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TNFSF15 variant predicts disease progression in Chinese patients with Crohn's disease

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Abstract

Background: The genetic variant of tumor necrosis factor superfamily member 15 (*TNFSF15*) is associated with Crohn's disease (CD) and the development of intestinal fibrosis and stricturing. We aimed to investigate its predictive role in disease progression and the impact of ileal fibrosis-associated protein expression in Chinese patients with CD.

Methods: We genotyped the single nucleotide polymorphism rs6478109 within the *TNFSF15* gene in 428 CD patients and 450 health controls to assess its association with CD. Genotype–phenotype correlation analyses were performed. Mucosal samples from non-diseased terminal ileum were analyzed for TL1A and fibrosis-associated protein expression using western blot and immunohistochemistry.

Results: The G allele frequency of rs6478109 was significantly higher among CD patients compared with health controls (63.3% vs. 46.7%, $P < 0.001$). Patients with GG genotype were more predisposed to develop the stricturing phenotype, compared with those with AA + AG genotypes with a hazard ratio of 1.426 (95% confidence interval: 1.029–1.977, $P = 0.033$). This trend was similarly observed in patients utilizing biological agents, with a hazard ratio of 4.396 (95% confidence interval: 1.780–10.854, $P = 0.001$). Furthermore, increased TL1A, pro-fibrotic proteins, and TGFβ1/Smad3 pathway activation were observed in non-diseased ileal mucosa of patients with GG genotype compared with those with AA genotype.

Conclusions: The *TNFSF15* risk genotype GG could promote the expression of pro-fibrotic proteins and may serve as a predictor for stricturing CD.

Keywords: *TNFSF15*; Crohn's disease; SNP; disease progression; fibrosis

Introduction

Crohn's disease (CD) is one of the major types of inflammatory bowel disease (IBD) involving chronic inflammation of the gastrointestinal tract and progressive bowel damage. Despite its unknown etiology, CD patients often experience intestinal fibrosis, manifesting as strictures and obstructive symptoms due to excess extracellular matrix accumulation [1]. More than 70% of patients will develop a stricturing or perforating complication, and over a third of CD patients will develop bowel strictures and obstructions requiring surgery [2]. Despite therapeutic advancements, the incidence of intestinal strictures and surgical needs has remained unchanged [3]. At present, there are no approved or effective medical therapies targeting intestinal fibrosis [4], but early identification of high-risk patients and modification of their treatment strategies are crucial to slowing disease progression, so it is important to identify some predictors of disease progression in CD patients [5].

Several clinical factors, such as CD diagnosis made under the age of 40 years, need for steroid therapy at diagnosis, and early use of azathioprine or anti-tumour necrosis factor therapy, have been suggested as predictors of a more aggressive and complicated disease, rather than as specific predictive factors of intestinal fibrosis, strictures, and obstruction [6]. In addition, genetic, epigenetic, and serological biomarkers have been evaluated as predictors of stricturing CD [6]. Among these factors, genetic biomarkers are present before disease onset and unaffected by disease flares, so they seem to be more suitable for predicting stricturing CD. Some genes, including *NOD2*, *ATG16L1*, *TLR4*, *CX3CR1*, *IL23R*, and *TNFSF15*, can be used as predictors of stricturing CD [6, 7].

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Tumor necrosis factor-like ligand 1A (TL1A) is a component of the tumor necrosis factor superfamily of ligands encoded by the *TNFSF15* gene [8]. Previous studies have reported that the *TNFSF15* gene is associated with a greater susceptibility to the development of CD, particularly in Asian patients [9–11]. A cross-ethnic study comparing Asian and European IBD patients found *TNFSF15* to have stronger IBD susceptibility in Asians [12]. Other studies disclosed the association between specific *TNFSF15* single nucleotide polymorphisms (SNPs) and disease progression in CD [7, 13]. In mice, constitutive TL1A expression leads to spontaneous intestinal inflammation and fibrosis [14]. Anti-TL1A treatment can reverse established colonic fibrosis, and a clinical trial has shown that an anti-TL1A monoclonal antibody can effectively alleviate intestinal inflammation by targeting tissue inflammation and fibrosis pathways [15, 16]. Hence, the *TNFSF15* gene may be a reliable predictor for stricturing CD.

Among the SNP sites within the TNFSF15 gene, the SNP rs6478109 stands out as being significantly associated with an elevated risk of CD compared with other SNPs in a Japanese population [17]. Furthermore, this particular SNP has been implicated in influencing the therapeutic outcomes of anti-TNF therapy among CD patients [18], which was the only *TNFSF15* SNP reported to be associated with the efficacy of anti-TNFα drugs. Therefore, the SNP rs6478109 may hold a more profound association with CD and its treatment response.

In this study, we aim to investigate the association between *TNFSF15* SNP (rs6478109) genotype with disease progression from inflammatory (B1) to stricturing (B2) or penetrating (B3) phenotype in Chinese CD patients, as well as the mechanism for the effects.

Materials and methods

Patient data and cohort design

This was a retrospective cohort study at a single center. We enrolled a total of 428 CD patients and 450 health controls at The Sixth Affiliated Hospital of Sun Yat-sen University (Guangzhou, P. R. China) between June 1999 and April 2023. Inclusion criteria were as follows: (i) patients were diagnosed with CD according to the current guidelines [19]; and (ii) patients were followed up for ≥6 months. Exclusion criteria were as follows: (i) patients with a history of malignancy; or (ii) patients with a history of bowel resection prior to the diagnosis of CD. Demographic and clinical data were collected from medical records, including age, sex, disease location, disease behavior, perianal disease, endoscopic findings and imaging results, Harvey–Bradshaw Index (HBI), treatment medications, disease progression events, and follow-up time.

Disease location and behavior were classified according to the Montreal classification [20]. Progression of disease behavior from onset to follow-up is defined as change from inflammatory (B1) to either stricturing (B2) or penetrating (B3) disease. Stricturing disease (B2) is defined as luminal narrowing (at least 50% reduction in luminal diameter), wall thickening (a 25% increase in wall thickness relative to the adjacent unaffected bowel), and proximal dilation of at least 3 cm on cross-sectional imaging (computed tomography enterography, magnetic resonance enterography or ultrasound), or inability to pass an adult colonoscope through the narrowed area without prior endoscopic dilation and with a reasonable amount of pressure applied [21]. Penetrating disease (B3) is defined as fistulizing disease with abscesses or fistulas in an adjacent organ (not perianal fistula) [22]. Clinical disease activity was determined using the HBI. A HBI score of ≤4 was defined as remission, 5–7 as mild activity, 8–16 as moderate activity, and ≥16 as severe activity [23].

Blood samples were drawn during clinical visits and stored at −80°C until used. Mucosal biopsies were collected from non-diseased terminal ileum of the resected tissue and during endoscopy.

The study was approved by the Sixth Affiliated Hospital of Sun Yat-sen University Research Ethics Board (approval no. 2024ZSLYEC-046).

Genotyping of *TNFSF15*

In this study, we genotyped *TNFSF15* (rs6478109) as a marker SNP for CD risk or non-risk haplotype. DNA was extracted from whole blood samples from all participants using a commercially available BIOG Blood DNA Isolate Kit (BAIDAI, 51018, Changzhou, Jiangsu, P. R. China). The amplification primer (forward: 5′-AACTGCAGG CAAAGCACATC-3′, reverse: 5′-TCAGTGGGTGGGGCAAATTA-3′) was designed based on rs6478109 sequence. The specificity of the primers was verified. The PCR conditions and parameters were performed as follows: preliminary denaturation was conducted at 94°C for 30 s, followed by 35 cycles of denaturation at 94°C for 10 s, with annealing at 60°C for 30 s and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were used for *TNFSF15* typing based on high-resolution Sanger sequencing.

SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot (WB) analysis

Total protein was extracted from intestinal tissues using RIPA lysis buffer (CWBIO, CW2333S, Beijing, P. R. China) supplemented with protease (CWBIO, CW2200S, Beijing, P. R. China) and phosphatase inhibitors (CWBIO, CW2383S, Beijing, P. R. China). Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific, USA). Protein was loaded onto and separated using a 10% PAGE gel (EpiZyme, PG212, Shanghai, P. R. China). It was then transferred onto polyvinylidene difluoride (PVDF) membranes (EMD Millipore, IPVH00010, Darmstadt, Germany). The membranes were blocked with nonfat dry milk in Tris-buffered saline with Tween-20 (TBST) for 1 h, then incubated with specific primary antibodies overnight at 4°C and the horseradish peroxidase-conjugated secondary antibody for 1 h subsequently. The blots were visualized using an ultra-high sensitivity chemiluminescence device (Bio-Rad, USA). The densities of the blots were analyzed using software ImageJ v1.8.0 software. The primary antibodies used are listed below: TNFSF15 (Bioss, BS-19940R, Beijing, P. R. China), collagen I (Bioss, BS-10423R, Beijing, P. R. China), MMP3 (Proteintech, 17873-1-AP, Wuhan, Hubei, P. R. China), TIMP1 (Bioss, BS-0415R, Beijing, P. R. China), transforming growth factor β1 (TGFβ1; proteintech, 21898-1-AP, Wuhan, Hubei, P. R. China), phospho-Smad2/3 (CST, 8828S, Boston, USA), Smad2/3 (CST, 5678S, Boston, USA) and β-actin (Servicebio, GB12001-100, Wuhan, Hubei, P. R. China).

Immunohistochemistry

Human non-diseased ileal tissues were fixed using 4% paraformaldehyde at 4°C overnight and then embedded in paraffin. They were cut into 3-μm-thick paraffin sections. The sections were dried at 60°C for 1 h, then deparaffinized in xylene, rehydrated with graded concentrations of ethanol, and rinsed with tap water for 3 min. The sections were incubated with 3% H_2O_2 for 15 min and then rinsed with distilled water for 5 min. The sections were placed in Tris-EDTA repair solution (pH 8.0) under high temperature and pressure for 15 min to repair antigenic sites in the tissue sections. After cooling to room temperature, the sections were washed with phosphate buffer solution (PBS)

buffer solution three times. After incubation with 0.5% Triton and 3% bovine serum albumin for 30 min, sections were then incubated with primary antibody (TL1A) at 4°C overnight. Rewarm the sections at 37°C for 30 min and washed three times with PBS. The tissues were then allowed to bind to the secondary antibodies and washed three times with PBS when they were incubated at 37°C for 1 h. A 3,3-diaminobenzidine color development kit (ORIGENE, ZLI-9017, Maryland, USA) was then used for color development of the tissue sections. Finally, the sections were stained with hematoxylin and dehydrated. The staining of positive areas was analyzed using ImageJ v1.8.0 software.

Statistical analyses

Statistical analysis was performed using the SPSS 25.0 statistical package (IBM SPSS, Chicago, IL, USA) and GraphPad Prism version 8 (GraphPad Software, California, USA). The Hardy–Weinberg equilibrium was tested using a chi-squared goodness-of-fit test to assess the *TNFSF15* allele frequencies. Statistical differences between *TNFSF15* genotypes were analyzed using Student's *t*-test for continuous variables and Fisher's exact test. Statistical analyses were performed by Student's *t*-test, chi-square analysis, or one-way analysis of variance followed by Bonferroni post-hoc test, depending on the type of data. Kaplan–Meier, log-rank tests, and Cox regression analysis were used to estimate clinical phenotype and genetic association. $P < 0.05$ was considered statistically significant.

Results

Baseline characteristics of enrolled subjects

Supplementary Table 1 summarizes the baseline characteristics of 428 CD patients (310 males) and 450 health controls (274 males). The mean age of onset was 26.6 (25.7–27.4) years in the CD patients. The disease location was ileal, colonic, and ileocolonic in 119 (27.8%), 14 (3.3%), and 295 (68.9%) patients, respectively. The disease behavior was inflammatory, stricturing, and penetrating in 139 (32.5%), 162 (37.8%), and 127 (29.7%) patients, respectively. 310 patients (72.4%) had perianal lesion. The mean time of follow-up was 48.1 (44.1–52.0) months. Significant differences in onset age, follow-up, and medication use were observed among different disease behaviors, suggesting variable patient impacts and potential medication effects on disease progression.

TNFSF15 (rs6478109) polymorphisms in study groups and clinical outcome

The SNP (rs6478109) followed Hardy–Weinberg equilibrium in our groups (Supplementary Table 2). The association of *TNFSF15* (rs6478109) with CD is shown in Supplementary Table 3. The G allele, identified as a risk factor for CD, was more frequent in CD patients than controls (63.3% vs. 46.7%, $P < 0.001$). The genotype of *TNFSF15* (rs6478109) in the study population was AA, AG, and GG in 55 (12.8%), 204 (47.7%), and 169 (39.5%) CD patients, as well as in 128 (28.4%), 224 (49.8%), and 98 (21.8%) controls, respectively. The odds ratio (OR) of rs6478109 for AA genotype compared with GG genotype was 0.249 [95% confidence interval (CI): 0.167–0.373, $P < 0.001$]. The OR of rs6478109 for AA + AG genotypes in comparison to GG genotype was 0.427 (95% CI: 0.317–0.574, $P < 0.001$). The *TNFSF15* (rs6478109) genotype was AA + AG and GG in 259 cases (60.5%) and 169 cases (39.5%). The percentage of B2 or B3 patients was significantly higher in the GG group compared with the AA + AG group ($P = 0.001$). Although HBI scores differed among genotypes, all were within mild activity, so it can be considered undifferentiated. Patients with different genotypes show no significant difference in the use of immunosuppressors (mainly including azathioprine) and biological agents (mainly including infliximab) before disease progression. Although usage of adalimumab showed differences between patients with different genotypes, it may have little impact on total medication because of the small amount (Table 1).

GG genotype of *TNFSF15* (rs6478109) predicts the progression to stricturing behavior

We have found that the percentage of B2 or B3 patients in the GG group was significantly higher than in the AA + AG group. To find out whether *TNFSF15* (rs6478109) genotype could predict disease progression from B1 phenotype to B2 or B3 phenotype, the analysis was performed according to two different possible outcomes, where time to progression was set as the exact time (in months) when progression occurred. The Kaplan–Meier survival curves of patients who remained free of stricturing or penetrating complications are shown in Figure 1.

Patients with *TNFSF15* (rs6478109) AA or AG genotypes both showed later disease progression (B1–B2) compared with patients with *TNFSF15* (rs6478109) GG genotype [mean: 95.4 months (95% CI: 65.1–125.6) vs. 112.6 months (95% CI: 92.5–132.6) vs. 63.8 months (95% CI: 54.2–73.4), $P = 0.044$ and $P = 0.001$, respectively; Figure 1B]. There was no significant difference in disease progression (B1–B3 and B1–B2 or B3) among the three groups (Figure 1A and C). Given the similar progression patterns in AA and AG patients, we combined them into a single group. The AA + AG group showed delayed disease progression from B1 to either B2 or B3 compared with the GG group [mean: 79.1 months (95% CI: 67.8–90.5) vs. 56.9 months (95% CI: 49.4–64.3), $P = 0.018$; Figure 1D], especially from B1 to B2 [mean: 109.0 months (95% CI: 91.7–126.4) vs. 63.8 months (95% CI: 54.2–73.4), $P < 0.001$; Figure 1E]. There was no difference in the disease progression (B1–B3) between the two groups (Figure 1F). Hazard ratios (HRs) for B1–B2 progression are summarized in Table 2, with the GG genotype emerging as an independent predictor of stricturing CD (HR: 1.426, 95% CI: 1.029–1.977, $P = 0.033$, Table 2). Additional predictors included onset age over 40 years, non-use of immunosuppressors, and non-use of biological agents. Details on medication use are provided in Supplementary Table 4. Additionally, the *TNFSF15* genotype demonstrated an interaction with the use of biological agents ($P = 0.039$, Table 2). In patients using biological agents, the GG genotype remained a significant predictor of stricturing CD (HR: 4.396, 95% CI: 1.780–10.854, $P = 0.001$, Table 3). Conversely, in patients not using biological agents, the GG genotype did not independently predict stricturing CD, while non-use of immunosuppressors emerged as an independent predictor (HR: 0.225, 95% CI: 0.129–0.394, $P < 0.001$, Table 3).

TNFSF15 genotype affects the protein expression levels of TL1A

As CD is a gastrointestinal inflammatory disease, we analyzed TL1A protein levels in ileal biopsies obtained from CD patients. WB revealed significantly elevated TL1A protein levels in the GG group compared with the AA group in non-diseased ileal tissue from CD patients (Figure 2A; $P < 0.05$). To exclude the influence of disease phenotype and activity, we also tested the TL1A protein levels in surgical samples from CD patients with stricturing or fistula and similar disease activity. Similar results were found in surgical samples (Figure 2B; $P < 0.05$).

Immunohistochemistry confirmed these findings, with TL1A staining primarily in the mucosal lamina propria and significantly higher protein levels in GG patients compared with AA or AG patients (Figure 2C and D; $P < 0.05$). The *TNFSF15* (rs6478109) genotype appears to modulate TL1A protein levels.

Table 1. Clinical characteristics of patients with various genotypes of *TNFSF15* (rs6478109)

Characteristic	Total (*n*=428)	Genotype AA (*n*=55)	Genotype AG (*n*=204)	Genotype GG (*n*=169)	P-value	
					AA vs. GG	AA + AG vs. GG
Male, *n* (%)	310 (72.4)	37 (67.3)	158 (77.5)	115 (68.0)	1.000	0.126
Age of onset, *n* (%)					0.382	0.602
A1 (≤16 years)	33 (7.7)	2 (3.6)	16 (7.9)	15 (8.9)		
A2 (17–40 years)	362 (84.6)	48 (87.3)	171 (83.8)	143 (84.6)		
A3 (>40 years)	33 (7.7)	5 (9.1)	17 (8.3)	11 (6.5)		
Mean age, years, mean (range)	26.6 (25.7–27.4)	27.1 (24.8–29.4)	26.7 (25.4–28.0)	26.3 (24.9–27.6)	0.544	0.540
Disease location, *n* (%)					0.740	0.415
L1 (ileal disease)	119 (27.8)	12 (21.8)	66 (32.4)	41 (24.3)		
L2 (colonic disease)	14 (3.3)	1 (1.8)	7 (3.4)	6 (3.5)		
L3 (ileocolonic disease)	295 (68.9)	42 (76.4)	131 (64.2)	122 (72.2)		
Disease behavior, *n* (%)					0.102	0.001
Montreal B1	139 (32.5)	21 (38.2)	77 (37.7)	41 (24.3)		
Montreal B2	162 (37.8)	19 (34.5)	62 (30.4)	81 (47.9)		
Montreal B3	127 (29.7)	15 (27.3)	65 (31.9)	47 (27.8)		
Perianal disease, *n* (%)	310 (72.4)	35 (63.6)	153 (75.0)	122 (72.2)	0.301	1.000
Mean follow-up period, months, mean (range)	48.1 (44.1–52.0)	47.0 (34.7–59.2)	49.9 (43.9–55.8)	46.3 (40.5–52.1)	0.910	0.472
HBI score, mean (range)	5.49 (5.28–5.70)	6.22 (5.56–6.87)	5.71 (5.42–5.99)	5.00 (4.66–5.34)	<0.001	<0.001
Medication used before B2 or B3, *n* (%)						
Immunosuppressors	131 (30.6)	17 (30.9)	68 (33.3)	46 (27.2)	0.722	0.339
Azathioprine	116 (27.1)	16 (29.1)	60 (29.4)	40 (23.7)	0.530	0.238
Methotrexate	24 (5.6)	4 (7.3)	13 (6.4)	7 (4.1)	0.566	0.396
Mercaptopurine	9 (2.1)	0 (0)	6 (2.9)	3 (1.8)	0.749	0.970
Biological agents	138 (32.2)	14 (25.5)	68 (33.3)	56 (33.1)	0.511	1.000
Infliximab	125 (29.2)	11 (20.0)	63 (30.9)	51 (30.2)	0.196	0.804
Adalimumab	17 (4.0)	4 (7.3)	11 (5.4)	2 (1.2)	0.051	0.047
Ustekinumab	14 (3.3)	1 (1.8)	8 (3.9)	5 (3.0)	1.000	1.000
Vedolizumab	10 (2.3)	1 (1.8)	4 (2.0)	5 (3.0)	1.000	0.514

B1 = inflammatory disease, B2 = stricturing disease, B3 = penetrating disease, HBI = Harvey–Bradshaw Index.

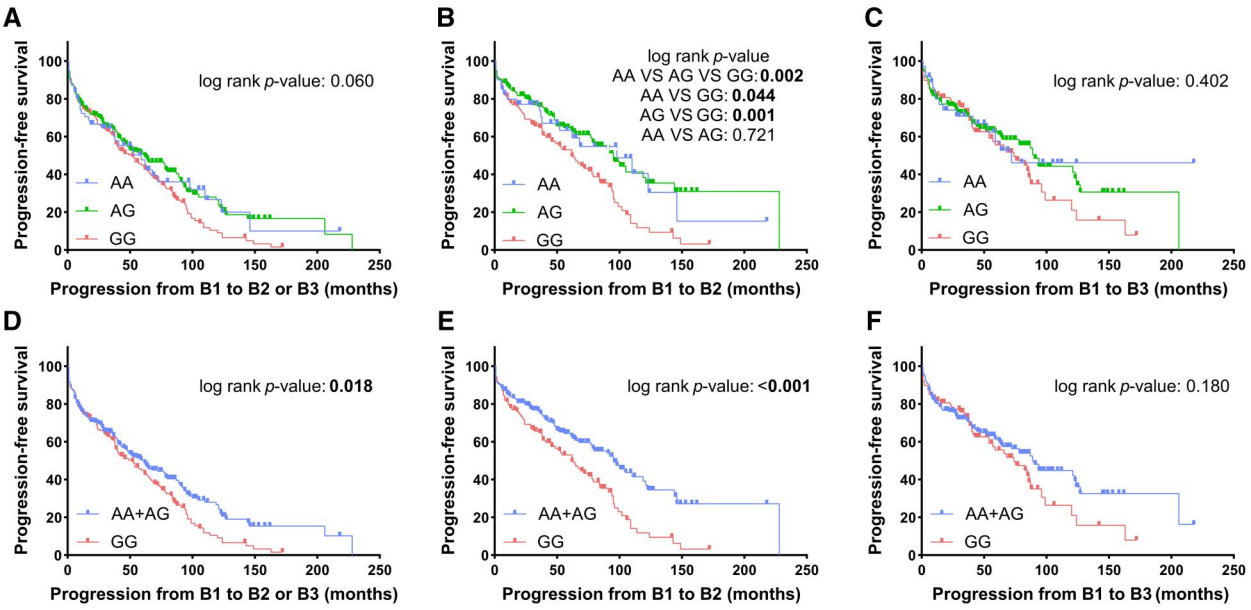

Figure 1. Kaplan–Meier estimates of patients with Crohn's disease who have remained free of behavior progression since diagnosis. (A) Association between *TNFSF15* genotypes (AA, AG, and GG) and disease progression (B1–B2 or B3). (B) Association between *TNFSF15* genotypes (AA, AG, and GG) and disease progression (B1–B2). (C) Association between *TNFSF15* genotypes (AA, AG, and GG) and disease progression (B1–B3). (D) Association between *TNFSF15* genotypes (AA + AG and GG) and disease progression (B1–B2 or B3). (E) Association between *TNFSF15* genotypes (AA + AG and GG) and disease progression (B1–B2). (F) Association between *TNFSF15* genotypes (AA + AG and GG) and disease progression (B1–B3). B1 = inflammatory disease, B2 = stricturing disease, B3 = penetrating disease.

TNFSF15 genotype affects the expression levels of fibrosis-associated proteins

As the *TNFSF15* genotype can predict the progression to complicated CD disease, especially bowel stricturing, it may affect the levels of fibrosis-associated proteins in non-diseased regions. Increased collagen is connected with down-regulated matrix metalloproteinase 3 (MMP3) and up-regulated tissue inhibitor metalloproteinase 1 (TIMP1). WB analysis revealed significantly higher collagen I protein levels in the GG group compared with the AA group. TIMP1 was also higher in the GG group, while

Table 2. Analysis of possible risk factors predicting stricturing Crohn's disease using the Cox model

Characteristic	Multivariable HR (95% CI)	*P*-value	*P*-value for interaction[a]
Genotype (AA + AG vs. GG)	1.426 (1.029–1.977)	0.033	–
Gender (male vs. female)	0.742 (0.517–1.164)	0.105	–
Age of onset (≤40 vs. >40)	1.741 (0.921–2.901)	0.034	0.652
Disease location			–
Ileal vs. colonic	0.924 (0.328–2.602)	0.881	–
Ileal vs. ileocolonic	1.321 (0.517–1.894)	0.130	–
Ileocolonic vs. colonic	0.699 (0.255–1.921)	0.488	–
Perianal disease	1.179 (0.820–1.695)	0.375	–
HBI score	0.999 (0.931–1.072)	0.945	–
Immunosuppressors	0.311 (0.204–0.475)	<0.001	0.172
Biological agents	0.320 (0.207–0.495)	<0.001	0.039

CI = confidence interval, HBI = Harvey–Bradshaw Index, HR = hazard ratio.
[a] Interaction between genotype and other factors.

Table 3. Analysis of possible risk factors predicting stricturing Crohn's disease using the Cox model (divided by use of biological agents)

Characteristic	Use of biological agents group		Non-use of biological agents	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Genotype (AA + AG vs. GG)	4.396 (1.780–10.854)	0.001	1.181 (0.825–1.692)	0.364
Gender (male vs. female)	0.809 (0.333–1.962)	0.639	0.815 (0.544–1.223)	0.323
Age of onset (≤40 vs. >40)	2.504 (0.791–7.929)	0.118	1.583 (0.886–2.826)	0.121
Disease location				
Ileal vs. colonic	1.273 (0.230–7.054)	0.782	0.770 (0.183–3.241)	0.722
Ileal vs. ileocolonic	1.736 (0.660–4.568)	0.263	1.369 (0.920–2.037)	0.121
Ileocolonic vs. colonic	0.733 (0.144–3.742)	0.709	0.733 (0.144–3.742)	0.709
Perianal disease (no vs. yes)	1.036 (0.379–2.828)	0.946	1.254 (0.846–1.861)	0.260
HBI score	1.082 (0.896–1.307)	0.411	0.997 (0.921–1.079)	0.937
Immunosuppressors (non-use vs. use)	0.481 (0.192–1.206)	0.119	0.225 (0.129–0.394)	<0.001

CI = confidence interval, HBI = Harvey–Bradshaw Index, HR = hazard ratio.

MMP3 levels showed no difference between the two groups (Figure 3A and B). This trend was consistent in both surgical and endoscopic samples (Figure 3C and D). Although MMP3 didn't decrease in the GG group, the increased expression of collagen I and TIMP1 also showed that the *TNFSF15* genotype may affect the expression of fibrosis-associated proteins before stricturing or fistula occurs.

TNFSF15 genotype affects the TGFβ1/Smad3 pathway

To explore the possible mechanism of TL1A regulation of fibrosis, we assessed the protein levels of the TGFβ1/Smad3 pathway. The TGFβ1/Smad3 pathway is a classical pathway involved in fibrosis. WB analysis results indicated that both TGFβ1 and p-smad3/smad3 were significantly higher in the GG group than in the AA group (Figure 4A and B; $P < 0.05$). It can also be identified in surgical samples (Figure 4C and D; $P < 0.05$). Our data showed that the GG genotype may activate the TGFβ1/Smad3 pathway and induce fibrosis.

Discussion

Intestinal stricturing and obstruction due to fibrosis remain challenging in IBD treatment. TL1A's crucial role in CD pathogenesis is well-documented [24–26]. This study showed that the *TNFSF15* (rs6478109) genotype can predict CD progression from inflammatory B1 to stricturing B2. Patients with GG genotype progress to stricturing behaviors earlier than those with AA + AG genotypes and the GG genotype was an independent predictor of stricturing CD, especially in patients using biological agents. Identifying this genotype at disease onset may aid in aggressive initial therapy to prevent complications. We also found that the *TNFSF15* (rs6478109) genotype may affect the TL1A expression in non-diseased ileal mucosa, as well as fibrosis-associated protein and TGF-β1/Smad3 pathway. Homozygous GG patients exhibited higher TL1A, collagen I, TIMP1, TGFβ1 and p-smad3/smad3. To the best of our knowledge, this is the first report describing the association between *TNFSF15* (rs6478109) genotype and stricturing CD at both clinical and molecular levels.

The results that *TNFSF15* (rs6478109) genotype can predict CD progression from B1 to B2 in this study were almost similar to the previous study. Previous studies have found that two other SNPs (rs4263839 and rs4246905) of *TNFSF15* can predict the disease progression in CD [7, 27]. Our study adds to this knowledge by discovering a new SNP, rs6478109, as a predictor that has not been previously reported. Furthermore, multivariate Cox regression analysis was utilized to validate the GG genotype as an independent predictor of stricturing CD. Early prediction of disease progression risk may aid in implementing optimal therapy strategies tailored to individual patients. Besides, we also found that age of onset (>40 years), non-use of immunosuppressors, and non-use of biological agents were identified as independent predictors of stricturing CD, offering potential utility in the identification of high-risk patient populations.

Pharmacological interventions targeting intestinal fibrosis are currently lacking in clinical practice, and the potential for reversibility of intestinal fibrosis is contingent upon the stage of fibrosis. Early-stage CD patients may respond to partial therapeutic medications, while those with advanced fibrosis may show complete resistance to treatment [28]. Immunosuppressors and biological agents have been recognized for their ability to reduce disease progression in CD, with combination therapy involving

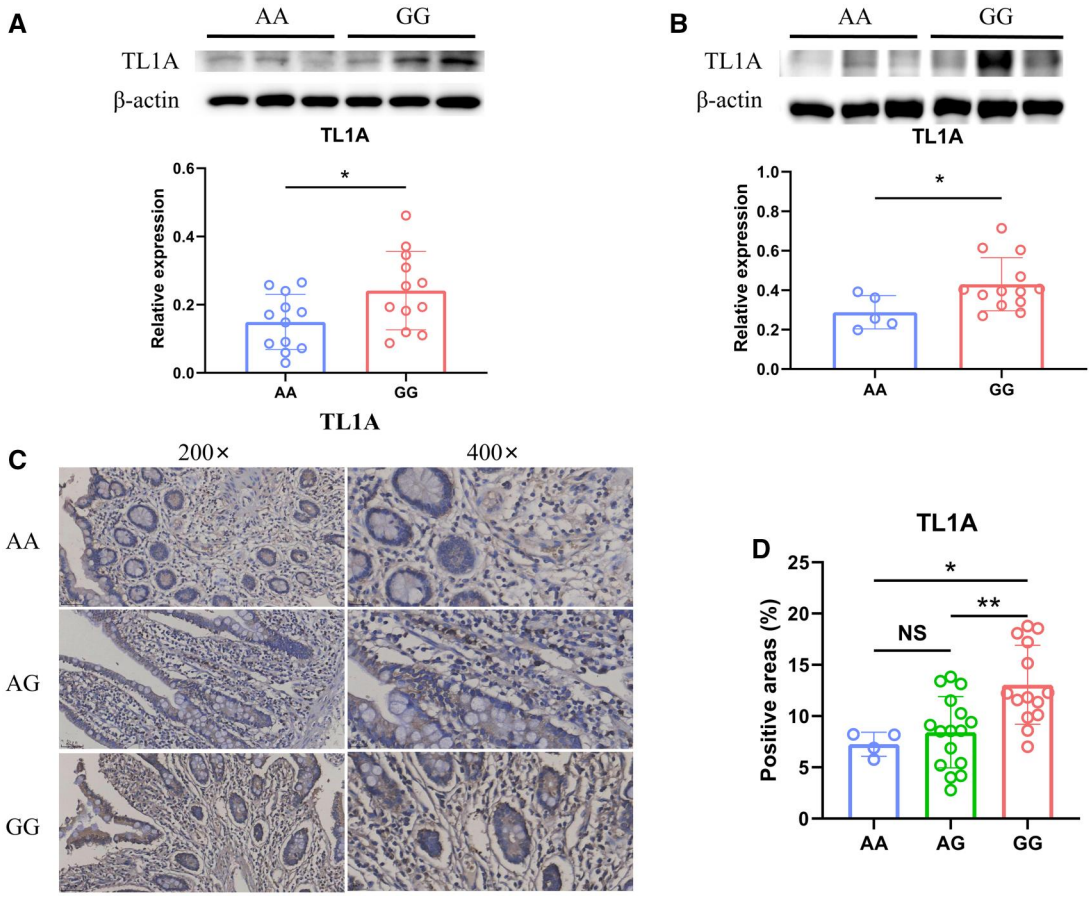

Figure 2. The expression of TL1A in non-diseased ileac tissues of patients with different *TNFSF15* genotypes. (A) Western blot analysis of TL1A in endoscopic biopsy tissues (AA, $n = 12$; GG, $n = 12$). (B) Western blot analysis of TL1A in surgical tissues (AA, $n = 5$; GG, $n = 13$). (C) Staining of TL1A in surgical tissues (immunohistochemistry; left, 200×; right, 400×). (**D**) Statistical analysis of TL1A expression in patients with different genotypes (AA, $n = 4$; AG, $n = 16$; GG, $n = 14$). Compared with the GG group: $^*P < 0.05$, $^{**}P < 0.01$. TL1A = tumor necrosis factor-like ligand 1A, NS = non-significant.

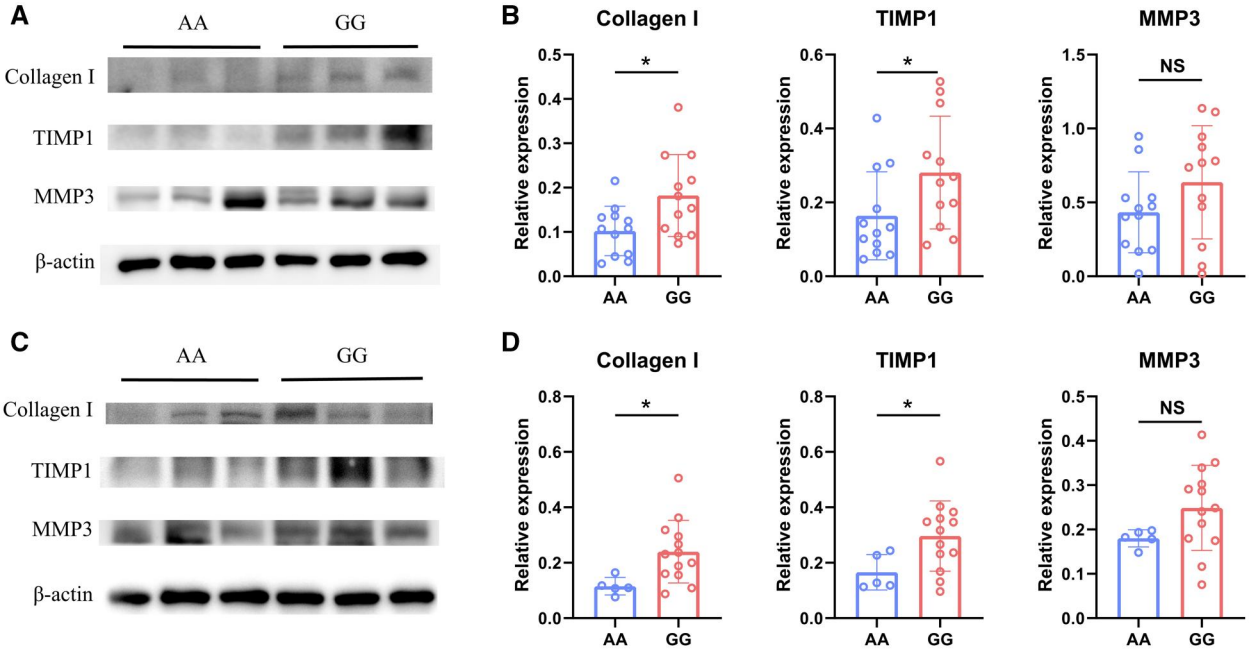

Figure 3. *TNFSF15* genotype changes fibrosis-associated protein expression in non-diseased ileac tissues. (A, B) Western blot analysis of collagen I, TIMP1, and MMP3 in endoscopic biopsy tissues ($n = 12$ in both AA and GG groups). (C, D) Western blot analysis of collagen I, TIMP1, and MMP3 in surgical tissues (AA, $n = 5$; GG, $n = 13$). Compared with the AA group: $^*P < 0.05$. TIMP1 = tissue inhibitors of metalloproteinase 1, MMP3 = metalloproteinase 3, NS = non-significant.

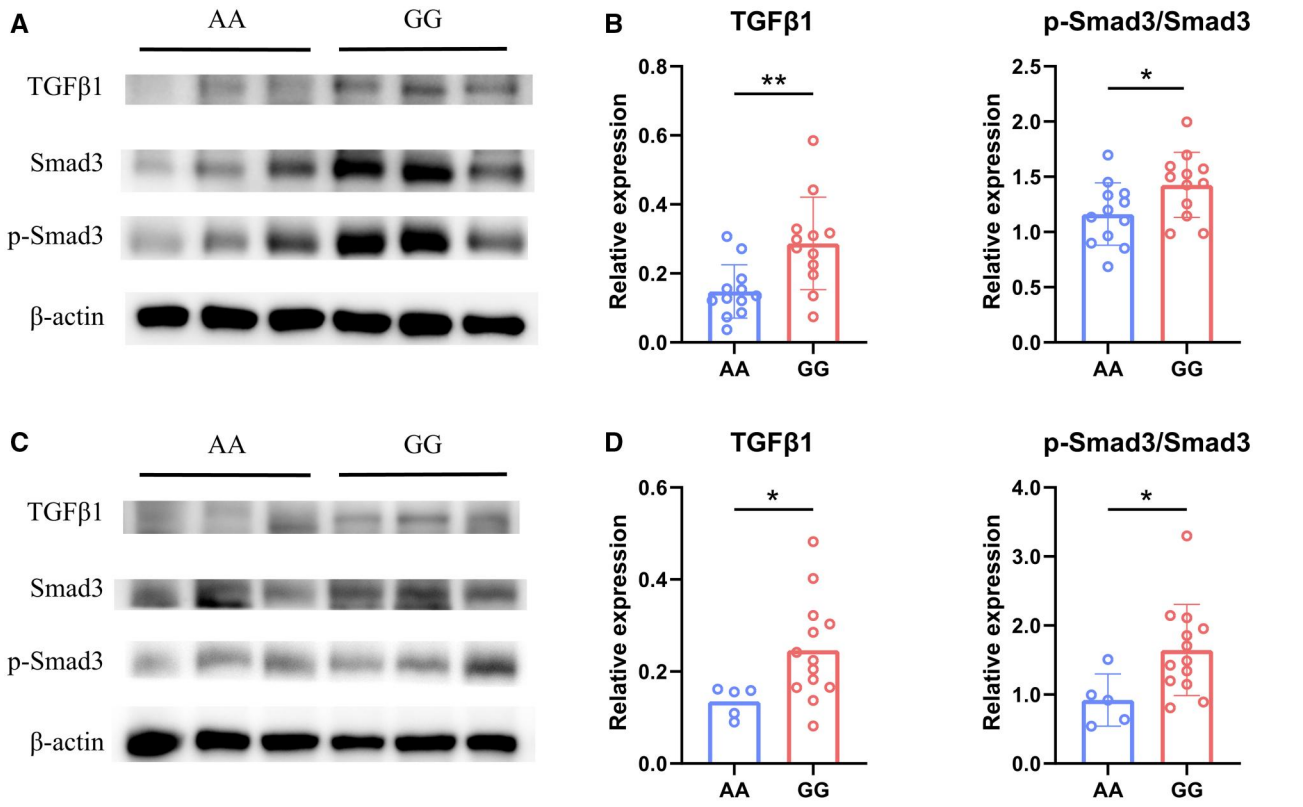

Figure 4. *TNFSF15* genotype GG activates TGFβ1/smad3 signal pathway in non-diseased ileac tissues. (**A**, **B**) Western blot analysis of TGFβ1, p-smad3/smad3 in endoscopic biopsy tissues (AA, $n = 12$; GG, $n = 12$). (**C**, **D**) Western blot analysis of TGFβ1, p-smad3/smad3 in surgical tissues (AA, $n = 5$; GG, $n = 13$). Compared with the AA group: *$P < 0.05$, **$P < 0.01$. TIMP1 = tissue inhibitors of metalloproteinase 1, MMP3 = metalloproteinase 3, TGFβ1 = transforming growth factor β1, NS = non-significant.

azathioprine and anti-TNF-α, as well as monotherapy with either, showing promise in delaying disease progression before behavioral modifications are implemented [5, 29, 30]. Our study identified the GG genotype as a potential independent predictor of stricturing CD in patients treated with biological agents, indicating a potential decrease in sensitivity to these medications. Biological agents exert their effects by precisely targeting and inhibiting essential inflammatory cytokines or cell surface molecules. However, the GG genotype appears to enhance the expression of TL1A, which in turn upregulates the expression of inflammatory factors like TNF-α and interleukin-12 (IL-12) [31]. Consequently, the GG genotype may undermine the targeted therapeutic potential of biological agents in these patients. Conversely, in patients not receiving biological agents, the GG genotype did not independently predict stricturing CD. This could be due to rapid progression from B1 to B2 in untreated CD patients, overshadowing the role of the *TNFSF15* genotype. Therefore, biological agents may be a more appropriate treatment option for patients with AA + AG genotype, while patients with GG genotype may require alternative therapeutic approaches at an early stage, such as the use of novel anti-TL1A medications and the combination of several treatments.

The most important finding of this study is the association between *TNFSF15* genotypes and TL1A expression in non-diseased ileal regions, along with other protein expressions linked to fibrosis. It means that even though there was no visible inflammation or fibrosis, patients in the GG group may have altered some fibrosis-related protein expressions prior to the onset of stricturing, which may lessen their sensitivity to biological agents and ultimately cause fibrotic stricturing and more severe disease progression than patients in the AA + AG group. Previous research has shown correlations between the TL1A expression in monocytes and macrophages and the *TNFSF15* genotype [32, 33]. However, the association between *TNFSF15* genotype and the expression of TL1A and other fibrosis-associated proteins in non-diseased regions has not been reported. In general, many studies have been performed on the role of TL1A in CD development. First, it was established that individuals with CD had elevated TL1A expression in their inflamed guts, primarily in mononuclear macrophages, cluster of differentiation 4 positive (CD4+), and cluster of differentiation 8 positive (CD8+) T cells [24]. Second, in murine models of TL1A overexpression, the colon may fibrose spontaneously and may worsen into ileum and colon fibrosis following dextran sulfate sodium (DSS) administration [34, 35]. In CD patients, many SNPs of *TNFSF15* have been linked to the development of the disease and clinical characteristics. The development of CD to stricturing or penetrating lesions was substantially associated with the SNPs of *TNFSF15*, rs4263839 and rs6478108, whereas the occurrence of anal fistula was associated with rs4574921 [7, 13]. Our findings, in conjunction with existing data, strongly suggest that the *TNFSF15* genotype is involved in CD fibrosis and stricturing.

Based on the significant role of TL1A in the development of intestinal fibrosis and stricturing, it is plausible that an anti-TL1A approach may prove to be efficacious in mitigating inflammation and fibrotic stricturing progression in the treatment of CD. Prior research has demonstrated that the use of anti-TL1A antibodies can reduce intestinal inflammation and fibrosis in murine models of colitis [36]. Additionally, a clinical trial involving patients with ulcerative colitis revealed that treatment with an anti-TL1A monoclonal antibody (PF-06480605) effectively alleviated intestinal inflammation and reduced the abundance of Th17 cells, monocytes, dendritic

cells, macrophages, and fibroblasts in the gastrointestinal tissues [15, 16]. Although current clinical trials investigating the use of anti-TL1A antibody treatment in patients with CD have not produced conclusive findings, available data indicates potential benefits of this therapy for individuals with CD, particularly those who are homozygous for the *TNFSF15* risk allele.

The limitations of this study include its retrospective design, the absence of WB results for patients with the AG genotype, and the lack of animal experiments to thoroughly verify the mechanisms underlying the impact of *TNFSF15* genotype on intestinal fibrosis and stricturing. In this study, the follow-up duration was significantly longer for patients without disease progression compared with those with progression, which could aid in identifying the progression of CD. However, despite the Kaplan–Meier analysis and immunohistochemistry results indicating a similar role between AA and AG, we did not assess protein levels by WB in patients with the AG genotype. Therefore, additional prospective cohort studies and animal experiments are necessary to confirm the observed relationship and clarify the mechanisms between *TNFSF15* genotypes and stricturing.

In conclusion, our study indicates an SNP (rs6478109) within the *TNFSF15* gene could potentially serve as a predictive marker for stricturing CD. Individuals who possess a homozygous genotype for the risk allele of *TNFSF15* may exhibit increased levels of TL1A and pro-fibrotic proteins in the non-diseased ileal region, potentially accelerating disease progression. Early detection of the *TNFSF15* genotype may facilitate the prediction of disease progression risk and the implementation of optimal treatment strategies for CD patients.

Supplementary Data

Supplementary data is available at *Gastroenterology Report* online.

Authors' Contributions

Q.Z., W.W., and M.Zhi conceptualized the project and designed all experiments. Q.Z. conducted experiments and drafted the manuscript. Q.Z., W.W., B.X., and J.L. collected clinical characteristics. Q.Z., W.W., D.L., J.Z., T.L., and J.D. collected clinical samples. Q.Z. and W.W. analyzed data and organized figures. Q.Z., W.W., and J.H. did the statistical analyses and data interpretation. W.W., M.Zhang, and M.Zhi supervised the project and contributed to revising the manuscript. All authors read and approved the final version of the manuscript.

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Conflicts of Interest

The authors declare that there are no conflicts of interest in this study.

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