF agent, wk (IQR)	194.5 (124.3-311.3)	122.5 (79.8-319.3)	0.318
Dose escalated anti-TNF therapy, *n* (%)	10 (27.0)	1 (9.1)	-
Concurrent steroids, *n* (%)	0 (0.0)	0 (0.0)	-
Concurrent aminosalicylates, *n* (%)	4 (10.8)	1 (9.1)	0.849
Combination with immunomodulator, *n* (%)	25 (67.6)	4 (36.4)	0.048
Methotrexate, *n* (%)	7 (18.9)	1 (9.1)	0.424
6-mercaptopurine, *n* (%)	6 (16.2)	1 (9.1)	0.537
Azathioprine, *n* (%)	12 (32.4)	2 (18.2)	0.336
Concurrent allopurinol, *n* (%)	4 (10.8)	0 (0.0)	0.248
Mean albumin, g/L (SD)	40.5 (4.5)	40.0 (5.7)	0.608
Median CRP, mg/L (IQR)	2.1 (1.0-4.5)	5.4 (1.7-9.3)	0.070
Median trough level (IQR)	9.2 (6.5-12.0)	5.4 (2.5-8.3)	0.004
Detectable antibody, *n* (%)	1 (2.7)	1 (9.1)	0.352

ADA: Adalimumab; BMI: Body mass index; CRP: C-reactive protein; IFX: Infliximab; IQR: Interquartile range; SD: Standard deviation; TNF: Tumor necrosis factor.

changing biologic therapy. Importantly, this study is the largest study to date assessing the relationship between adalimumab trough levels and clinical fistula healing. This data adds to the growing body of evidence that fistula healing improves with higher anti-TNF trough levels, and that higher levels may be required for perianal fistula healing than for mucosal healing in luminal CD[12-14,20].

This study did not show an association between infliximab and adalimumab trough levels and fistula closure. Not all previous studies have assessed fistula closure, but some have found that patients with fistula closure had significantly higher maintenance infliximab and adalimumab trough levels[13,14]. Our results may have been limited by inadequate power due to relatively small numbers of patients who achieved fistula closure in our cohort. We had a high fistula healing rate in this study, with 72.7% and 77% of patients on maintenance infliximab and adalimumab achieving fistula healing respectively. This finding was possibly due to high rates of combination therapy with an immunomodulator (69.7% and 60.4% in the infliximab and adalimumab groups respectively).

Randomised controlled trials have shown that infliximab is effective at both inducing and maintaining fistula healing[7,8]. Our study found that fistula healing was associated with higher infliximab trough levels. This finding is supported by a post-hoc analysis of ACCENT II which found that higher infliximab trough levels during induction were associated with a complete absence of draining fistulas at week 14[12], as well as similar findings in other studies assessing induction and maintenance infliximab therapy[11,13]. In the future, there may be a role for the infliximab biosimilar CT-P13 in order to achieve these high infliximab levels required for perianal fistula healing; with recent randomised controlled trials demonstrating higher trough levels from subcutaneous administration of CT-P13 compared to intravenous administration[21]. Interestingly, our study found that fistula healing was associated with younger age in both univariate and multivariate analyses. Whilst patient factors including albumin and body weight have previously been shown to affect infliximab trough levels[22], the influence of age is unclear. This finding may be due to the relatively younger age at diagnosis of CD for patients with fistula healing or longer duration of infliximab therapy. Five patients in this study had been changed from infliximab to adalimumab or vice versa and were included in both groups, however the anti-TNF level and anti-TNF antibody levels at the time of changing treatment were not collected. Reassuringly, previous studies have demonstrated that the presence of infliximab antibodies does not decrease future response rates to adalimumab and vice versa[23].

Adalimumab has also been shown to be effective in both inducing[9] and maintaining fistula healing [24]. Our study found that fistula healing was associated with higher adalimumab trough levels. Whilst there is limited data on the association between adalimumab trough levels and fistula healing, our findings are consistent with two smaller retrospective studies that showed that patients with fistula healing had higher adalimumab trough levels compared to those without fistula healing[14,20]. On multivariate logistic regression analysis, adalimumab trough levels ≥ 7.05 mg/L and concurrent immunomodulator therapy both remained significantly associated with healing. This reflects how concomitant immunosuppressive therapy can be used to decrease the immunogenic response and therefore improve fistula healing rates[25].

This study has several limitations. Assessment of fistula healing was based on clinical assessment, which may not be as accurate as an objective assessment such as with magnetic resonance imaging of the pelvis. A recent study has demonstrated that higher anti-TNF trough levels are associated with improved rates of radiological healing in perianal fistulising CD[26]. However, the absence of drainage remains a clinically relevant endpoint that impacts on patient quality of life. In order to provide an objective marker of response, biochemical markers of disease activity including CRP and albumin were analysed and found not to correlate with fistula healing. Data was retrospectively collected, so in order to address this we only included patients with documented perianal exams and definitions for fistula healing and closure that were in line with previous randomised controlled trials[8]. We found that fistula healing is associated with higher infliximab and adalimumab trough levels, however further randomised controlled trials are required to assess whether dose escalation to higher levels improves healing and the optimal method for dose escalation. Whilst reactive TDM with dose escalation at the time of loss of response is effective, it remains unknown whether proactive TDM with subsequent dose modification improves outcomes. Notably, all previous studies on proactive TDM have focused on luminal disease with no prospective studies evaluating proactive TDM in perianal fistulising CD.

CONCLUSION

Our study showed that higher infliximab and adalimumab trough levels are associated with perianal CD fistula healing, with higher rates of healing in higher tertiles of infliximab and adalimumab levels. However, no association with fistula closure was observed. Further prospective studies are required to confirm target infliximab and adalimumab trough levels and determine the optimal dose escalation method to achieve these target levels.

ARTICLE HIGHLIGHTS

Research background

Anti-tumor necrosis factor (anti-TNF)-alpha agents, including infliximab and adalimumab, are effective medical treatments for perianal fistulising Crohn's disease (CD), but not all patients achieve fistula healing with up to 60% of patients treated with maintenance infliximab lose response within one year.

Research motivation

Accumulating evidence suggests that this loss of response is partly due to sub-therapeutic anti-TNF trough levels. Retrospective studies and post-hoc analyses of prospective data have identified that higher infliximab trough levels are associated with fistula healing and closure compared to what is observed for mucosal healing in luminal disease, with emerging data suggesting similar results for

adalimumab. Quantitative assays for therapeutic drug monitoring permits individualisation of infliximab and adalimumab dosing, however there are very few studies on perianal fistulising CD and the optimal target levels for perianal fistulising CD remains unclear.

Research objectives
This study aims to assess the association between serum trough infliximab and adalimumab levels and perianal fistula healing and closure and identify optimal target levels.

Research methods
In this multi-centre retrospective study conducted across four tertiary inflammatory bowel disease centres in Australia, we identified CD patients with perianal fistulae on maintenance infliximab or adalimumab who had a trough level within twelve weeks of clinical assessment. The primary outcome was fistula healing, defined as cessation in fistula drainage. The secondary outcome was fistula closure, defined as healing and closure of all external fistula openings. Differences between patients who did or did not achieve fistula healing were compared using the Chi-square test, *t*-test or Mann-Whitney *U* test.

Research results
Out of a total of 114 patients (66 infliximab, 48 adalimumab), 48 (72.7%) patients and 37 (77%) patients on maintenance infliximab and adalimumab respectively achieved fistula healing. Patients who achieved fistula healing had significantly higher infliximab and adalimumab trough levels compared to patients who did not [infliximab: 6.4 (3.8-9.5) *vs* 3.0 (0.3-6.2) mg/L, $P = 0.003$; adalimumab: 9.2 (6.5-12.0) *vs* 5.4 (2.5-8.3) mg/L, $P = 0.004$]. Serum trough levels for patients with and without fistula closure were not significantly different for infliximab [6.9 (4.3-10.2) *vs* 5.5 (2.5-8.3) mg/L, $P = 0.105$] or adalimumab [10.0 (6.6-12.0) *vs* 7.8 (4.2-10.0) mg/L, $P = 0.083$].

Research conclusions
Higher maintenance infliximab and adalimumab trough levels are associated with perianal fistula healing in CD.

Research perspectives
Our study showed that higher infliximab and adalimumab trough levels are associated with perianal CD fistula healing, with higher rates of healing in higher tertiles of infliximab and adalimumab levels, but no association with fistula closure was observed. Further prospective studies are required to confirm target infliximab and adalimumab trough levels and determine the optimal dose escalation method to achieve these target levels.

FOOTNOTES

Author contributions: Gu B, Williams AJ, Ng W and Connor S conceived concept and design of study; Gu B and Venkatesh K collected the data; Gu B analysed the data; Gholamrezaei A and Xuan W provided statistical support; Gu B prepared the first draft of the manuscript; and all authors provided edits and critiqued the manuscript for intellectual content.

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Conflict-of-interest statement: Gu B has nothing to disclose. Williams AJ has received honoraria from Takeda, Janssen and Abbvie and honoraria and grant support from Ferring. Ng W received grants from Janssen and Pfizer, during the conduct of the study; grants and personal and speaker fees from Abbvie, Takeda, Grants from Ferring and Shire. Corte C has received unrestricted educational grants from Ferring, Janssen, Shire and GESA. Corte C has received honoraria from Janssen, Ferring, Astra-Zeneca, Abbvie and Shire. Travel support and conference registration from Takeda, Shire, Janssen and Nycomed. Advisory board fees from Celgene and Gilead. Ghaly S has received educational grants from Janssen, Ferring, Pfizer and Takeda. Honoraria from Janssen, Ferring, Takeda, AbbVie, Shire. Advisory board fees from Pfizer, AbbVie, Gilead, Ferring and MSD. Paramsothy S is a consultant for Finch Therapeutics and has received speaker fees from Ferring, Janssen and Takeda. Connor S has received honoraria, speaker fees, educational support and/or grant funding from Abbvie, Aspen, BMS, Celgene, Celltrion, Chiesi, DrFalk, Ferring, Fresenius Kabi, Gilead, Janssen, MSD, Novartis, Pfizer, Takeda, Vifor, Agency for Clinical Innovation, Gastroenterological Society of Australia, Medical Research Future Fund and The Leona M and Harry B Helmsley Charitable Trust. This research project did not receive any grant funding.

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ORIGINAL ARTICLE

Retrospective Study

Whole lesion histogram analysis of apparent diffusion coefficient predicts therapy response in locally advanced rectal cancer

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Abstract

BACKGROUND

Whole-tumor apparent diffusion coefficient (ADC) histogram analysis is relevant to predicting the neoadjuvant chemoradiation therapy (nCRT) response in patients with locally advanced rectal cancer (LARC).

AIM

To evaluate the performance of ADC histogram-derived parameters for predicting the outcomes of patients with LARC.

METHODS

This is a single-center, retrospective study, which included 48 patients with LARC. All patients underwent a pre-treatment magnetic resonance imaging (MRI) scan for primary tumor staging and a second restaging MRI for response evaluation. The sample was distributed as follows: 18 responder patients (R) and 30 non-responders (non-R). Eight parameters derived from the whole-lesion histogram analysis (ADCmean, skewness, kurtosis, and ADC10th, 25th, 50th, 75th, 90th percentiles), as well as the ADCmean from the hot spot region of interest (ROI), were calculated for each patient before and after treatment. Then all data were compared between R and non-R using the Mann-Whitney U test. Two measures of diagnostic accuracy were applied: the receiver operating characteristic curve and the diagnostic odds ratio (DOR). We also reported intra- and interobserver variability by calculating the intraclass correlation coefficient (ICC).

RESULTS

Post-nCRT kurtosis, as well as post-nCRT skewness, were significantly lower in R

than in non-R (both $P < 0.001$, respectively). We also found that, after treatment, R had a larger loss of both kurtosis and skewness than non-R (Δ%kurtosis and Δskewness, $P < 0.001$). Other parameters that demonstrated changes between groups were post-nCRT ADC10th, Δ%ADC10th, Δ%ADCmean, and ROI Δ%ADCmean. However, the best diagnostic performance was achieved by Δ%kurtosis at a threshold of 11.85% (Area under the receiver operating characteristic curve [AUC] = 0.991, DOR = 376), followed by post-nCRT kurtosis = 0.78 × 10^{-3} mm^2/s (AUC = 0.985, DOR = 375.3), Δskewness = 0.16 (AUC = 0.885, DOR = 192.2) and post-nCRT skewness = 1.59 × 10^{-3} mm^2/s (AUC = 0.815, DOR = 168.6). Finally, intraclass correlation coefficient analysis showed excellent intraobserver and interobserver agreement, ensuring the implementation of histogram analysis into routine clinical practice.

CONCLUSION

Whole-tumor ADC histogram parameters, particularly kurtosis and skewness, are relevant biomarkers for predicting the nCRT response in LARC. Both parameters appear to be more reliable than ADCmean from one-slice ROI.

Key Words: Apparent diffusion coefficient; Diffusion-weighted imaging; Histogram analysis; Magnetic resonance imaging; Locally advanced rectal cancer

Core Tip: Whole-tumor apparent diffusion coefficient (ADC) histogram analysis is an emergent imaging analysis in which every voxel is used to obtain a histogram; it thus provides statistical information about tumors. Our study revealed that ADC histogram profiling is a valuable approach that can help differentiate treatment response in locally advanced rectal cancer. When determining tailored treatments that are associated with minimal morbidities, such as the watch and wait method, an accurate treatment response prediction is critical. Given the limitations of this study, more research is needed to establish the clinical utility of our findings.

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INTRODUCTION

Neoadjuvant chemoradiation therapy (nCRT) is the gold standard treatment for patients with locally advanced rectal cancer (LARC), followed by surgical resection and adjuvant chemotherapy[1,2]. After nCRT, the ability to achieve tumor reduction or even a pathological complete response (pCR) is observed in approximately 75% of treated patients, whereas the remainder exhibited no treatment response[3,4]. The ability to predict the response to nCRT is important for patients with potentially curable LARC who wish to explore personalized treatment to expand their therapeutic outcomes[5].

Functional magnetic resonance imaging (MRI) techniques, such as diffusion-weighted imaging (DWI), can provide additional physiological information about a tumor's cellular environment, offering great potential to evaluate the therapeutic response to nCRT[5]. This is because the apparent diffusion coefficient (ADC), a quantitative parameter used to assess water diffusion through tissue in DWI, shows an inverse relationship with tissue cellularity[6]. Viable tumor cells restrict the mobility of water, whereas necrotic tumor cells allow the increased diffusion of water molecules[7].

The possibility that ADC may be associated with the nCRT response has been amply investigated in LARC[8-12]; however, significant correlations have not been found in any studies to date[10]. Inconsistencies in previous findings may be due to a lack of standardized imaging and acquisition techniques[5,11], but they may also be due to the fact that the ADC measurements were performed using a manually drawn region of interest (ROI) from a single slice of the ADC map, which holds limited ability to reflect the actual whole-tumor characteristics[13-15].

In the case of whole-lesion histogram analysis of the ADC, a volumetric ROI is positioned on the entire lesion over contiguous slices and a histogram of ADC values reflecting voxel frequency is constructed, leading to the improved evaluation of heterogeneity[16]. Based on this method, first-order heterogeneity parameters can be obtained, which assess the spectrum of ADC values gained from all

voxels within a volume of interest[17]. A growing number of studies have used ADC histogram parameters, as these analyses provide additional information that can aid in the discrimination between benign and malignant regions, or they can help to better characterize the response to treatment in different tumors, such as ovarian, prostate, and breast cancer[18-21]. The application of whole-volume ADC histogram analysis in rectal tumors is increasing in frequency as well, and the role of this parameter in predicting nCRT is promising but limited[22-25].

The purpose of this study was to investigate the imaging response to nCRT using DWI in patients with LARC. We hypothesized that the ADC histogram-derived parameter might better predict treatment responses to nCRT compared with ADC from the hotspot ROI, as histogram parameters can display the heterogeneous features of tumors.

MATERIALS AND METHODS

Patients

The institutional review board approved this retrospective study, and the requirement to obtain informed consent was waived given the study's retrospective nature. The study population was selected from LARC patients at our institution between February 2015 and October 2020. According to Enkhbaatar *et al*[23], we defined the inclusion criteria as follows: (1) Proven histopathology of rectal adenocarcinoma; (2) greater than stage T2 on pre-nCRT MR imaging; with or without regional lymph node metastases and no distant metastases; (3) pre- and post-nCRT rectal MRI imaging with diffusion-weighted (DW) imaging; (4) long-course nCRT; and (5) surgical resection. Mucinous tumors were excluded from this study.

Forty-eight patients were enrolled in the study (34 men and 14 women; age range: 28–84 years). All patients were further divided into two subgroups based on the pathological response of the primary tumor: responders (R) and non-responders (non-R). Only patients with grade 0 according to the TRG-Ryan system were regarded as patients with a complete pathological response (R), while patients with TRG 1-3 were non-R.

MRI protocol

All images were obtained on a 3T MRI system (Discovery MR 750w GEM®; General Electric Healthcare, Milwaukee, WI, United States) using a phased-array body coil. Intravenous antispasmodic agents were not administered, and patients received no bowel preparation before the MRI examination. Our study groups comprised patients who underwent pre-treatment MRI for primary tumor staging, and a second restaging MRI for response evaluation 6 wk after the completion of nCRT. The scanning protocol is listed in Table 1[23]. In brief, we obtained standard T2-weighted (T2W) spin-echo sequences in axial, coronal, and sagittal directions. To improve tumor tissue visualization (including the delineation of the muscular layer), these planes were planned perpendicular to the main axis of the tumor. Moreover, a T1W spin-echo sequence in an axial direction, as well as an axial non-enhanced DWI with b = 1200 s/mm^2, were acquired. ADC maps were automatically generated using the in-line software provided by the vendor during image acquisition. Additionally, axial, sagittal, and coronal fat-suppressed contrast T1W sequences were acquired and used to suppress the signal from adipose tissue. A gadolinium-based contrast agent (Gd-DTPA, Magnevist; Bayer Schering, Berlin, Germany) was used to enhance the quality of MRI. Representative images of our MRI protocol are provided in Figure 1.

Image analysis

Two radiologists (JARP and MEJ, with 10 years and 5 years of experience in gastrointestinal imaging, respectively) reviewed the imaging studies and performed all tumor measurements on the pre- and post-nCRT images. At the initial review, each radiologist was blinded to the other radiologist's opinion. Also, they were blinded to the pathology results to assess interobserver and intraobserver variability. After that, the two radiologists would hold a discussion to arrive at a final decision by consensus. If a disagreement occurred, another radiologist with 25 years of experience (YVN) aided in making the final decision.

DWI analysis was performed with a workstation using the GE Advantage Workstation 4.6 software featuring the READYVIWER application (2006–2010; General Electric, Boston, MA, United States). On the pre-nCRT b1200 diffusion images, the tumor was defined as a focal mass with high signal intensity in comparison with the signal of the normal adjacent rectal wall. More precisely, the delineated ROIs covered the edge of each lesion, and the ROIs were drawn along the inner margin of the rectal walls to avoid intraluminal gas, water, and other contents. Further, necrotic areas, cysts, and vessels related to each lesion at the corresponding slice were also avoided, as identified on T2WI images. In addition, the highest and lowest slices of the DWI images were excluded given their partial volume effects[24]. After nCRT, the tumor was defined by focal areas of residual high signal, as identified on the b1200 images within the location of the primary tumor bed and/or corresponding with the residual tumor on T2WI MRI images as a reference standard. To compare and identify the tumor location, the pre-treatment images were at the readers' disposal when analyzing the post-treatment images.

Table 1 Magnetic resonance imaging sequences and data acquisition parameters

Parameter	Magnetic resonance imaging sequences							
	T2 FSE sagittal	T2 FSE axial	T2 FSE coronal	T1 FSE axial	DWI axial	T1 + GD axial	T1 + GD coronal	T1 + GD coronal
Repetition time in ms	5325	9890	7509	850	7750	435	295	265
Echo time in ms	102	102	102	Min	Min	Min	Min	Min
Slices, *n*	30	40	30	40	40	40	40	30
FOV	24	20	20	20	20	20	20	24
Slices thickness in mm	4	4	4	4	4	4	4	4
Broadband in Hz/Px	62.5	62.5	50	62.5	-	50	50	50
Phase	384	384	416	384	60	320	320	320
Acquisition time in min:s	2:35	3:08	2:45	3:53	5:18	2:31	2:16	2:02

DWI: Diffusion-weighted imaging; FSE: Fast spin-echo; GD: Gadolinium; MRI: Magnetic resonance imaging.

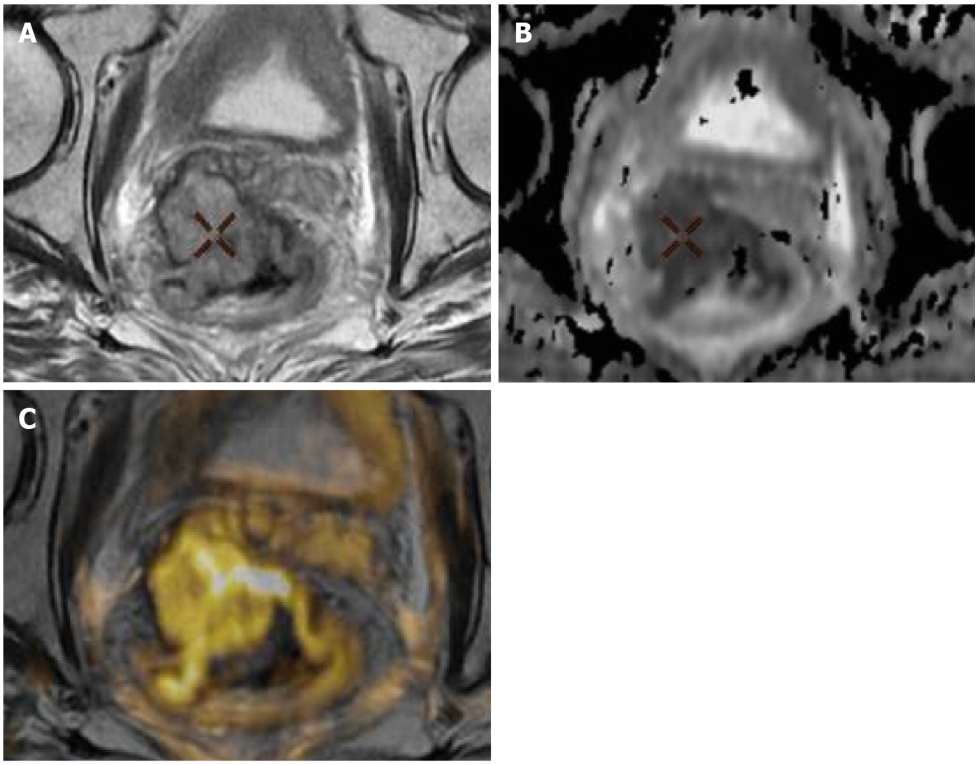

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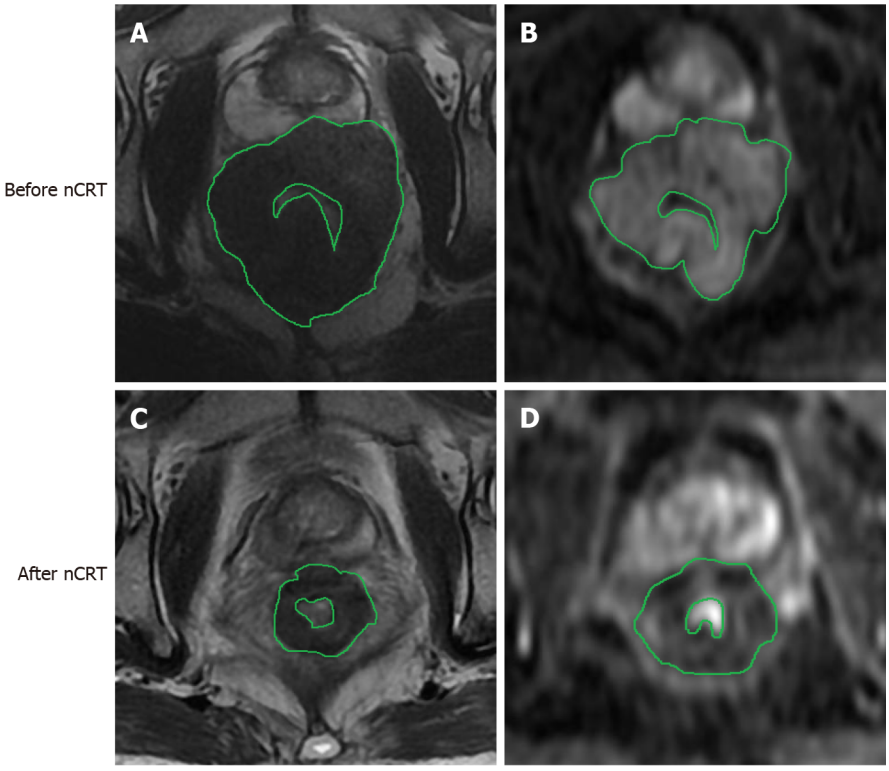
Figure 1 Representative images of magnetic resonance imaging protocol. A-C: Axial T2 (A), apparent diffusion coefficient (ADC) map and T2 fusion ADC map color (B) and images of bulky tumor (C), showing tumor extending more than 5 mm into the mesorectal fat and invading the mesorectal fascia.

It should be noted that, in the first instance, one large ROI was placed to cover most of the largest axial tumor cross-section, which facilitated the calculation of the ADCmean values (ROI ADCmean). Thereafter, a volume of interest (VOI) was manually created on the ADC maps, where ROIs were drawn on all tumor slices (whole-lesion measurement). Within this VOI, the following parameters were calculated: (1) ADCmean, the average ADC value of all voxels within the VOI; (2) ADCn% (10^{th}, 25^{th}, 50^{th}, 75^{th}, and 90^{th} percentiles), the point at which the n% of the voxel values that formed the histogram were found to be at the left; (3) skewness, which measures the asymmetry of the distribution of values about the mean value; and (4) kurtosis, which is a measure of the 'peakedness' of the distribution of values in the ROI image. The corresponding frequency table for each lesion was exported, and the histogram parameters were computed by SPSS v. 26.0 (IBM Corporation, Armonk, NY, United States). Figure 2 is a schematic illustration of a representative ROI.



DOI: 10.3748/wjg.v28.i23.2609

Figure 2 Images of rectal tumor before and after neoadjuvant chemoradiation therapy. A, C: T2-weighted magnetic resonance images obtained in 67-year-old man with a rectal tumor (histopathologic response Ryan 1) to evaluate tumor volume; B, D: Diffusion-weighted images (DWI) that were obtained from the same case. As we can see in the present case, regions of interest were drawn manually slice by slice on DWI images along the edge of the lesion to cover as much tumor area as possible without excluding cystic or necrotic areas. nCRT: Neoadjuvant chemoradiation therapy.

Histopathologic review

Specimens were evaluated according to an established protocol that was previously described by our research team[26]. In brief, fresh surgical specimens were evaluated to determine the quality of the mesorectal excision before being fixed in 4% formaldehyde for 48 h prior to sectioning. After fixation, the specimens were serially sectioned (in slices of 1 cm), and the mesorectal boundary was linked. When the residual tumor was visible, a minimum submission of four blocks was recommended. All mesorectal lymph nodes were histologically examined, as was the involvement of the circumferential resection margin. When no residual tumor cells were identified, each block was cut into 3 level sections, and immuhistochemistry for keratin was done. All hematoxylin and eosin slides were reviewed by an experienced pathologist (EHB, with 15 years of experience examining rectal cancer).

The pathologic response of the primary tumor was estimated using the modified Ryan's classification as follow[26,27]: TRG0, complete response with no viable cancer cells; TRG1 moderate response with single cancer cells or small groups of cancer cells; TRG2, minimal response with residual cancer outgrown by fibrosis, and TRG3, poor response with minimal or no tumor killing and extensive residual cancer.

Statistical analyses

The following formula was used to calculate changes in all metrics included in the current study: *PerC* = (Parameter post-treatment - Parameter pre-treatment) / Parameter pre−treatment × 100.

It must be noted that when pre- and post-nCRT kurtosis values were obtained, a result of +3.00 indicated the absence of kurtosis. To simplify the interpretation, we adjusted this result to 0 (*i.e.* kurtosis of –3 = 0). Thus, any reading other than 0 was referred to as an excess of kurtosis. On the other side, to negate division by 0 when calculating the percentage change in kurtosis, we added 3, *i.e.* [(Kurtosis post-treatment + 3) – (Kurtosis pre-treatment + 3) / (Kurtosis pre-treatment + 3)] × 100.

In the case of skewness, and to avoid dividing by 0, only change (not the percentage change) was used (*i.e.* skewness post treatment – skewness pre-treatment)[28]. As skewness did not have a lower bound such as kurtosis, the +/– sign was considered to calculate changes in this parameter. To compare variables among R and non-R, a Mann–Whitney *U* test (MWU) was applied, as the Kolmogorov-Smirnov test confirmed the non-normal distribution of any parameter included here. Accordingly, the data were presented as medians and interquartile ranges (IQR)[29]. When the differences in a variable were significant ($P < 0.05$) in the MWU test, the cut-off value, sensitivity, specificity, positive predictive

value, negative predictive value, area under the receiver operating characteristic (ROC) curve (AUC), and accuracy, were analyzed. The optimal cut-off values of ADCmean from the hot spot ROI and parameters derived from the histogram analysis of DWI were determined *via* the Youden index, while differences in the AUC were analyzed according to the method described by DeLong *et al*[30]. Furthermore, the diagnostic odd ratio (DOR) was designed to provide an additional measure of the performance of our potentially useful biomarkers to predict treatment response in LARC.

Finally, the intraobserver variability and interobserver variability were assessed using the intraclass correlation coefficient (ICC). For the agreement analysis, the outcomes were interpreted as follows, in accordance with Cicchetti (1994): 0.2 or less, poor agreement; 0.21–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.74, good agreement; and 0.75–1.00, excellent agreement[31]. Statistical analyses were performed using SPSS v. 26. $P < 0.05$ was considered statistically significant.

RESULTS

Among the 58 patients that were originally included in this study, 10 had severe imaging artifacts. Thus, our final sample included 48 patients whose clinical and pathological characteristics are described in Table 2.

The median values and IQRs for the ROI ADCmean values and parameters derived from the histogram analysis of DWI are described in Table 3. Accordingly, post-nCRT kurtosis, as well as post-nCRT skewness, were significantly lower in R than in non-R (both $P < 0.001$, respectively). Furthermore, our results showed significant differences in the relative changes of kurtosis (Δ%kurtosis) between R and non-R ($P < 0.001$), with the largest loss of kurtosis in R. Additionally, median Δskewness displayed lower values in R than in non-R ($P < 0.001$).

We also found that patients with a favorable response (R) had higher post-nCRT ADC10th values than did non-R ($P = 0.036$). Correspondingly, the median values of Δ%ADC10th, Δ%ADCmean, and ROI Δ%ADCmean were also higher in R than in non-R, ($P = 0.020$, $P = 0.032$ and $P = 0.020$, respectively).

Receiver operating characteristics of those parameters that exhibited significant differences in the MWU test are reported in Table 4. The highest AUC values for predicting the treatment response in LARC were demonstrated by Δ%kurtosis, post-nCRT kurtosis, Δskewness and post-nCRT skewness (AUCs = 0.991, 0.985, 0.885, and 0.815, respectively). Meanwhile, the lowest diagnostic accuracy was observed in post-nCRT ADC10th (AUC = 0.681), Δ%ADCmean (AUC = 0.686), Δ%ADC10th (AUC = 0.589) and ROI Δ%ADCmean (AUC = 0.583).

The ROC curves for Δ%kurtosis, post-nCRT kurtosis, Δskewness, and post-nCRT skewness are displayed in Figure 3, while the comparison of AUC values between all of our potentially useful biomarkers for predicting the treatment response in LARC are presented in Supplementary Table 1.

It is important to mention that according to the DeLong analysis, no significant differences were found in the diagnostic accuracy of Δ%kurtosis and post-nCRT kurtosis. As well, no differences were demonstrated between Δskewness and post-nCRT skewness. However, the latter two parameters had lower accuracy than kurtosis-derivate metrics.

Finally, to verify the diagnostic accuracy of all metrics reported in Table 4, we calculated DORs. The DOR of a test is the ratio of the odds of positivity if a patient has a disease relative to the odds of positivity when a patient does not have a disease. The value of DOR ranges from 0 to infinity, with higher values indicating better discriminatory test performance[32,33]. As demonstrated in Table 5, Δ%kurtosis and post-nCRT kurtosis had the highest power of discrimination for treatment response by using DORs (approximately 376), followed by Δskewness (192.2) and post-nCRT skewness (168.6). Meanwhile, the lowest power of discrimination was observed in post-CRT ADC10th (5.48), Δ%ADCmean (4.26), Δ%ADC10th (3.65), and ROI Δ%ADCmean (3.47).

Regarding interobserver and intraobserver variability, the parameters derived from the histogram analysis of DWI, as well as the ADC values from the hotspot ROI, had an excellent agreement. The ICC measuring intraobserver variability ranges from 0.777-0.931 (Table 6), while the ICC measuring intraobserver variability ranges from 0.889-0.993 (Table 7).

DISCUSSION

Heterogeneity of malignant lesions is a feature that can be determined by characterizing changes in the histogram analysis of ADC values, which is recognized as a promising tool in cancer research when discerning between benign and malignant tumors or to better characterize the response to anti-cancer treatments[34-38].

This study focused on the ADCmean from the hot-spot ROI and a series of parameters corresponding to certain points on the ADC histogram using DWI, which have been proposed to predict treatment response in patients with rectal cancer[39,40]. As our results demonstrated, the parameters that changed significantly in response to nCRT were Δ%kurtosis, post-nCRT kurtosis, Δskewness, post-nCRT skewness, post-nCRT ADC10th, Δ%ADCmean, Δ%ADC10th, and ROI Δ%ADCmean. However, the

Table 2 Clinical and pathological characteristics of the patients' studies

Characteristics	*n* (%)
Sex	
Female	23 (48)
Male	25 (52)
RECIST 1.1	
Partial response	22 (46)
Stable disease	13 (27)
Progressive disease	13 (27)
Ryan's classification	
0	18 (38)
1	10 (21)
2	9 (19)
3	11 (22)
Treatment response	
Complete responders after nCRT	18 (38)
Non-responders' patients after nCRT	30 (62)
Tumor location	
Upper third	7 (15)
Middle third	14 (29)
Lower third	20 (41)
Diffuse	7 (15)
ypT stage	
T0	4 (8)
T1s	4 (8)
T1a	2 (4)
T2	8 (17)
T3	20 (42)
T4b	10 (21)
ypN stage	
N0	20 (42)
N1a	14 (29)
Nic	14 (29)
Degree of differentiation	
Well-differentiated adenocarcinoma	6 (13)
Moderately differentiated adenocarcinoma	35 (73)
Poorly differentiated adenocarcinoma	7 (14)
Surgical approach	
Low anterior resection	16 (33)
Intersphincteric resection	26 (54)
Abdominoperineal resection	6 (13)

nCRT: Neoadjuvant chemoradiation therapy.

Table 3 Median and interquartile range of pre- and post-neoadjuvant chemoradiation therapy parameters, as well as of changes between pre- and post-treatment values

	Responders	Non-responders	*P* value
Pre-nCRT parameters			
pre-nCRT ADCmean	0.75 (0.60-0.90)	0.85 (0.70-0.90)	0.146
10^{th} percentile	0.20 (0.17-0.23)	0.20 (0.17-0.26)	0.812
25th percentile	0.32 (0.29-0.35)	0.32 (0.23-0.41)	1.000
50^{th} percentile	0.40 (0.35-0.40)	0.40 (0.30-0.50)	0.698
75^{th} percentile	0.56 (0.47-0.58)	0.57 (0.45-0.63)	0.391
90^{th} percentile	0.71 (0.55-0.77)	0.76 (0.50-0.80)	0.556
Skewness	1.10 (0.90-1.14)	1.19 (0.88-1.37)	0.135
Kurtosis	0.89 (0.83-0.92)	0.92 (0.83-0.95)	0.296
ROI ADCmean	0.92 (0.80-1.20)	0.91 (0.83-0.93)	0.562
Post- nCRT parameters			
post- nCRT ADC_{mean}	1.20 (0.98-1.52)	1.10 (0.90-1.30)	0.065
10^{th} percentile	0.36 (0.30–0.37)	0.32 (0.31–0.34)	0.036[1]
25^{th} percentile	0.41 (0.40-0.52)	0.42 (0.41-0.50)	0.476
50^{th} percentile	0.66 (0.56-0.70)	0.65 (0.51-0.66)	0.127
75^{th} percentile	0.71 (0.67-0.80)	0.70 (0.66-0.75)	0.050
90^{th} percentile	0.89 (0.80-0.95)	0.80 (0.79-0.89)	0.105
Skewness	0.92 (0.60–1.14)	2.00 (1.15–2.67)	< 0.001[1]
Kurtosis	0.65 (0.59–0.72)	0.90 (0.80–0.90)	< 0.001[1]
ROI ADCmean	2.50 (1.50-2.70)	2.00 (1.80-2.30)	0.056
Changes between pre-treatment and post-treatment			
Δ%ADCmean	57% (14%–103%)	27% (0%–59%)	0.032[1]
Δ%$ADC10^{th}$	86% (37%–118%)	48% (11%–88%)	0.020[1]
Δ%$ADC25^{th}$	39% (19%-57%)	22% (0%-58%)	0.905
Δ%$ADC50^{th}$	70% (31%-86%)	31% (2%-65%)	0.067
Δ%$ADC75^{th}$	40% (20%-61%)	37% (0%-57%)	0.288
Δ%$ADC90^{th}$	27% (11%-58%)	9% (7%-119%)	0.061
Δskewness	–0.20 (–0.40–0.00)	0.49 (0.10–0.50)	< 0.001[1]
Δ%kurtosis	41% (18%–54%)	2.5% (1.4%–5.9%)	< 0.001[1]
ROI Δ%ADCmean	55% (48%–60%)	23% (15%–30%)	0.020[1]

[1]Statistically significant difference. ADC: Apparent diffusion coefficient; nCRT: Neoadjuvant chemoradiation therapy; ROI: Region of interest.

highest diagnostic accuracy was obtained for Δ%kurtosis, post-nCRT kurtosis, post-nCRT skewness, and Δskewness, suggesting that these metrics might be useful when selecting responders (TRG 0) for an organ preservation approach with either 'watch-and-wait' or local excision[39,40].

The results derivate from parameters with the highest diagnostic accuracy in predicting treatment response to nCRT in the current work are reviewed below.

First, we demonstrated that both post-nCRT kurtosis and post-nCRT skewness were significantly lower in R than in non-R. The overall trends from the histogram studies have shown that, following treatment, the histogram analysis of *DWI* and diffusion kurtosis imaging (DKI) shifted to the right upon decreased kurtosis and skewness in rectal cancer[39-43]. For example, in 2017, Hu *et al*[39] reported that the post-treatment mean kurtosis derived from DKI showed reduced values in R when compared with non-R patients, whereas Enkhbaatar *et al*[23] (2019) documented that the histogram of R presented negative changes in skewness following a loss of this parameter after therapy.

Table 4 Diagnostic performance of the best magnetic resonance imaging histogram derived parameters to detect responder patients

	Cutoff	Sensitivity	Specificity	PPV	NPV	Accuracy	AUC (95%CI)
Δ%kurtosis	11.85%	94.4%	96.7%	94.4%	96.7%	96.0%	0.991 (0.925-1.000)
Post-nCRT kurtosis	0.78	93.3%	99.0%	90%	99.0%	96.0%	0.985 (0.957-1.000)
Δskewness	0.16	66.7%	99.0%	64.3%	99.0%	79.2%	0.885 (0.795-0.975)
Post-nCRT skewness	1.59	63.3%	99.0%	62.0%	99.0%	77.1%	0.815 (0.795-0.634)
Post-nCRT ADC10th	0.34×10^{-3} mm^2/s	66.7%	73.3%	60.0%	79.0%	71.0%	0.681 (0.509-0.852)
Δ%ADCmean	56.00%	56.0%	77.0%	56.0%	73.3%	66.7%	0.686 (0.500-0.820
Δ%ADC10th	74.21%	61.1%	70.0%	55.0%	75.0%	66.7%	0.589 (0.483-0.815)
ROI Δ%ADCmean	55.00%	61.0%	69.0%	52.0%	72.0%	65.3%	0.583 (0.425-0.715)

ADC: Apparent diffusion coefficient; AUC: Area under the receiver operating characteristic curve; nCRT: Neoadjuvant chemoradiation therapy; NPV: Negative predictive value; PPV: Positive predictive value; ROI: Region of interest.

Table 5 Diagnostic odds ratios of magnetic resonance imaging parameters in differentiating respond and non- respond patients in locally advanced rectal cancer

	Diagnostic odds ratio	95%CI
Δ%kurtosis	376.0	228.9-842.1
Post-nCRT kurtosis	375.3	225.7-887.7
Δskewness	192.2	69.0-253.3
Post-nCRT skewness	168.6	54.0-251.7
Post-nCRT ADC10th	5.48	1.0-19.6
Δ%ADCmean	4.26	1.0-14.4
Δ%ADC10th	3.65	1.0-12.5
ROI Δ%ADCmean	3.47	1.0-11.2

ADC: Apparent diffusion coefficient; nCRT: Neoadjuvant chemoradiation therapy; ROI: Region of interest.

In the same way, kurtosis from R had greater reductions than from non-R, which indicates Gaussian or flatter distributions in patients with a complete response to the therapy. In biological tissues, it is believed that the non-Gaussian behavior (more precisely, a platykurtic curve) of water might occur because of a heterogeneous environment characterized by multiple compartments, organelles, and semipermeable membranes[44]. Thus, when an important reduction in kurtosis is noticed, a higher displacement of water molecules in DWI is assumed.

Furthermore, as mentioned above, negative changes of skewness after nCRT were seen in R, while non-R exhibited positive changes in this parameter. Negatively skewed curves show the majority of scores above the mean, and positively skewed curves are just the opposite[44]. In physiology, the association between changes in skewness and responses to antineoplastic therapy have not been fleshed out, but a curve negatively skewed suggests a loss of cellular structure[23]. Therefore, favorable treatment response is suspected.

Our MWU analysis also found differences between R and non-R across other parameters, such as ADC10th, Δ%ADCth, Δ%ADCmean and ROIΔ%ADCmean, as stated in our results section. However, both the ROC curve analysis and the DOR calculation indicated that only Δ%kurtosis, post-nCRT kurtosis, Δskewness, and post-nCRT skewness appear to predict a favorable response to the therapy, whereas the other metrics did not possess that predictive property.

Briefly, the Youden index calculation indicated that post-nCRT kurtosis, post-nCRT skewness, and Δskewness values below 0.78×10^{-3} mm^2/s, 1.59×10^{-3} mm^2/s, and 0.16, respectively, might be significant indicators of the occurrence of pCR. Meanwhile, Δ% changes above 11.85% also indicated a positive treatment effect with high accuracy. It is important to remember that, according to the DeLong analysis, the kurtosis-related parameters exhibit a better diagnostic performance than do skewness-related parameters.

Table 6 Intraobserver variability

		ICC	95%CI
Basal			
Test1 and test2, reader1	ROI ADCmean	0.850	0.742–0.800
Test1 and test2, reader2	ROI ADCmean	0.890	0.850-0.820
After treatment			
Test1 and test2, reader1	ROI ADCmean	0.800	0.750-0.819
Test1 and test2, reader2	ROI ADCmean	0.823	0.800-0.850
Basal			
Test1 and test2, reader1	ADCmean	0.850	0.756-0.920
Test1 and test2, reader2	ADCmean	0.777	0.745-0.812
After treatment			
Test1 and test2, reader1	ADCmean	0.845	0.830-0.850
Test1 and test2, reader2	ADCmean	0.823	0.800-0.833
Basal			
Test1 and test2, reader1	10^{th} percentile	0.820	0.880-0.950
Test1 and test2, reader2	10^{th} percentile	0.880	0.800-0.920
After treatment			
Test1 and test2, reader1	10^{th} percentile	0.780	0.740-0.853
Test1 and test2, reader2	10^{th} percentile	0.853	0.723-0.901
Basal			
Test1 and test2, reader1	25^{th} percentile	0.803	0.800-0.922
Test1 and test2, reader2	25^{th} percentile	0.863	0.801-0.895
After treatment			
Test1 and test2, reader1	25^{th} percentile	0.788	0.750-0.837
Test1 and test2, reader2	25^{th} percentile	0.820	0.780-0.846
Basal			
Test1 and test2, reader1	50^{th} percentile	0.850	0.840-0.920
Test1 and test2, reader2	50^{th} percentile	0.845	0.790-0.860
After treatment			
Test1 and test2, reader1	50^{th} percentile	0.821	0.800-0.913
Test1 and test2, reader2	50^{th} percentile	0.833	0.800-0.897
Basal			
Test1 and test2, reader1	75^{th} percentile	0.821	0.790-0.860
Test1 and test2, reader2	75^{th} percentile	0.859	0.820-0.920
After treatment			
Test1 and test2, reader1	75^{th} percentile	0.851	0.790-0.880
Test1 and test2, reader2	75^{th} percentile	0.837	0.791-0.856
Basal			
Test1 and test2, reader1	90^{th} percentile	0.850	0.820-0.890
Test1 and test2, reader2	90^{th} percentile	0.880	0.850-0.960
After treatment			
Test1 and test2, reader1	90^{th} percentile	0.831	0.800-0.902

Test1 and test2, reader2	90th percentile	0.901	0.850-0.975
Basal			
Test1 and test2, reader1	Skewness	0.920	0.900-0.940
Test1 and test2, reader2	Skewness	0.901	0.880-0.923
After treatment			
Test1 and test2, reader1	Skewness	0.931	0.920-0.950
Test1 and test2, reader2	Skewness	0.889	0.877-0.910
Basal			
Test1 and test2, reader1	Kurtosis	0.920	0.890-0.950
Test1 and test2, reader2	Kurtosis	0.910	0.850-0.960
After treatment			
Test1 and test2, reader1	Kurtosis	0.890	0.850-0.960
Test1 and test2, reader2	Kurtosis	0.880	0.840-0.982

ADC: Apparent diffusion coefficient; ICC: Intraclass correlation coefficient; ROI: Region of interest.

Table 7 Interobserver variability (intraclass correlation coefficient and 95% confidence intervals)

Pre-treatment	Reader one *vs* reader two	Post-treatment	Reader one *vs* reader two
ROI ADCmean	0.985 (1.900-0.999)	ROI ADCmean	0.889 (0.850-0.950)
ADCmean	0.989 (0.980–0.994)	ADCmean	0.990 (0.985-0.995)
10th percentile	0.972 (0.951–0.984)	10th percentile	0.992 (0.986–0.996)
25th percentile	0.970 (0.947–0.983)	25th percentile	0.950 (0.940-0.982)
50th percentile	0.986 (0.976–0.992)	50th percentile	0.987 (0.945-0.995)
75th percentile	0.989 (0.980–0.994)	75th percentile	0.990 (0.982–0.994)
90th percentile	0.989 (0.980–0.994)	90th percentile	0.972 (0.987–0.996)
Skewness	0.990 (0.982–0.994)	Skewness	0.993 (0.987–0.996)
Kurtosis	0.992 (0.986–0.995)	Kurtosis	0.972 (0.951–0.984)

ADC: Apparent diffusion coefficient; ROI: Region of interest.

Aligned with this finding, numerous authors have documented that kurtosis is more directly correlated to the underlying structural, physiological, molecular, and metabolic changes that occur during tumor progression than skewness[45]. This may be the reason why the kurtosis of ADC values has been used to indicate deviations from Gaussianity, even in the most challenging mathematical designs that predict the response to chemotherapy, such as radiomics analysis[46-48].

The results obtained from the ROC curved are partially supported by the estimated DORs, which were approximately 376 for both Δ%kurtosis and post-nCRT kurtosis. This means that for the cut-off points of Δ%kurtosis and post-nCRT kurtosis calculated here, the odds for positivity among subjects with a non-pCR was 376 times higher than the odds for positivity among subjects with a pCR. In the same way, Δskewness and post-nCRT skewness demonstrated respectable diagnostic performances with DOR values of 192.17 and 168.56, respectively. Although these values appear to be lower than DORs of Δ%kurtosis and post-nCRT kurtosis, the confidence intervals for these metrics clearly overlap, so we cannot conclude that the kurtosis-related parameters were statistically better than the skewness-related parameters using DOR.

Finally, this study confirm that ADC histogram analysis is a reproducible technique. Similarly, van Heeswijk *et al*[49] demonstrated that histogram-derived parameters had good interobserver agreement, with ICC values ranging from 0.80-0.98. This result supports the method's validity and suggests that it can be used in clinical practice. Furthermore, we utilized non-precise tumor delineation, which was quicker and produced comparable findings to those obtained by an expert radiologist's measurement, suggesting that this technique could be performed semiautomatically with an excellent interobserver agreement. This finding is very important when considering the implementation of histogram analysis

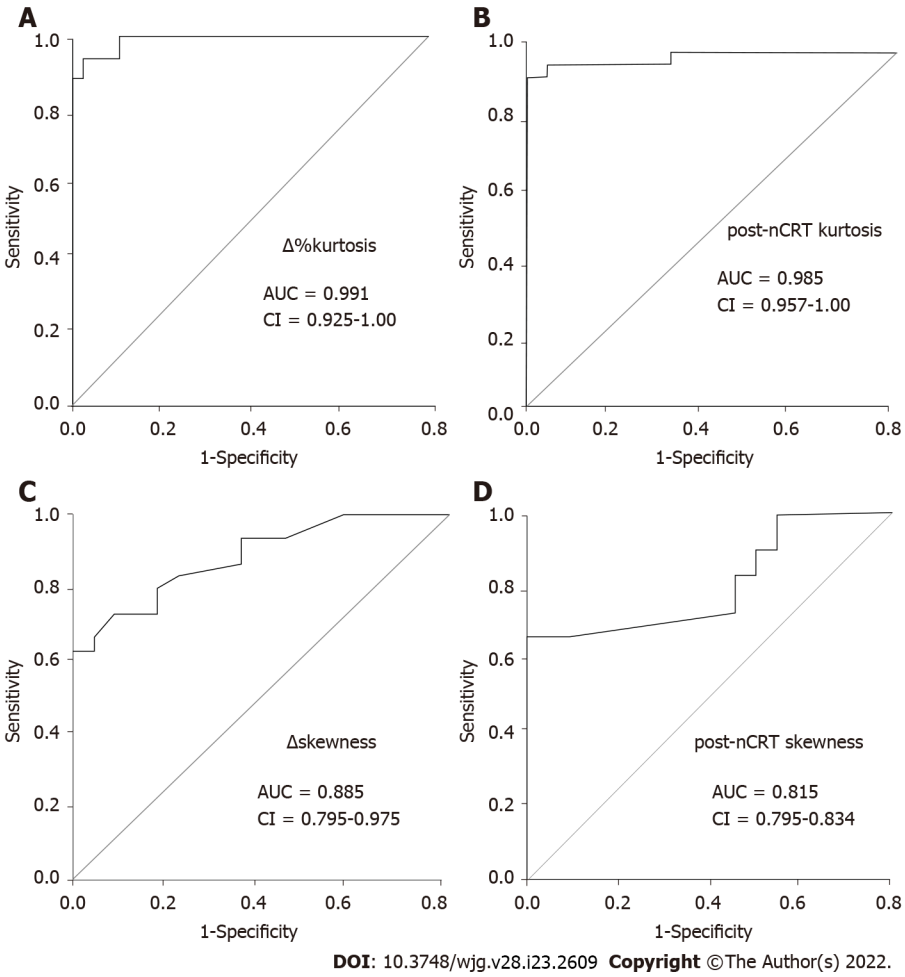


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Figure 3 Receiver operating characteristic curves displaying the diagnostic performances of the four histogram parameters derived from apparent diffusion coefficient values with the highest accuracy. A: Δ%kurtosis; B: Post-neoadjuvant chemoradiation therapy (nCRT) kurtosis; C: Δskewness; D: Post-nCRT skewness. AUC: Area under the receiver operating characteristic curve.

in routine clinical practice.

Our study had important limitations. First, this was a retrospective, single-center evaluation. We believe that the present study might serve as a foundation for larger prospective studies in the future. Second, we included only a small number of patients (n = 48), while no validation group was included (both restricting the conception of a predictive model by using a multivariate logistic regression analysis). Third, the patient numbers among the different histopathologic TRGs were not well balanced. Only 18 patients (38%) achieved a histopathologic complete response, which may have introduced an element of statistical bias. However, these patients achieved a strict pCR, underlying the high degree of accuracy of our metrics. Fourth, the parameters obtained from the hotspot ROI were not conclusive enough to predict treatment response in the present study. This result is still in significant disagreement with our prior work where we demonstrated a high diagnostic accuracy of the Δ%ADCmean when distinguishing a pCR in rectal cancer by choosing a cutoff value of 55%[26]. Differences in research methods might explain this discrepancy, but we sustain that it is more reliable to use volumetric ROIs than one slice ROIs.

In summary, although further studies are needed to address the limitations of the current work, we demonstrated the benefits of considering measures other than the ROI ADCmean to evaluate the response to therapy in patients with LARC. Moreover, kurtosis and skewness have been selected by many radiomics studies of rectal cancer, emphasizing the importance of first-order statistics features for the assessment of therapy response[47,50]. Our results support the importance of these parameters, but they also helped us to standardize both the extraction and analysis of the data collected, which is a crucial step when developing and validating our own multiparametric model to predict treatment outcomes.

CONCLUSION

Based on the DWI technique, some whole-lesion histogram parameters could provide valuable information when diagnosing rectal cancer. In particular, kurtosis and skewness might be a useful indicator in the preoperative evaluation of a pCR in rectal cancer. Understanding skewness and kurtosis of the ADC parameters is the simplest way to recognize the deviation of Gaussianity, which indicates tumor heterogeneity. Moreover, we demonstrated high interobserver reliability for measurements of all of the histogram-derived parameters analyzed in the current work, addressing the challenges associated with replication that are well-known among more complex predictive models. Further long-term studies are needed to determine the ultimate clinical utility of our results.

ARTICLE HIGHLIGHTS

Research background

Studies have shown that successful treatment of many tumors can be detected using diffusion-weighted magnetic resonance imaging (MRI) as an increase in the apparent diffusion coefficient (ADC). However, findings from rectal cancer have been limited. Therefore, the criteria used for tumor staging and surveillance are largely based on anatomic criteria at this time. Broadly, whole lesion histogram analysis of ADC aims to fill this gap, extracting and analyzing the higher quantitative data with the aim of more accurate, tumor-specific evaluation and characterization.

Research motivation

ADC histogram parameters reflect the distribution and variation of all voxels within the entire lesion, which reduce the subjectivity of region of interest (ROI) placement and improves repeatability in the quantitative ADC analysis. Previous studies have applied volumetric ADC histogram analysis to predict treatment response of squamous carcinoma, breast cancers, and ovarian cancers. No ADC histogram study thus far has focused on locally advanced rectal cancer (LARC).

Research objectives

We aim to evaluate the effectiveness of whole lesion histogram analysis of ADC in the prediction to neoadjuvant chemoradiation therapy (nCRT) response in patients with LARC.

Research methods

This was a retrospective study. We collected data of 48 consecutive patients with histologically confirmed LARC. All patients underwent a pre-treatment MRI for primary tumor staging and a second restaging MRI for response evaluation. The sample was distributed as follows: responders (R), n = 18; and non-responders (non-R), n = 30. Eight parameters derived from the histogram analysis of ADC, as well as the ADCmean from the hot spot ROI, were obtained and compared between R and non-R. The diagnostic accuracy in the prediction of treatment response of all variables included in the present study was calculated as well.

Research results

Post-nCRT kurtosis, Δ%kurtosis, post-nCRT skewness an Δskewness exhibited the highest diagnostic performance in predicting a good response to nCRT.

Research conclusions

The results of our study support that histogram-parameters derived from ADC values can be used to stratify good responders into studies exploring individualized, less extensive treatment regimens, such as the omission of radiotherapy and less extensive surgery, or even deferral of surgery.

Research perspectives

We need to expand the sample size to confirm further the diagnostic accuracy of kurtosis and skewness. In addition, the long-term outcome of this analysis should be a radiomic model for predict treatment response in rectal cancer.

FOOTNOTES

Author contributions: Sollozo-Dupont I designed the study; Sollozo-Dupont I and Domínguez Osorio V analyzed the data; Domínguez Osorio V and Vela-Sarmiento I collected the data; Sollozo-Dupont I, Jiménez de los Santos ME, and Reyes-Pérez JA wrote the paper; Villaseñor-Navarro Y and Moreno-Astudillo L reviewed the study; Jiménez de los Santos ME and Reyes-Pérez JA contributed equally to this work; All authors contributed to the manuscript for

important intellectual content and approved the submission.

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

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

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CASE REPORT

Primary gastric dedifferentiated liposarcoma resected endoscopically: A case report

Joon Hyun Cho, Jun Hyeon Byeon, Si Hyung Lee

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Abstract

BACKGROUND

Liposarcoma is one of the most common adult mesenchymal tumors but is uncommon in the gastrointestinal tract and extremely rare in the stomach. Furthermore, the histological subtypes of liposarcoma usually reported in the stomach are well-differentiated or myxoid, and few reports have been issued on small-sized gastric liposarcomas resected endoscopically and followed up. Herein, we report a case of primary gastric dedifferentiated liposarcoma (DL) that was resected endoscopically.

CASE SUMMARY

A 67-year-old female Korean patient was referred to our institution for further evaluation of a gastric submucosal tumor (SMT) located in the lesser curvature of the gastric body by esophagogastroduodenoscopy. Endoscopic ultrasound revealed a well-circumscribed, slightly heterogeneous, isoechoic, 17 mm × 10 mm sized mass originating from the third sonographic layer. Computed tomography showed no evidence of significant lymph node enlargement or distant metastasis. Endoscopic resection was undertaken using the snare resection technique after mucosal precutting to provide a definitive histopathologic diagnosis, which proved to be consistent with DL, based on its morphology and the immunoexpressions of MDM2 and CDK4. The patient was planned for surgery because the deep resection margin was positive for malignancy. After declining any invasive procedure or adjuvant treatment, the patient was placed under close follow-up, and at one year after endoscopic resection, remained disease free.

CONCLUSION

This is the first reported case of a small primary gastric DL resected endoscopically and followed up. This report demonstrates that when diagnosis of a SMT is uncertain, the use of invasive techniques, including endoscopic resection, should be considered.

Key Words: Gastric liposarcoma; Dedifferentiated liposarcoma; Submucosal tumor; Endoscopic resection; Case report

Core Tip: Liposarcoma is uncommon in the gastrointestinal tract and rarely encountered in the stomach. Furthermore, the dedifferentiated histologic subtype has not been previously reported in the stomach in the English literature. We experienced a case of a small (1.7 cm) primary gastric dedifferentiated liposarcoma, which was resected endoscopically. This report cautions that if a diagnosis of submucosal tumor is uncertain, the use of aggressive techniques, including endoscopic resection, should be considered.

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INTRODUCTION

Liposarcoma is one of the most common adult soft tissue sarcomas and has a peak incidence between the ages 50 and 65 and a prevalence of 15%-20% among all sarcoma patients[1]. There are four histological subtypes of liposarcomas[2]. Atypical lipomatous tumor/well-differentiated liposarcoma (WDL) is the most common subtype followed by dedifferentiated liposarcoma (DL), which frequently occurs in retroperitoneum[3,4]. The myxoid and pleomorphic types usually present in the extremities [5]. Thus, liposarcoma usually arises in deep soft tissues of the proximal extremities, retroperitoneum, or trunk[6], and is encountered in the gastrointestinal tract in only 2% of cases[7].

The majority of liposarcomas of the alimentary tract arise in the esophagus[8], and liposarcomas originating at more distal sites, such as stomach, small intestine, and large intestine, are rare[9]. Less than 40 cases of primary liposarcoma of the stomach have been described in the medical literature, and the most reported histological subtypes of gastric liposarcoma are WDL and myxoid liposarcoma[10]. In fact, no report on DL of the stomach has been published in the English literature, though one case report on DL of the gastroesophageal junction was issued in 2018[11]. Here, we describe the first case of a small primary gastric DL resected endoscopically and provide a review of the literature.

CASE PRESENTATION

Chief complaints

A 67-year-old female Korean patient was referred to our institution for further evaluation and treatment of a gastric submucosal tumor (SMT) incidentally discovered by esophagogastroduodenoscopy (EGD) during a routine medical check-up.

History of present illness

The patient had experienced no abdominal pain or discomfort.

History of past illness

She had a history of breast conserving surgery due to breast cancer 6 years previously.

Personal and family history

The patient had diabetes and was being treated with oral hypoglycemic agents. She was a non-smoker and non-alcohol drinker and had no significant family history.

Physical examination

Physical examination was unremarkable, and her abdomen was soft, nontender, and nondistended with no palpable mass.

Laboratory examinations

Laboratory tests, which included common serum tumor markers such as carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9), were normal.

Imaging examinations

EGD revealed a SMT-like protruding lesion of approximately 15 mm diameter located in the posterior wall of the lesser curvature of the distal part of the gastric body (Figure 1A). The lesion was covered with normally appearing mucosa except for focal mucosal erythema and depression over the lesion. Gastric endoscopic ultrasonography (EUS) examination demonstrated a well-demarcated mass, which measured 17 mm × 10 mm, located in the third layer of the gastric wall. The echo pattern of the mass was slightly heterogeneous and isoechoic (Figure 1B), but its EUS appearance was insufficient for diagnosis. The differential diagnosis included lipoma, neuroendocrine tumor, and ectopic pancreas. Initial biopsy specimens obtained at the site of the focal mucosal erythema and depression were negative for a neoplasm. Computed tomography (CT) of the abdomen showed a well-enhanced, intraluminal protruding polypoid lesion arising from the gastric body but no evidence of lymph node enlargement or distant metastasis (Figure 2).

FINAL DIAGNOSIS

After initial work-up, endoscopic resection was conducted using the snare resection technique after mucosal precutting for a definitive histopathologic result (Figure 3). At gross examination, the tumor was whitish yellow, well-shaped, and solid. Histopathologic examination showed the tumor was located submucosally and primarily composed of spindle-shaped atypical cells with pleomorphic and hyperchromatic nuclei and indistinct pale cytoplasm (Figure 4A-D). Deep resection margins were positive for tumor. Immunohistochemical staining performed for the differential diagnosis of gastrointestinal stromal tumor, schwannoma, leiomyosarcoma, malignant melanoma, and any poorly or undifferentiated sarcoma, revealed tumor cells were negative for CD117, DOG1, CD34, SMA, S-100, Desmin, HMB45, and SOX10, but positive for MDM2 and CDK4 nuclear staining (Figure 4E and F). Based on these findings, the histopathological diagnosis was consistent with DL.

TREATMENT

The patient was planned for surgery because the deep resection margin was positive for DL, but declined any invasive procedure or adjuvant treatment.

OUTCOME AND FOLLOW-UP

She remains under close follow-up, which includes biannual CT scanning and EGD, and at one year after endoscopic resection remained disease free.

DISCUSSION

Although they are the most common mesenchymal neoplasms, liposarcomas are rarely found in the gastrointestinal tract. Since the disease was first described by Abrams *et al*[12] in 1941, fewer than 40 cases of gastric liposarcoma have been reported in the literature worldwide[10]. The etiology of gastric liposarcoma remains uncertain, though some patients have a family history of soft tissue neoplasms, which suggests the involvement of genetic factors[13]. Gastric liposarcomas originate due to the proliferation of undifferentiated mesenchymal cells within submucosa and the tunica muscularis layer of stomach, and an exophytic growth is typical[10,14]. Most gastric liposarcomas are located in the antrum or lesser curvature.

As is the case for other soft tissue sarcomas, there are no characteristic clinical findings[15], and patients may remain asymptomatic for years. Symptoms depend primarily on tumor location and size and the presence or absence of ulceration. The most common symptoms are a palpable mass, mechanical obstruction, and gastrointestinal bleeding. In general, nonspecific symptoms are the main cause of delayed diagnoses, and thus, most liposarcomas are large at diagnosis. However, our patient underwent regular biennial EGD under a national cancer screening program, and the small SMT was detected early. Furthermore, endoscopic resection performed for a definite diagnosis resulted in the diagnosis of a small, asymptomatic gastric liposarcoma.

The characteristic histological features of liposarcoma are the presence of immature fat cells and lipoblasts[14]. Liposarcomas are classified histologically as WDL, DL, myxoid/round cell liposarcoma, or pleomorphic liposarcoma[2], and considerations of histological subtype are important during the disease course. While DL, round-cell, and pleomorphic liposarcomas are high-grade aggressive tumors with the ability to metastasize, WDL and myxoid liposarcomas are low-grade tumors that progress

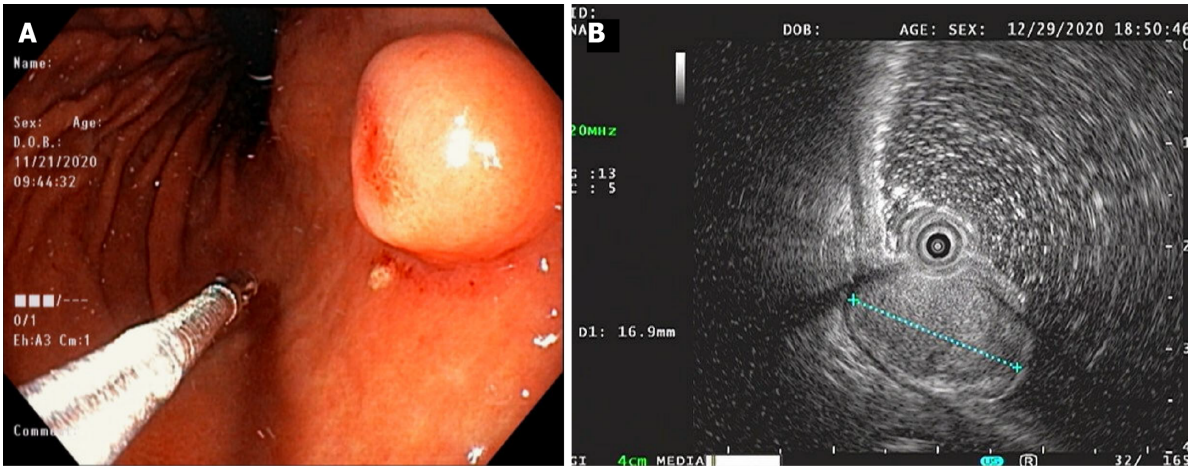

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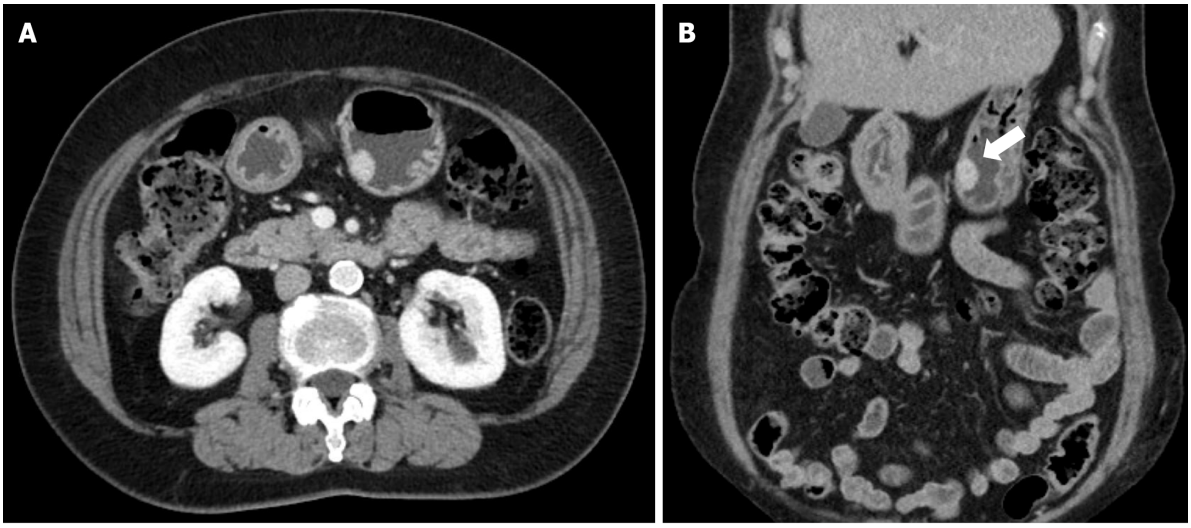
Figure 1 Endoscopy and endoscopic ultrasound images. A: Endoscopic image showing a 15-mm-sized, submucosal tumor-like, protruding lesion with focal mucosal erythema and depression of overlying mucosa; B: Endoscopic ultrasound image showing a well-circumscribed, slightly heterogeneous, 17 mm × 10 mm sized, isoechoic mass originating from the third sonographic layer.



DOI: 10.3748/wjg.v28.i23.2625

Figure 2 Abdominal computed tomography images. A and B: Axial and coronal computed tomography images showing a well-enhanced and protruding intraluminal mass in the gastric body.

more slowly[3,5]. Most of the gastric liposarcomas reported have been of the well-differentiated or myxoid histologic subtypes[10], and DL arising in the stomach has not been previously reported in the English literature; although a case of DL of heart and stomach was reported in 2017[16]. Notably, in this report, histopathological examination failed to determine that the primary lesion was located in the stomach. In addition, one case of primary DL of the gastroesophageal junction has been reported[11]. Thus, to the best of our knowledge, this is the first reported case of primary gastric DL.

DL is defined as a combination of WDL and high-grade sarcoma that displays evidence of non-lipogenic differentiation like undifferentiated high-grade pleomorphic sarcoma, fibrosarcoma, or myxofibrosarcoma[17,18]. DL can occur *de novo* (90% of cases) or as recurrence from a preexisting WDL (10% of cases)[19]. If DL develops from the transformation of a preexisting WDL into non-lipogenic sarcoma, dedifferentiation develops in 20% of the first and 44% of the second local recurrences and has been shown to be associated with poor progression and metastasis[5]. The histologic diagnosis of DL is generally based on the identification of WDL areas, which were scarce in our patient. In these cases, the differential diagnosis of any poorly-differentiated or undifferentiated sarcoma is important, and MDM2 and CDK4 immunohistochemical staining or FISH testing for amplification of the MDM2 and CDK4 genes is diagnostically helpful[20]. In our patient, DL was confirmed by CDK4 and MDM2 immunostaining.

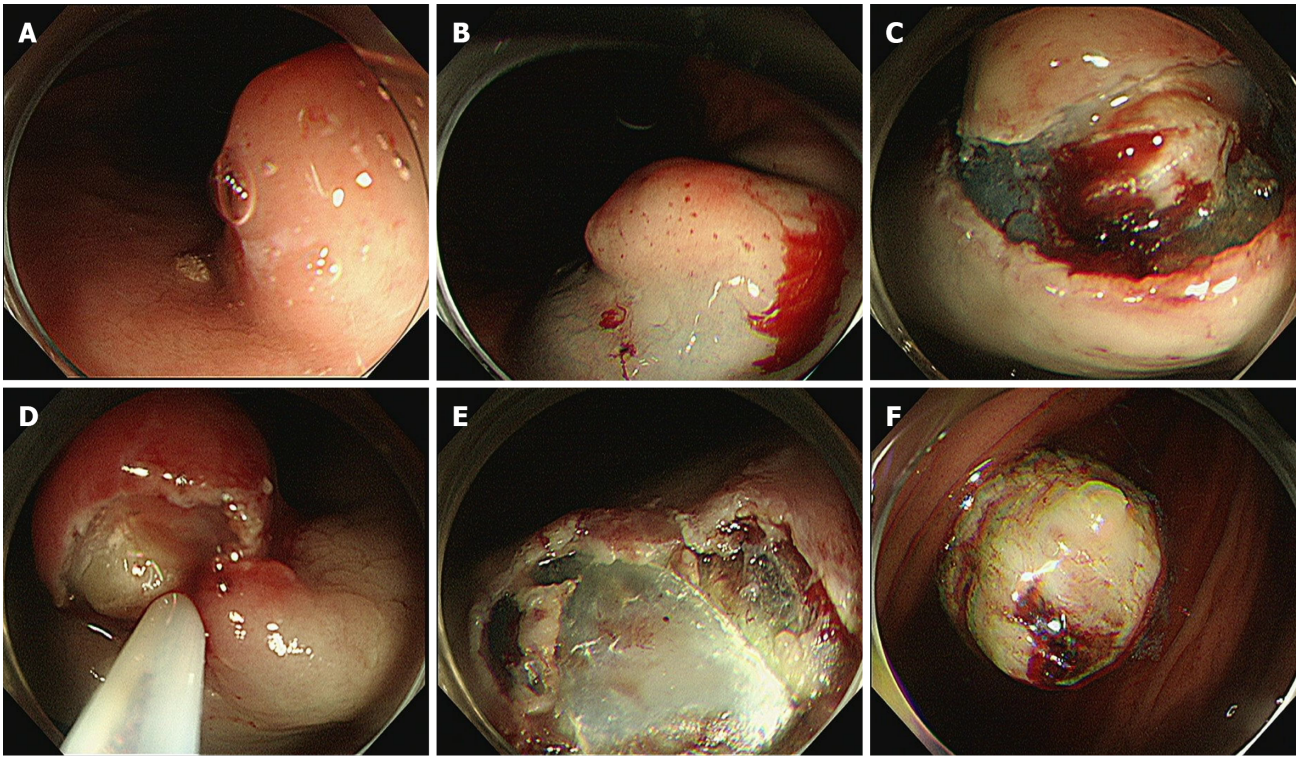


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Figure 3 Process of the endoscopic resection. A: Marking outside the lesion; B: Injection of saline–epinephrine for submucosal lifting; C: Circumferential mucosal incision and partial submucosal dissection; D and E: Resection using a snare; F: Resected tumor.

DL often exhibits abdominal cavity involvement usually of retroperitoneum, and an intraperitoneal origin is extremely rare[3,21,22]. When DL occurs inside the abdominal cavity it presents as a space-occupying mass lesion. The pathognomonic findings of DL in CT and magnetic resonance images are a heterogeneous, non-lipogenic, encapsulated mass[23], which are sufficient for diagnosis and obviate the need for needle biopsy. In our patient, because DL arising from the gastric wall presented as a small gastric SMT, CT was not diagnostically useful. Although EUS is considered the most useful modality in terms of defining the presumptive nature of SMT, our case demonstrates the difficulty of differentiating benign and malignant SMT by EUS alone, particularly when a lesion is small. Lesion echogenicity is also worth mentioning. Unlike the hyperechogenic EUS feature of other pathologic types[10,24,25], our case showed isoechoic EUS features, which appeared to be related to a sparsity of immature lipocytes or lipoblasts as the WDL area was extremely limited in our patient. DL seems to have a better prognosis than other high-grade sarcomas, especially in terms of its metastatic potential. Yet, careful long-term follow-up is essential because approximately 40% of DLs recur locally, 17% metastasize, and 28% of patients eventually die of the disease[1]. Surgery remains the treatment of choice for DL, and it is crucial that the tumor be completely removed[4], though the efficacies of targeted chemotherapy and radiotherapy are being investigated[26].

CONCLUSION

Liposarcoma is uncommon in the gastrointestinal tract and rare in the stomach, and primary DL in the stomach is extremely rare but should be considered in the differential diagnosis of any poorly-differentiated or undifferentiated sarcoma. The reasons why this case report is valuable are: (1) It presents the first reported case of a small primary gastric DL, resected endoscopically, and followed up; and (2) it cautions that if a diagnosis of SMT is uncertain after initial examination by EGD, EUS, and/or CT, the use of aggressive techniques, including endoscopic resection, should be considered.

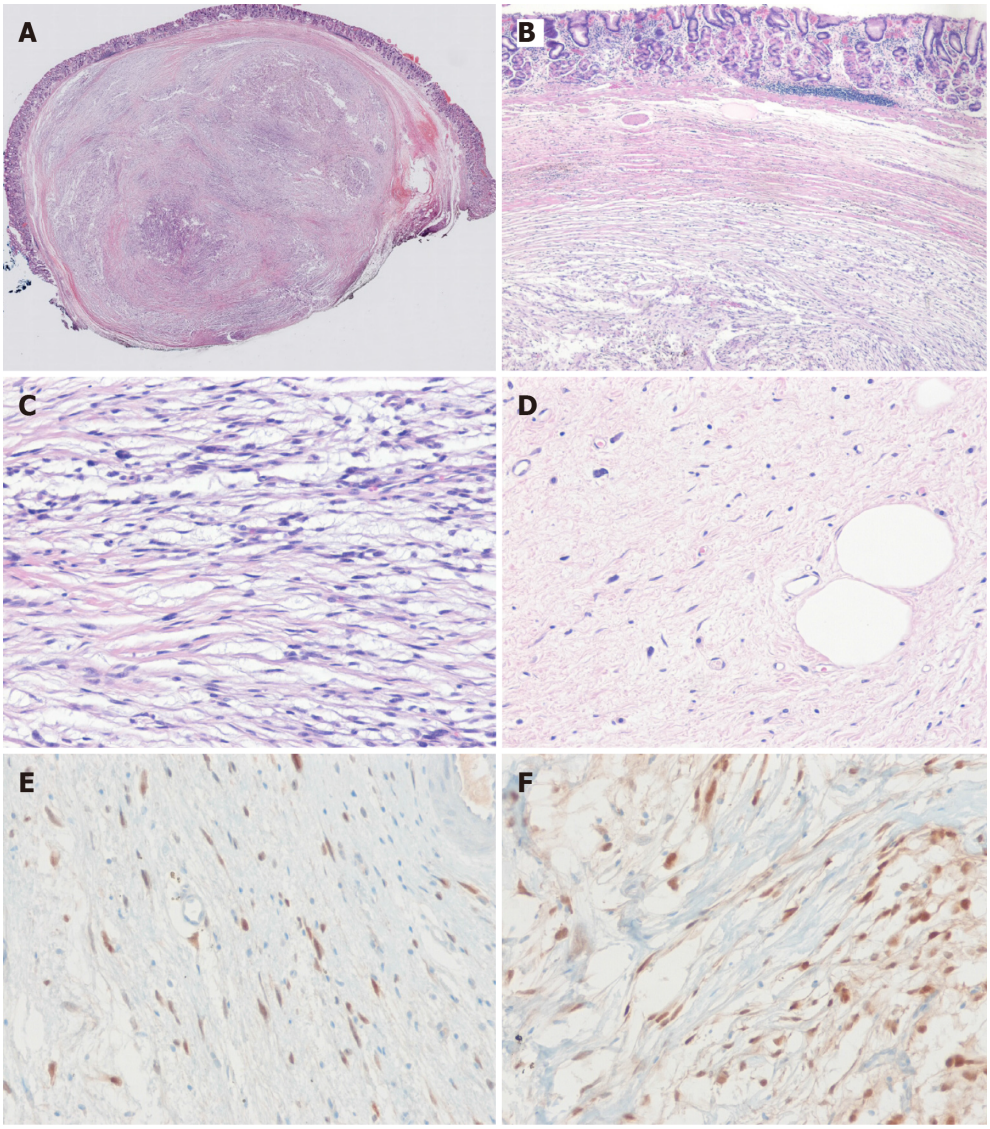

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Figure 4 The histopathological diagnosis of the endoscopically resected lesion. A and B: The tumor had a smooth margin, an internal heterogeneous morphology, and was located in submucosa (H & E, original magnification × 10, × 40); the deep resection margin was positive for tumor; C: The neoplasm consists of infiltrated, atypical spindle-shaped tumor cells with nuclear hyperchromasia (H & E, original magnification × 100); D: Photomicrograph showing highly pleomorphic spindle cells with some rare lipoblasts (H&E, original magnification × 100); E and F: Immunohistochemical staining for tumor cells show diffuse positivity for MDM2 (E) and CDK4 (F).

FOOTNOTES

Author contributions: Cho JH, Byeon JH, and Lee SH were responsible for acquiring clinical data and for manuscript writing and revision.

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LETTER TO THE EDITOR

Reconstructing the puzzle of the role of therapeutic endoscopy in the management of post-bariatric surgery complications

Konstantinos Argyriou, Adolfo Parra-Blanco

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Abstract

We have recently read with interest the mini-review article "Therapeutic endoscopy for the treatment of post-bariatric surgery complications". The abovementioned article is a brief overview of the different endoscopic modalities employed in the management of bariatric surgery complications and represents an important decision support tool for clinicians to improve their current practice. Although we appreciate the endeavor of Larsen and Kozarek, based on our in-depth analysis, we came across several minor issues in this article; thus, we present our comments in this letter. In case the authors contemplate these comments in their relevant research, we believe that their contribution would be considerable for future studies.

Key Words: Endoscopic treatment; Bariatric surgery; Complications; Obesity; Sleeve gastrectomy; Roux-en-Y gastric bypass

Core Tip: Over the last decade, the incidence of bariatric surgery has substantially increased. Despite advances in surgical techniques, postoperative complications emerge and require a multidisciplinary approach. Currently, there is no standardized guideline-based algorithm for managing bariatric complications (BC); however, minimally invasive treatments are generally preferred over reoperations. Endoscopic procedures provide minimally invasive options to manage BC. However, their exact role has not been completely delineated. The article by Larsen and Kozarek successfully addressed this issue; however, we identified several limitations that require further consideration. Therefore, we would like to share our views on this interesting review.

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TO THE EDITOR

We read with great interest the mini-review article "Therapeutic endoscopy for the treatment of post-bariatric surgery complications"[1]. In this article, Larsen and Kozarek[1] provided a concise overview of the role of endoscopy in the management of adverse events complicating the three most common types of the currently performed bariatric surgeries including Roux-en-Y gastric bypass, laparoscopic adjustable gastric band, and sleeve gastrectomy. From the extensive list of bariatric complications (BC), the authors confined their analysis only to those that are amenable to endoscopic treatment such as postoperative anastomotic strictures, leaks, fistulae, choledocholithiasis, weight regain, and band erosion. The salient highlights of this review were that the authors, by summarizing the relevant literature and incorporating their own clinical experience, were able to not only delineate the role of therapeutic endoscopy in the BC management but to also provide clinicians with practical tips that are expected to improve their daily practice. However, the most striking point of this article was that the authors holistically approached every referred complication from epidemiology to endoscopic treatment, highlighting areas that need to be further investigated. Therefore, we believe that this article has strong reference and practical value for future studies. Nonetheless, through our in-depth reading, we came across several limitations and anticipate a discussion with the authors.

First, by carefully analyzing the author's list of BC, we noticed that the endoscopic management of post-operative gastrointestinal bleeding (GIB) was not discussed in this review. The reason behind this exclusion was not mentioned by the authors. However, we regard this omission as a limitation of this article because the endoscopic management of GIB is challenging in bariatric patients. This occurs because the altered postoperative anatomy and the time interval of the bleeding episode from the operation impose restrictions not only on the type of the endoscopic equipment that would be used to approach the site of bleeding but also on the modality that would be used to achieve hemostasis. For example, standard endoscopes may not be able to reach sites of bleeding at the biliopancreatic limb or beyond the gastro-jejunal anastomosis in patients who underwent gastric bypass, whereas thermal ablation methods may cause unfavorable outcomes such as perforation in patients with freshly stapled anastomosis[2,3]. Considering these challenges, we believe that the endoscopic management of GIB has particular importance for the clinicians involved in the management of bariatric patients, and we suggest it to be supplemented in this mini-review.

Another limitation of this article is that the authors did not make clear to the reader the way they selected the studies included in this review. Although they successfully summarized the major findings of several reference studies, by performing our own literature search, we identified several omissions. For example, in the management of bariatric leakage and fistulae, the authors did not discuss the results of the most recent meta-analysis written by Rogalski *et al*[4] on the effectiveness of self-expandable stents, clipping, and tissue sealants. As a result, the authors did not make any reference to the use of fibrin glue as an alternative modality for fistulae closure in their review[4]. Likewise, by not including in their summary of evidence two reference studies on the effectiveness and safety of bougie dilations in the management of anastomotic stenosis, the authors did not discuss all available modalities that could be used as alternative options to balloon dilations[5,6]. We believe that the abovementioned information is important for the reader to acquire a complete overview of the pleiotropic role that endoscopy can play in the management of BC and, thus, needs to be supplemented.

The final limitation of this article refers to the different endoscopic techniques that can be used by clinicians to achieve biliopancreatic access in bariatric patients who underwent gastric bypass. Based on the included studies and their own experience, the authors referred to three techniques for performing endoscopic retrograde cholangiopancreatography (ERCP) in bariatric patients, including the overtube-assisted enteroscopy technique, the lap-assisted transgastric, and the endoscopic ultrasound-directed transgastric technique, with the first technique being their first-line option for most indications. However, considering that not all centers managing bariatric patients can perform these techniques, we performed our own literature search and came across an additional option. Specifically, we found that in bariatric patients who underwent gastric bypass, the biliopancreatic access to the excluded gastrointestinal part can be also achieved through the gastrocutaneous tract created after the removal of a gastrostomy tube without the need for reoperation or special equipment. This technique is known as gastrostomy-assisted ERCP, and it is performed in 3 steps. The first step includes the endoscopic insertion of the gastrostomy tube, which stays in situ for 5–14 d until the maturation of the tract. Then, the tube is removed, and the tract is dilated with a balloon to an extent that will allow the passage of the duodenoscope. After completion of the dilation of the tract, ERCP can be repeatedly performed[7]. Given the wide availability of gastrostomy tubes, we believe that the abovementioned technique has

particular value for the clinicians involved in the management of bariatric patients and should be supplemented in this review.

In summary, despite the abovementioned limitations, we believe that this article can be a valuable reference study, guiding clinicians in their daily practice. Thus, we offer our evidence-based considerations in this review to expand the value of the research basis that this article sets, leading to more comprehensive future studies.

FOOTNOTES

Author contributions: Argyriou K and Parra-Blanco A designed and performed the research; Argyriou K wrote this comment; Parra-Blanco A revised the manuscript.

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