Transcribed ultraconserved region (T-UCR) uc.261 expression is closely correlated with disease activity and intestinal permeability in Crohn's disease

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Xiao-Xian Qian*, Chen-Wen Cai*, Han-Yang Li, Li-Jie Lai, Dong-Juan Song, Yu-Qi Qiao, Jun Shen and Zhi-Hua Ran

Correspondence to:
Xiao-Xian Qian
State Key Laboratory for Oncogenes and Related Genes, Key Laboratory of Gastroenterology and Hepatology, Ministry of Health, Division of Gastroenterology and Hepatology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai Cancer Institute, Shanghai Institute of Digestive Disease, Shanghai Inflammatory Bowel Disease Research Center, 160 Pu Jian Ave, Shanghai 200127, China.

Minhang Hospital, Fudan University, Institute of Fudan-Minhang Academic Health System, Minhang Hospital, Fudan University, 170 Xin Song Rd, Shanghai 201199, China
qxx1011@qq.com

Jun Shen
State Key Laboratory for Oncogenes and Related Genes, Key Laboratory of Gastroenterology and Hepatology, Ministry of Health, Division of Gastroenterology and Hepatology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai Cancer Institute, Shanghai Institute of Digestive Disease, Shanghai Inflammatory Bowel Disease Research Center, Shanghai, 160 Pu Jian Ave, Shanghai 200127, China.
rjyysj@163.com

Zhi-Hua Ran
State Key Laboratory for Oncogenes and Related Genes, Key Laboratory of Gastroenterology and Hepatology, Ministry of Health, Division of Gastroenterology and Hepatology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai Cancer Institute, Shanghai Institute of

Abstract

Objectives: Transcribed ultraconserved region (T-UCR) uc.261 is reported to participate in intestinal mucosa barrier damage in Crohn's disease (CD). The aim of this study was to determine the association with disease activity and intestinal permeability.

Methods: Uc.261 level in colon mucosa and Harvey-Bradshaw Index (HBI) were evaluated in 20 active CD patients. Uc.261 expression and transepithelial electrical resistance (TEER) were determined in Caco2 and T84 cells treated with tumor necrosis factor alpha (TNF-α), respectively. Body weight, disease activity index (DAI), colon length, histological index (HI), intestinal permeability to FITC-dextran, uc.261, and tight junction proteins (TJPs) levels were evaluated in BALB/C mice treated with saline enema, trinitrobenzene sulfonic acid (TNBS)/ethanol enema, and anti-TNF-α monoclonal antibody injection, respectively.

Results: Uc.261 expression was overexpressed in CD patients, TNF-α treated cells, and colitis mice. Uc.261 expression was positively correlated with HBI ($r = 0.582$, $p = 0.007$) in CD patients, and positively correlated with TNF-α concentration and negatively correlated TEER in Caco2 and T84 cells (all $p < 0.05$). Furthermore, uc.261 was positively correlated with DAI ($r = 0.824$, $p = 0.008$), HI ($r = 0.672$, $p = 0.021$), and intestinal permeability ($r = 0.636$, $p = 0.012$), while negatively correlated with body weight ($r = –0.574$, $p = 0.035$), colon length ($r = –0.866$, $p = 0.017$), and TJP expression (all $p < 0.05$) in colitis mice.

Conclusions: Uc.261 expression was closely correlated with disease activity and intestinal permeability in CD. Anti-TNF-α treatment may play its role through suppressing uc.261 expression in colitis mice.

Keywords: Crohn's disease, disease activity, intestinal permeability, T-UCR, uc.261

Introduction

In the human genome, 481 ultraconserved regions (UCRs) have been discovered that are highly conserved in the human, rat, and mouse genomes.[1] Most UCRs can be transcribed to RNAs, and those UCRs are called transcribed ultraconserved regions (T-UCRs).[2] In our previous report, we investigated T-UCR expression profiles in colon mucosa epithelial cells in active Crohn's disease (CD) patients with the Arraystar Human T-UCR Microarray, and we found eight T-UCRs with altered expression in CD patients.[3] By further experiments in Caco2 and T84 monolayer cell models, we demonstrated that uc.261, a specific T-UCR in colon mucosa epithelial cells, participated in intestinal mucosa barrier damage by changing the activity of Cdc42 and PKCζ, and was highly expressed in active CD patients.

Some indices have been developed to evaluate the activity and severity of CD, such as Crohn's Disease Activity Index (CDAI),[4] simplified

Digestive Disease, Shanghai Inflammatory Bowel Disease Research Center, Shanghai, 160 Pu Jian Ave, Shanghai 200127, China.
rjyyrzh@163.com

Xiao-Xian Qian
State Key Laboratory for Oncogenes and Related Genes, Key Laboratory of Gastroenterology and Hepatology, Ministry of Health, Division of Gastroenterology and Hepatology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai Cancer Institute, Shanghai Institute of Digestive Disease, Shanghai Inflammatory Bowel Disease Research Center, Shanghai, Shanghai, China

Minhang Hospital, Fudan University, Shanghai, China

Institute of Fudan-Minhang Academic Health System, Minhang Hospital, Fudan University, Shanghai, China

Chen-Wen Cai
Han-Yang Li
Li-Jie Lai
Dong-Juan Song
Yu-Qi Qiao
State Key Laboratory for Oncogenes and Related Genes, Key Laboratory of Gastroenterology & Hepatology, Ministry of Health, Division of Gastroenterology and Hepatology, Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai Cancer Institute, Shanghai Institute of Digestive Disease, Shanghai Inflammatory Bowel Disease Research Center, Shanghai, Shanghai, China

*Xiao-Xian Qian and Chen-Wen Cai had equal contribution to this article.

endoscopic score for CD (SES-CD),[5] capsule endoscopy scoring index (Lewis score),[6] and some serological parameters such as C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR).[7,8] To date, the simplified CDAI, or Harvey-Bradshaw Index (HBI) is still the tool used most comprehensively to evaluate the activity and severity of CD and to monitor the effect of treatment.[9] Although we have discovered that uc.261 expression was elevated in active CD patients,[3] we aimed to further investigate the correlation of uc.261 expression with disease activity of CD.

The colonic mucosa forms an intrinsic barrier against the colonic luminal environment.[10] Tight junction proteins (TJPs) are components of the mucosa barrier, which comprise transmembrane proteins, including junctional adhesion molecules (JAMs), occludin, and claudins, as well as cytoplasmic proteins such as zonula occludens (ZOs).[11] In CD patients, a subtype of inflammatory bowel disease (IBD), the intestinal mucosa barrier is segmentally impaired.[12,13] A two-fold increase in intestinal permeability was discovered in patients with CD and their relatives, indicating that permeability changes may occur even before the onset of intestinal inflammation. Therefore, it was proposed that the primary intestinal defect may be a genetic predisposition to CD, making it another etiologic factor in CD.[14] A significant correlation was also found between the value of the intestinal permeability index and the probability of relapse of CD, showing that increasing in intestinal permeability precedes clinical relapses in CD, and, consequently, can be considered as an indicator of subclinical disease.[14] One study using the lactulose/mannitol (L/M) test also supported the view that increased intestinal permeability was a predictor of clinical relapse in CD.[15] In our previous study, we found that overexpression of uc.261 caused damage to permeability of the intestinal mucosa in human CD.[3] Our previous study also connected uc.261 with tight junction in CD. Interestingly, we also found that tumor necrosis factor alpha (TNF-α), a therapeutic keypoint, could damage tight junction of intestinal mucosa through uc.261.[3] Thus, in this study, we also aimed to verify the correlation of uc.261 expression with intestinal permeability of CD.

Methods

Subjects and tissue specimens

A total of 20, newly diagnosed, active CD patients with colonic involvement were enrolled in this study from October 2014 to July 2015 in Renji Hospital, School of Medicine, Shanghai Jiao Tong University. The diagnosis of CD was made according to the Chinese consensus on diagnosis and management of IBD (Guangzhou).[16] Colonic pinch biopsies from inflamed sites were obtained during colonoscopy. The activity assessment of CD was based on the HBI,[17] and a score $\geqslant$5 indicated clinically active disease. The baseline characteristics of all CD patients recruited are listed in Supplemental Digital Content 1. This study was approved by the ethics committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University, with a formal approval document (approval date 12 March 2014). All subjects provided written informed consent for taking and analyzing their biopsy samples and the publication of their information.

Polymerase chain reaction

Polymerase chain reaction (PCR) assay was performed to explore RNA expression of uc.261 and TJPs in human colon, Caco2, and T84 cells, and in mouse colon. The steps were as described in our previous report.[3] For more details of PCR, see Supplemental Digital Content 1. Primers for PCR are listed in Table S1 in Supplemental Digital Content 2.

Cell culture, TNF-α stimulation, and transepithelial electrical resistance assays

Caco2 and T84 cell can form monolayers in plates, and are used widely as colon epithelium models to mimic the epithelium of the human colon mucosa.[3] Caco2 and T84 cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). For more details of cell culture, see Supplemental Digital Content 1.

Following establishment of monolayer of Caco2 and T84 cells at about 12 days after seeding, TNF-α (Sigma, St Louis, MO, USA) was added to the medium at three different concentrations (5 ng/ml, 10 ng/ml, and 20 ng/ml) to mimic different disease

activity in CD. Another group of cells had double distilled water added as a control. After culturing with TNF-α for 24 h, cells in six-well plates were harvested to determine the RNA expression of uc.261 by PCR.

Transepithelial electrical resistance assays (TEER) values of Caco2 and T84 cells in Transwell plates were determined with Millicell ERS-2 (Millipore, Billerica, MA, USA), in units of $\Omega \cdot cm^2$. For more details of TEER assays, see Supplemental Digital Content 1.

Mice

A total of 70 female BALB/C mice (18–23 g) were obtained from Shanghai Slike Experimental Animal Company Ltd. (Shanghai, China) and were kept in the animal facility of Shanghai Mei-Xuan Biological Technology Company (Shanghai, China) under specific pathogen-free conditions with a 12-h light/dark cycle for 1 week before use. All mice were 6–8 weeks of age, and had free access to standard mouse chow and water. All animal experiments were approved by the Renji Hospital Ethical Committee for Animal Welfare on animal care and experimentation (approval date: 12 March 2014). Mice were divided randomly into three groups and treated with either saline enema (normal control group, NC group, 20 mice), TNBS (Sigma-Aldrich, St Louis, MO, USA)/ethanol enema (TNBS group, 25 mice), and anti-TNF-α mAb (BIOSS, Woburn, Massachusetts, USA) injection 7 days and 10 days after TNBS/ethanol enema (anti-TNF group, 25 mice).

Induction of colitis and anti-TNF-α mAb treatment in mice

Colitis in mice was induced according to a standard protocol,[18] with slight modifications (Figure 1a). For more details of induction of colitis, see Supplemental Digital Content 1.

On days 8 and 11, anti-TNF-α mAb was administered twice by intraperitoneal injection with a dosage of 5 mg/kg to mice in the anti-TNF group, while saline was injected to mice in the NC and TNBS groups. On days 1, 4, 7, 9, 12, and 15, three mice in each group were sacrificed (Figure 1a). Their colons were removed from the colon–cecal junction to the anus and colon length was measured. The colons were then cut open longitudinally, cleaned of feces, subjected to macroscopic evaluation, and processed for RNA or protein isolation and histology.

Disease activity index determination

Disease activity index (DAI) was used to evaluate the grade and extent of intestinal inflammation according an established index system (Table S2 in Supplemental Digital Content 2).[19] The scores ranged from 0 to 4, including three parameters: weight loss, stool consistency, and rectal bleeding.

Histological evaluation

Stained sections of mouse colon were examined for evidence of colitis using an established histological colitis scoring system (Table S3 Supplemental Digital Content 2).[19] For more details of induction of histological evaluation, see Supplemental Digital Content 1.

Intestinal permeability

The fluorescein isothiocyanate (FITC)-Dextran 4000 (Sigma-Aldrich, St. Louis, MO, USA) test was employed to evaluate the colonic permeability in mice, as described by Volynets and colleagues and Williams and colleagues.[20,21] For more details of induction of intestinal permeability, see Supplemental Digital Content 1.

Western blotting

Conventional western blotting (WB) was performed to investigate the protein expression of TJPs, including JAM-A, occludin, Claudin-1, and Zo-1 in mouse colon mucosa. Pictures of films were analyzed with ImageJ 1.45 software (National Institutes of Health, Bethesda, MD, USA). The antibodies used are listed in Table S4 in Supplemental Digital Content 2.

Statistical analysis

The results are expressed as means ± SEM. Statistical analysis was performed using the Wilcoxon matched paired test, and $p < 0.05$ was taken as significant. Pearson correlation was used to test correlation between two parameters. All statistical analyses were performed using SPSS 15.0 software (SPSS, Chicago, IL, USA).

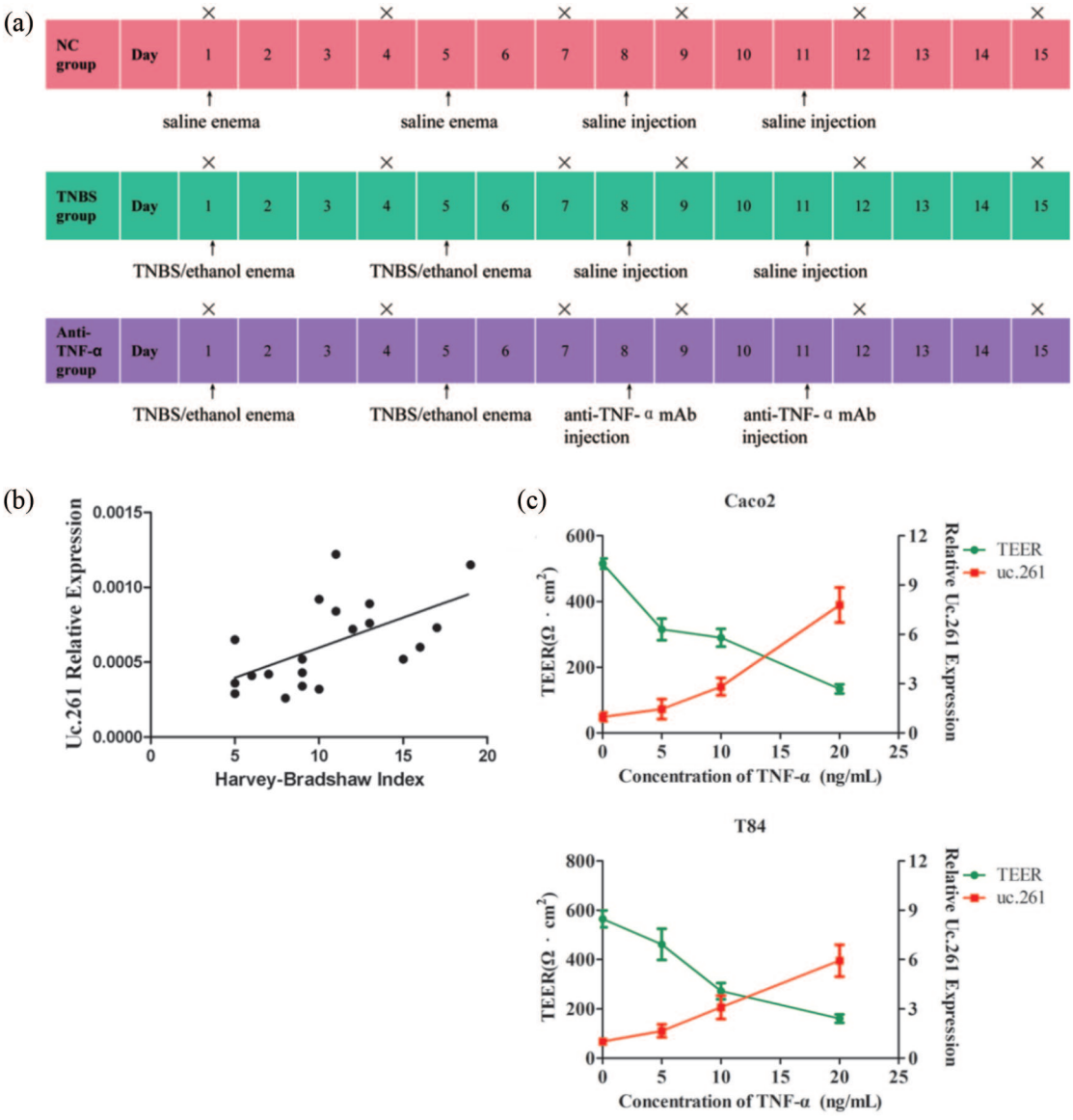

Figure 1. (a) Procedure of experiments. Colitis in mice was induced by twice TNBS/ethanol enema. ×, sacrifice. (b) Correlation of uc.261 expression in colon mucosa with HBI in 20 active CD patients. A positive correlation was found between uc.261 expression and HBI. (c) Changes in TEER values and uc.261 expression in Caco2 and T84 monolayer cells. TNF-α concentration was negatively correlated with TEER values, and positively correlated with uc.261 expression. A negative correlation was found between uc.261 expression and TEER values.

CD, Crohn's disease; HBI, Harvey-Bradshaw Index; mAb, monoclonal antibody; TEER, transepithelial electrical resistance assays; TNBS, trinitrobenzene sulfonic acid; TNF-α, tumor necrosis factor alpha.

Results

Uc.261 expression was positively correlated with HBI in CD patients

A total of 9 male and 11 female active CD patients were enrolled in this study, ranging from 15 to 39 years, with a mean age of 28.1 ± 7.5 years. The HBI of the active CD patients ranged from 5 to 19, with a mean score of 10.5 ± 4.8. Pearson correlation analysis showed a positive correlation between uc.261 expression and HBI ($r = 0.582$, $p = 0.007$) in active CD patients (Figure 1b). This result indicated that uc.261 expression may be a potential predictor of CD activity.

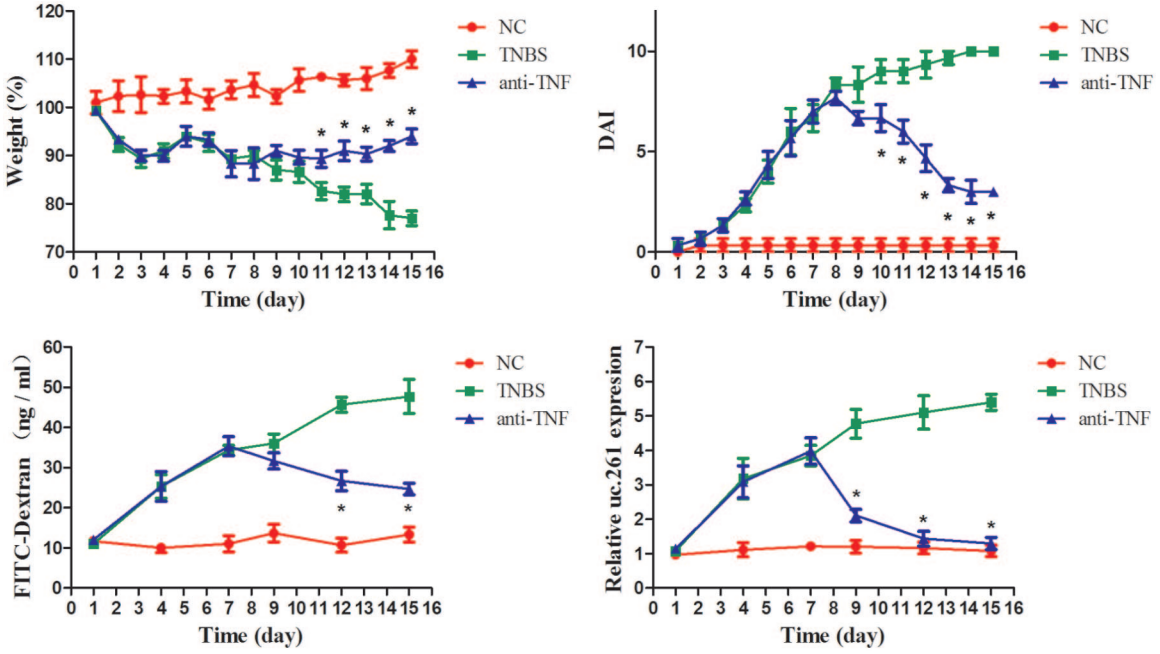

Figure 2. Changes in body weight, DAI, intestinal colon permeability to FITC-dextran, and colon uc.261 expression in mice. Mice in the NC group gained weight slightly. Mice in the TNBS and anti-TNF groups decreased body weight, especially after the second TNBS/ethanol enema. Mice in the anti-TNF group partly recovered their weight after anti-TNF mAb injections. Mice in the NC group had a DAI of zero. Mice in the TNBS and anti-TNF groups had elevated DAI. Mice in the anti-TNF group showed decreased DAI after anti-TNF mAb injections. Mice in the TNBS and anti-TNF groups showed greatly increased intestinal permeability. Mice in the anti-TNF group showed decreased intestinal permeability after anti-TNF mAb injections. Mice in the TNBS and anti-TNF groups showed greatly elevated uc.261 expression. Mice in the anti-TNF group showed decreased uc.261 expression after anti-TNF mAb injections. *$p < 0.05$, comparing the TNBS group with anti-TNF group.
DAI, disease activity index; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; NC, normal control; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor.

Uc.261 expression is positively correlated with TNF-α concentration and negatively correlated with TEER in cells

TEER values of the monolayers formed by Caco2 and T84 cells dropped significantly after TNF-α stimulation for 24 h (Figure 1c). A negative correlation was found between TNF-α concentration and TEER values in Caco2 and T84 cells ($r = –0.943$, $p = 0.000$; $r = –0.949$, $p = 0.000$). Uc.261 RNA expression rose significantly after TNF-α stimulation for 24 h. A positive correlation was found between TNF-α concentration and uc.261 expression in Caco2 and T84 cells ($r = 0.946$, $p = 0.000$; $r = 0.956$, $p = 0.000$). When studying the relationship of uc.261 expression with TEER values, a negative correlation was found between these values in Caco2 cells and T84 cells ($r = –0.846$, $p = 0.000$; $r = –0.915$, $p = 0.000$). These results indicated that uc.261 expression was closely correlated with the severity of inflammation and permeability of monolayer cells.

Uc.261 expression was overexpressed in TNBS-induced colitis mice

Colitis mice models were built successfully using TNBS/ethanol enema in the TNBS and anti-TNF groups; eight mice died, six in the TNBS group and two in the anti-TNF group. Mice in NC group showed slight increase of body weight, no changes in DAI, length of colon, HI, intestinal permeability to FITC-Dextran, uc.261 and TJPs expression in colon mucosa. However, from day 2, mice in TNBS group showed significant decrease of body weight, colon length, and TJPs expression in colon mucosa, and significant increase of uc.261 expression, DAI, HI, and intestinal permeability to FITC-Dextran (Figures 2–6). Pearson correlation analysis showed a negative correlation between uc.261 expression and body weight ($r = –0.574$, $p = 0.035$), length of colon ($r = –0.866$, $p = 0.017$), TJPs RNA expression (JAM-A: $r = –0.754$, occludin: $r = –0.873$, Claudin-1: $r = –0.935$, ZO-1: $r = –0.899$; all

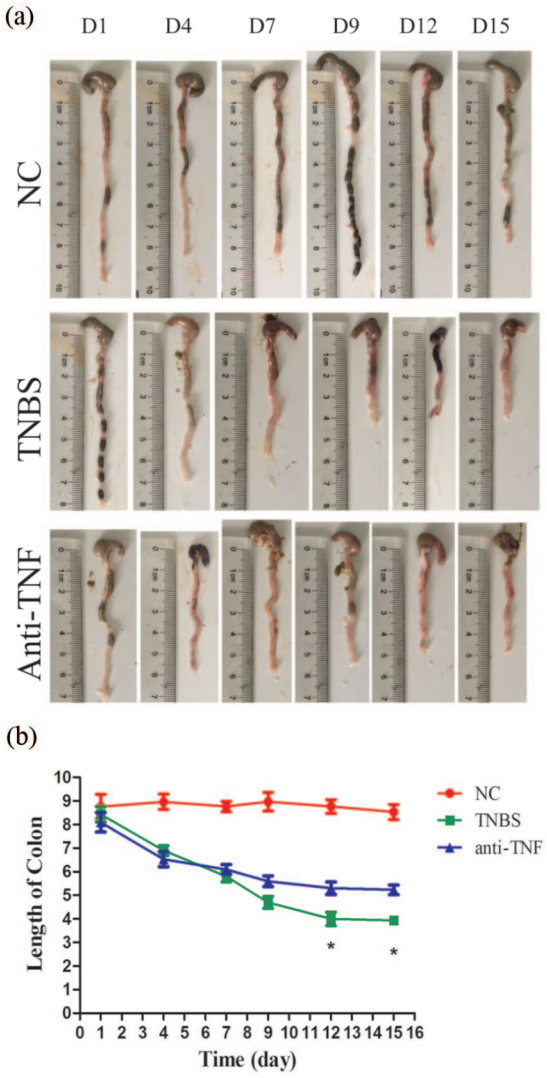

Figure 3. Changes of colon length in mice. (a) Pictures of resected colons of mice. The bulging end of each colon is the mouse cecum. (b) Changes in colon length. Mice in the NC group had constant colon length. Mice in the TNBS and anti-TNF groups showed greatly decreased colon length. Mice in the anti-TNF group showed less decrease in colon length after anti-TNF mAb injections compared with the TNBS group, but colon length did not increase. *$p < 0.05$, comparing the TNBS group with the anti-TNF group.
mAb, monoclonal antibody; NC, normal control; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor.

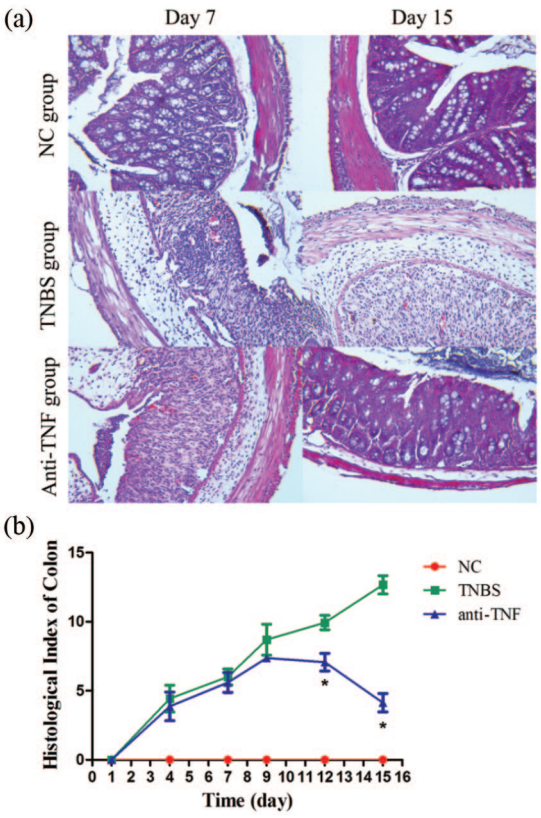

Figure 4. Histological changes in murine colon mucosa. (a) Pictures of colon sections stained with hematoxylin and eosin. (b) Changes in HI. Mice in the NC group had no inflammation in colon mucosa and a HI of zero. Mice in the TNBS and anti-TNF groups showed severely impaired structure of mucosa, abundant infiltration of inflammatory cells, and greatly elevated HI. Mice in the anti-TNF group partly recovered the normal structure of mucosa, had less infiltration of inflammatory cells, and had decreased HI after anti-TNF mAb injections compared with the TNBS group. *$p < 0.05$, comparing the TNBS group with the anti-TNF group.
HI, histological index; mAb, monoclonal antibody; NC, normal control; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor.

$p < 0.05$), and protein expression (JAM-A: $r = -0.796$, occludin: $r = -0.975$, Claudin-1: $r = -0.854$, ZO-1: $r = -0.932$; all $p < 0.05$); a positive correlation between uc.261 expression and with DAI ($r = 0.824$, $p = 0.008$), HI ($r = 0.672$, $p = 0.021$), and intestinal permeability to FITC-Dextran ($r = 0.636$, $p = 0.012$). These results suggested that uc.261 expression was overexpressed in TNBS-induced colitis mice, and it was closely correlated with disease activity and intestinal permeability.

Anti-TNF treatment suppresses uc.261 expression in TNBS-induced colitis mice

Mice in the anti-TNF group were administered anti-TNF-α mAb by intraperitoneal injection twice, on day 8 and day 11, with a dosage of

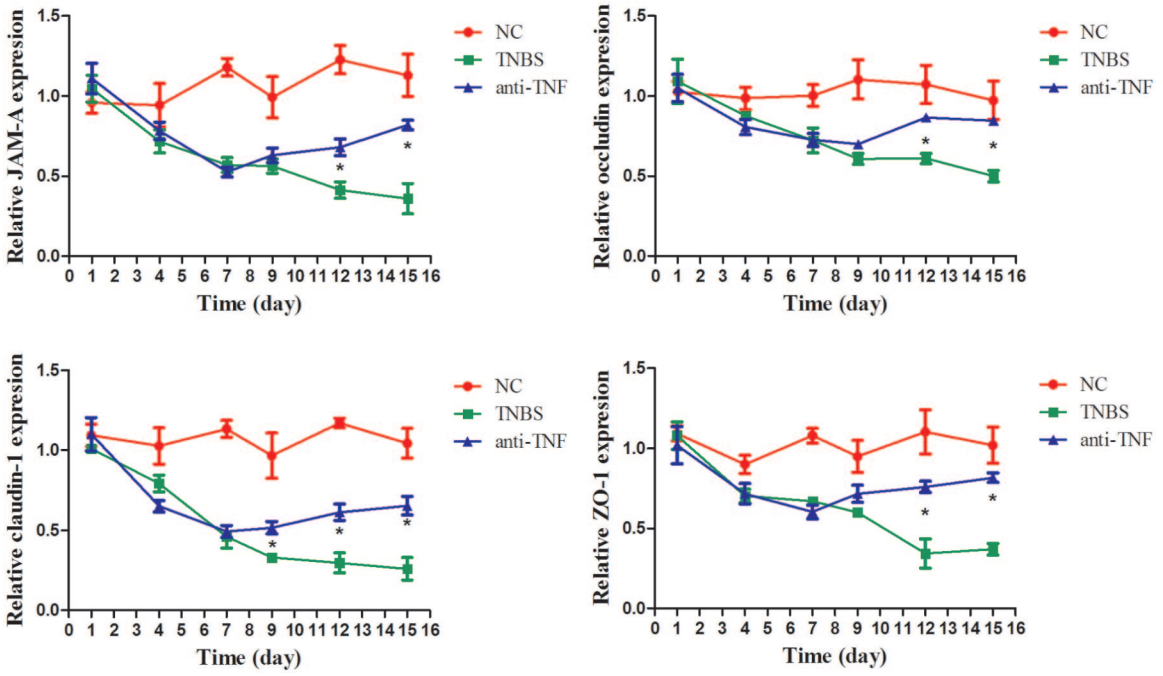

Figure 5. Changes in mRNA levels of TJPs in murine colon mucosa. Mice in the NC group had high and constant mRNA expression of TJPs. Mice in the TNBS and anti-TNF groups showed greatly decreased mRNA expression of TJPs. Mice in the anti-TNF group showed elevated mRNA expression of TJPs after anti-TNF mAb injections. *$p < 0.05$, comparing the TNBS group with the anti-TNF group.
mAb, monoclonal antibody; NC, normal control; TJP, tight junction protein; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor.

5 mg/kg. Loss of body weight slowed down after the first injection, and mice began to gain weight after the second injection. Besides, we observed that uc.261 expression, DAI, HI, and intestinal permeability to FITC-Dextran decreased after injections, while TJP mRNA/protein expression increased and length of colon decreased less after injections. However, anti-TNF treatment did not restore the health of mice, and the data collected still differed from that of NC mice. Thus, anti-TNF treatment could partly reverse the changes seen in TNBS-induced colitis mice, and anti-TNF-α treatment may play its role by suppressing uc.261 expression.

Discussion

Inspired by our previous study,[3] we investigated more characteristics of T-UCR uc.261 and its potential correlations with clinical and pathological parameters. This is the first study to demonstrate the association of uc.261 RNA expression with disease activity and intestinal permeability in human CD and mice colitis models.

HBI is the tool most widely used to evaluate the activity and severity of CD.[3] HBI includes five items: general situation, abdominal pain, abdominal mass, diarrhea, and associated conditions. In this study, Pearson correlation analysis showed a positive correlation between uc.261 expression and HBI in active CD patients. It indicated that uc.261 expression can be used as a potential biomarker of the activity of CD.

Hollander and colleagues discovered that permeability changes may occur even before the onset of CD.[14] Another observation suggested that increased intestinal permeability is an early step in the pathogenesis of CD.[22] Intestinal permeability in healthy population and IBD can be measured by the sugar absorption test.[23] Two newly published studies employed different methods to measure intestinal permeability by iohexol test or endoscopic confocal laser endomicroscopy.[24,25] Increased intestinal permeability is a new target for disease prevention and therapy of CD in humans.[26] On the other hand, TEER values of monolayer cells can directly reflect permeability

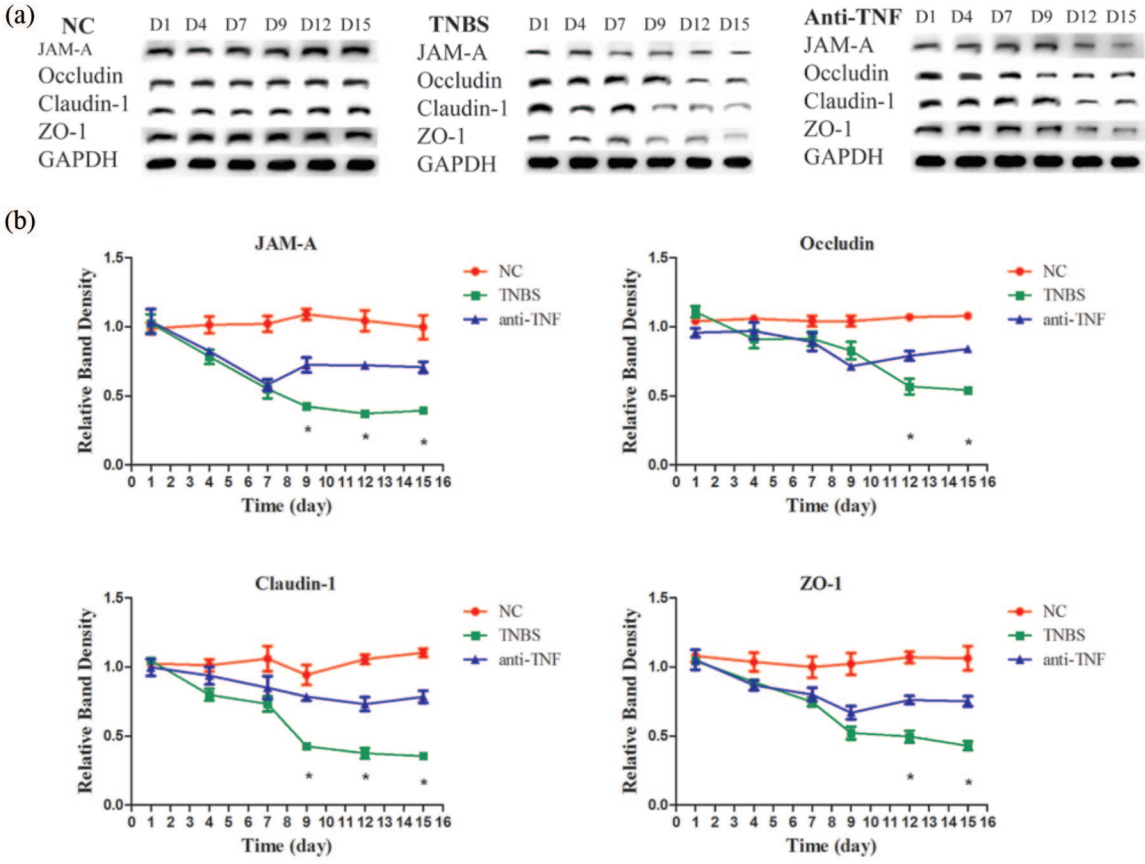

Figure 6. Changes in protein levels of TJPs in murine colon mucosa. (a) Pictures of protein electrophoresis bands. (b) Relative band densities. Mice in the NC group had high and constant protein expression of TJPs. Mice in the TNBS and anti-TNF groups showed greatly decreased protein expression of TJPs. Mice in the anti-TNF group showed elevated protein expression of TJPs after anti-TNF mAb injections. *$p < 0.05$, comparing the TNBS group with the anti-TNF group.
mAb, monoclonal antibody; NC, normal control; TJP, tight junction protein; TNBS, trinitrobenzene sulfonic acid; TNF, tumor necrosis factor.

of the epithelium, and a decrease in TEER value often indicates damage to the epithelium.[27] In this study, we added TNF-α to the medium of Caco2 and T84 cells, and determined the RNA expression of uc.261 and their TEER values. Results showed that uc.261 expression had not only a positive correlation with the concentration of TNF-α, but also a negative correlation with TEER values. These findings suggest that, in Caco2 and T84 cells, uc.261 expression is closely correlated with the severity of inflammation and permeability of monolayers. These findings in cells were consistent with our previous findings in human CD,[3] suggesting that the relationship of uc.261 expression with disease activity and intestinal permeability is a common phenomenon both *in vivo* and *in vitro*.

TNBS-induced colitis mice is often used to simulate pathological changes in human CD. In other studies,[28] changes in several parameters have often been investigated in TNBS-induced colitis mice, including body weight, DAI, length of colon, HI of colon, intestinal permeability to FITC-Dextran, and TJP mRNA/protein expression. Body weight, DAI, length of colon, and HI of colon can reflect disease activity. Intestinal permeability to FITC-Dextran is a direct measurement to study colonic permeability.[20] TJPs are important components of the mucosal barrier. Impaired mucosal barrier has lower expression of TJPs. The results of our study were similar to those previous studies. TNBS-induced colitis mice showed decreased body weight, length of colon, and TJP mRNA/protein expression, while

their DAI, HI of colon, and intestinal permeability to FITC-Dextran greatly increased.[21] In addition, we studied the expression of uc.261 in colon mucosa and found that TNBS-induced colitis mice overexpressed uc.261. Furthermore, we studied the association of uc.261 expression with other parameters, and found that uc.261 had a negative correlation with body weight, length of colon, and TJP mRNA/protein expression, and a positive correlation with DAI, HI, and intestinal permeability to FITC-Dextran. These findings indicated that uc.261 was closely associated with disease activity and intestinal permeability in TNBS-induced colitis mice. Combined with our previous findings in Caco2 and T84 cells,[3] we suggested that overexpressed uc.261 may participate in some signal pathways associated with inflammatory reactions and damage to epithelial integrity in colitis mice.

Pro-inflammatory cytokines play roles in the pathogenesis of human CD and TNBS-induced colitis mice. TNF, IFN-γ, and IL-18 mRNAs in colon mucosa were unregulated in TNBS colitis, and anti-TNF therapy significantly reduced the levels of TNF and IL-18.[28] Anti-IL-18 mAb was shown to result in a dramatic attenuation of TNBS colitis,[29] and anti-TNF therapy was also shown to significantly reduce IL-18.[28] Pentoxifylline showed therapeutic effects in human IBD and an experimental modal of colitis,[30,31] by inhibiting the synthesis and IFN-γ-inducing activity of IL-18.[32] Moreover, anti-TNF therapy can decrease cell infiltration in the bowel after TNBS application by inducing apoptosis in lamina propria macrophages.[28] In our previous study,[3] we found that TNF-α treated Caco2 and T84 monolayer cells showed upregulated expression of uc.261. Downregulation of uc.261 expression in cells also decreased trans-monolayer permeability, and unregulated both mRNA expression of TJPs and their assembly to the cell membrane. In this study, we applied anti-TNF-α mAb to treat TNBS-induced mice colitis and found similar results in mice to those in cell modals. Anti-TNF treated mice were shown to exhibit rapid recoveries of body weight, colon length, and TJP mRNA/protein expression, and a gradual decrease in disease activity, mucosal inflammation, intestinal permeability, and, most importantly, downregulated uc.261 expression. Therefore, we propose that anti-TNF treatment can partly reverse the inflammation seen in TNBS-induced colitis mice, and that anti-TNF-α treatment may play its role by suppressing uc.261 expression.

There are several limitations to this study. First, we studied only the correlation of colon mucosal uc.261 expression with disease activity in active CD patients. Its correlation with disease activity in inactive CD patients has not yet been studied. Second, we were unable to use the sugar absorption test to study the correlation of uc.216 expression with intestinal permeability because of the low willingness of patients to take this test. Third, we administered anti-TNF mAb to treat TNBS-induced colitis mice and found downregulation of uc.261. It remains unclear that whether the downregulation of uc.261 had a direct therapeutic effect on colitis mice. Further studies are needed.

In conclusion, uc.261 expression was closely correlated with disease activity and intestinal permeability in human CD, colon epithelium modal cells, and TNBS-induced colitis mice. Anti-TNF-α treatment may play its role by suppressing uc.261 expression in colitis mice. Downregulation of uc.261 may be a promising choice of therapy for CD in the future.

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Xiao-Xian Qian and Chen-Wen Cai are co-first authors.

Specific author contributions

X-XQ performed most of the experiments and wrote the manuscript. CWC help write and revise the manuscript. H-YL, L-JL and D-JS performed the statistical analysis. JS and Z-HR critically revised with manuscript. X-XQ, Y-QQ, JS, and Z-HR obtained the funding. All authors contributed to data interpretation and revision of the manuscript and approved the final manuscript.

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the study design, the collection, analysis, and interpretation of data, or the writing of the article and the decision to submit it for publication.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Guarantor of the article

Jun Shen

ORCID iD

Jun Shen https://orcid.org/0000-0001-7206-1847

Supplemental material

Supplemental material for this article is available online.

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