

Original Article

Implications of Notch signaling in anastomotic recurrence of patients with Crohn's disease

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Abstract: Crohn's disease (CD) is frequently recurred in the site of the anastomosis after bowel resection. However, the molecular mechanisms underlying this phenomenon are poorly investigated. Notch signaling plays an important role in maintaining intestine epithelial homeostasis and barrier function. In this study, we assessed whether Notch signaling is involved in the pathological processes of anastomosis. By an integrated informatics analysis, we found Notch-1 expression was dramatically increased in the CD tissues compared with disease-unaffected ileum. And this alternation was confirmed by real-time PCR and western blotting, respectively. Furthermore, we demonstrated that activation of Notch signaling is specially distributed in the mesenteric side compared with the anti-mesenteric side of ileum in CD patients. And up-regulated expression of NICD (intracellular activation fragment of Notch-1) in the mesenteric side was closely associated with increased recurrence rate of CD. In conclusion, Notch signaling is significantly activated in the mesenteric side of the diseased ileum, and it will be reasonable to construct anastomosis on the anti-mesenteric side of the ileum to prevent CD recurrence.

Keywords: Notch, Crohn's disease, anastomosis, recurrence

Introduction

Crohn's Disease (CD) is a kind of chronic inflammatory bowel disease, which usually occurs in the terminal ileum and right colon, and likely involves the whole gastrointestinal tract. However, the pathogenesis of CD remains largely unexplored. In the recent 10 years, the domestic incidence of CD has increased about 10 to 20 times [1]. Although there are some biological agents can be available for treatment, there are still as much as 70%-90% of patients with CD due to drug treatment failure or complications have to surgical resection [2]. The postoperative recurrence rate of CD is extremely high and mainly in the site of the anastomosis [3, 4]. It is reported the recurrence rate of CD is 50% a year after surgical resection, this rate rise to 73% at the 5 years, and 39% of the patients need to further surgical therapy [5].

The cause of anastomosis appears to be still controversial. Some researchers think that several local factors such as suture material, local

ischemia, and anastomotic obstruction might play a major role in the pathogenesis of anastomotic recurrence [6, 7]. And through statistical analysis of clinical data, some others found that compared with end-to-end anastomosis, side-to-side anastomosis obtained less anastomotic recurrence rate after ileo-colonic resection [8, 9]. However, the underlying mechanisms involved in this phenomenon remain a large area to investigate.

Notch signaling pathway plays an important role in maintaining intestine epithelial homeostasis and barrier function [10, 11]. During the differentiation of intestinal tissue stem cells, Notch-1 signaling also exhibit critical roles. The expression of Notch-1 downstream target gene, Hes-1 (Hairy and enhancer of split-1) and Atoh-1 (Atonal homolog-1), are essential to intestine epithelial homeostasis through guiding intestinal stem cells differentiate into absorptive function cells or differentiate into secretory cells, including Paneth cells, goblet cells [12, 13]. Notch signaling has been widely shown to be involved in the pathogenesis of Crohn's dis-

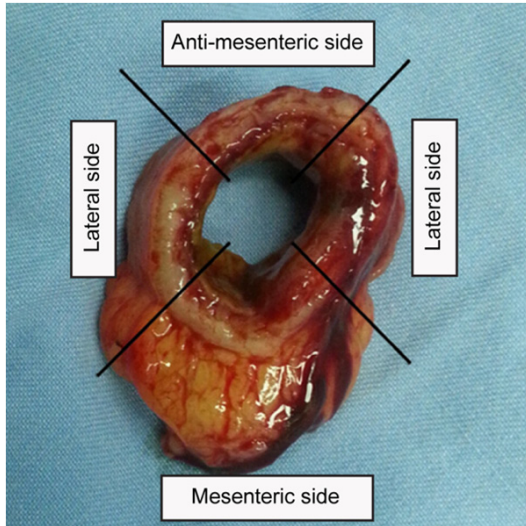


Figure 1. Complete “ring” of ileal wall from diseased ileum. The ring was divided in the four quadrants, including the mesenteric side, anti-mesenteric side, and two lateral sides.

ease. However, the expression pattern of Notch signaling in the site of anastomosis remains unclear.

In this study, an integrated bioinformatics analysis identified Notch mRNA was up-regulated in the CD specimens compared with normal intestinal samples. By immunohistochemistry, we evaluated the expression of molecules involved in Notch signaling in the different (mesenteric, lateral, and anti-mesenteric) sides of the ileum wall, which might critically involved in the anastomosis in patients with CD. Finally, our results implied that high activation of Notch signaling in the mesenteric side was associated to increased recurrence rate, and provided evidence to construct anastomosis on the anti-mesenteric side of the intestine in CD patients.

Materials and methods

Clinical tissue samples

A total of thirteen patients suffered from CD who underwent ileocolonic resection from June 2014 to July 2005 in our department were collected. This study was performed according to the Helsinki declaration principles and all patients were well informed and the process was approved by Ethics Committee of Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, China. The diagnosis was con-

firmed based on clinical manifestation, pathological and serological examinations. The follow-up time was calculated from the date of surgery to the last known follow-up. From the longitudinal section of the ileum, the ileum wall can be divided into four parts, including the mesenteric side (M), anti-mesenteric side (AM), and left and right lateral side (L) (**Figure 1**). Each sample was divided in two parts, and one was stored in liquid nitrogen for molecular analysis, and one was preserved in paraffin-embedded for histological analysis.

Quantitative real-time PCR

Total RNA from different (mesenteric, lateral, and anti-mesenteric) sides of the ileum wall was extracted by RNA Extraction Kit (SLNco, Cinoasia, China), and cDNA was synthesized using PrimeScript RT reagent Kit (TaKaRa Biotechnology, Japan). The primers used in this study were shown as follows. Notch-1, forward 5'-TGGACCAGATTGGGGAGTTC-3', reverse 5'-GCACACTCGTCTGTGTTGAC-3'; Hes-1, forward 5'-CCTGTCATCCCCGTCTACAC-3', reverse 5'-CATGGAGTCCGCCGTAA-3'; TGF- β , forward 5'-CTAATGGTGAAACCCACAACG-3', reverse 5'-TATCGCCAGGAATTGTTGCTG-3'; IGF1, forward 5'-GCTCTTCAGTTCGTGTGTGGA-3', reverse 5'-GCCTCCTTAGATCACAGCTCC-3'; NF- κ B, forward 5'-GTGGGGACTACGACCTGAATG-3', reverse 5'-GGG-GCAGATTGTCAAAGATG-3'; Expression of indicated genes was conducted on a Real-time Thermo Cycler (FTC3000, Funglyn, Canada) with SYBR Green Real-time PCR Master Mix (QPK-201, TOYOBO, Japan). Relative expression of indicated genes were determined by normalizing expression of each Ct value to β -actin Ct value and data were analyzed according to the $2^{-\Delta\Delta Ct}$ formula.

Immunohistochemistry

Immunohistochemical analysis was performed as previously described [14]. Briefly, CD or normal tissue sections were deparaffinized in xylene and rehydrated with graded ethanol. Then sections were incubated with 0.3% hydrogen peroxide and boiled in a microwave for 15 min to unmask antigen epitopes, followed by blocking with 10% BSA. After rinsing three times with phosphate-buffered saline (PBS), the sections were blocked with 10% BSA (Sangon, Shanghai). After washing three times with phosphate-buffered saline (PBS), slides were first incubated using the antibody for

Implications of Notch signaling in Crohn's disease

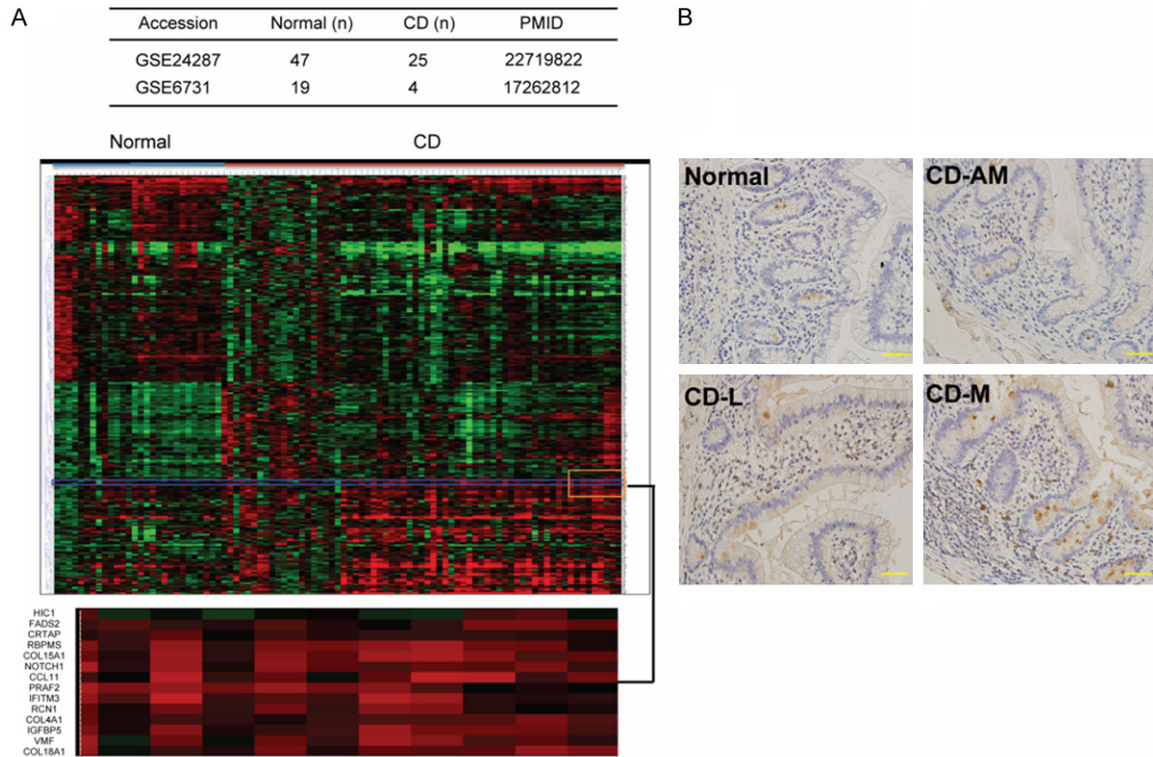


Figure 2. Notch-1 is differently expressed in CD. A. Heatmap analysis of the genetic profile between CD and diseased-unaaffected ileum tissues. Red to green scale represents high to low gene expression. B. Immunohistochemical analysis of Notch-1 expression in the mesenteric side (M), anti-mesenteric side (AM), lateral side (L) of CD tissues and diseased-unaaffected ileum tissues (Normal). Scale bar: 50 μ m.

Notch-1 (Abcam, ab52627, US), NCID (Abcam, ab8925, US), and Hes-1 (Abcam, ab71559, US) at 4°C overnight with optimal dilution. After washing three times with PBS, slides were incubated with second antibody labeled by HRP (rabbit) (Proteintech, US) at room temperature for 1 h. Finally, the bound antibodies were visualized with 3,3'-diaminobenzidine tetrahydrochloride and counterstained by hematoxylin. Scoring was conducted according to the percent of positive cells: < 5% scored 0; 6%-25% scored 1; 25%-50% scored 2; more than 50% scored 3 and staining intensity: no staining scored 0, weakly staining scored 1, moderately staining scored 2 and strongly staining scored 3, respectively. The final score was designated as low or high expression group using the percent of positive cell score \times staining intensity score as follows: low expression was defined as a total score < 4 and high expression with a total score \geq 4. These scores were determined independently by two senior pathologists.

Western blotting

Notch-1, IGF-1 and TGF- β expression in the mesenteric and anti-mesenteric side of ileum

tissues were detected by immunoblotting. Briefly, total cellular proteins were harvested in accordance with the manufacturer's instructions (Sangon Biotech, Shanghai, China). The lysates were separated by SDS-PAGE and transferred to nitrocellulose filter membranes (Millipore, USA). After blocking with 5% skim milk, the membrane was incubated in primary antibodies against Notch 1 (Abcam, ab52627, US), IGF-1 (Abcam, ab9572, US), and TGF- β (Abcam, ab66043, US). Blots were then incubated with HRP-tagged secondary antibody (Abmart, China) and visualized using electrochemiluminescence (Millipore, Billerica, MA, USA).

Statistical analysis

Data were presented as the means \pm SD of three independent experiments. The SPSS software program (version 17.0; IBM Corporation) was used for statistical analysis. Graphical representations were performed with GraphPad Prism 5 (San Diego, CA) software. The Student's t-test was used to analyze differences between two groups, and one-way

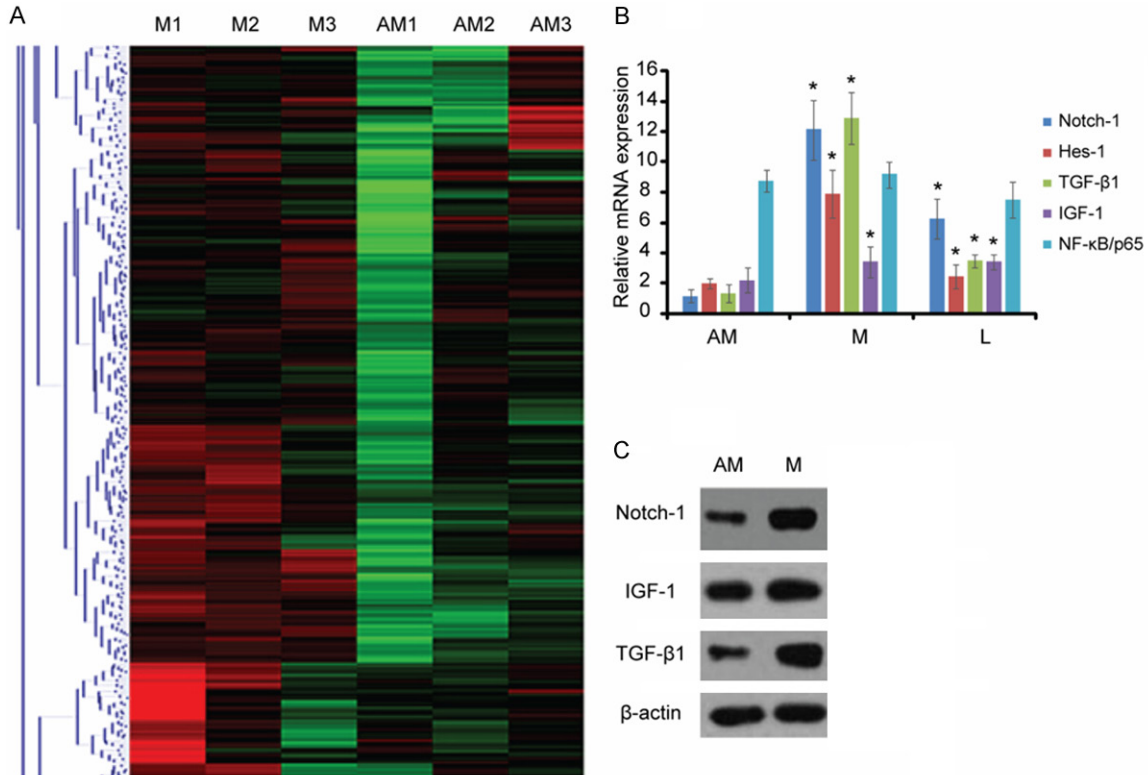


Figure 3. Enhanced Notch-1 signaling in the anti-mesenteric side of ileum. A. Heatmap analysis of differently expressed genes between the mesenteric side (M) and anti-mesenteric side (AM) of CD tissues. Red to green scale represents high to low gene expression. B. The mRNA expression of Notch-1, Hes-1, TGF-β1, IGF-1 and NF-κB in the mesenteric side (M), anti-mesenteric side (AM), and lateral side (L) of CD tissues was detected by real-time PCR. C. The protein expression of Notch-1, TGF-β1 and IGF-1 in the mesenteric side (M) and anti-mesenteric side (AM) of CD tissues was detected by western blotting.

ANOVA was used to determine the significance of differences among multiple groups. *P* values less than 0.05 were considered statistically significant.

Results

Notch-1 is differently expressed in CD

In order to identify critical factors involved in CD, we evaluated the changes in gene expression occurring in conditions of CD and disease-unaffected ileum from two independent GEO datasets (Figure 2A, GSE24287 and GSE6731). The expression of Notch-1 was dramatically increased in the CD tissues compared with disease-unaffected ileum. By immunohistochemical analysis, we confirmed elevated Notch-1 protein expression in CD tissues. Notably, we also found enhanced immunoreactivity of Notch-1 in the mesenteric side than the anti-mesenteric side of ileum (Figure 2B). This

indicates Notch-1 might be involved in the outcome of anastomosis in patients with CD.

Enhanced Notch-1 signaling in the anti-mesenteric side of ileum

Next, to determine why side-to-side anastomosis are associated with lower recurrence rate compared with end-to-end anastomosis, a expression profile chip technology on both AM and M specimens was performed (Figure 3A). In the differently expressed genes, we further detected the mRNA expression of Notch signaling molecules (Notch-1 and Hes-1) by real-time PCR. The results showed that Notch-1 and Hes-1 in anti-mesenteric side was significantly lower than the mesenteric and lateral side (Figure 3B). The mRNA expression of several other factors, including TGF-β, IGF-1 and NF-κB, which play critical roles in the development of CD, was also measured. Consistent with previous report [15], the mRNA expression level of

Implications of Notch signaling in Crohn's disease

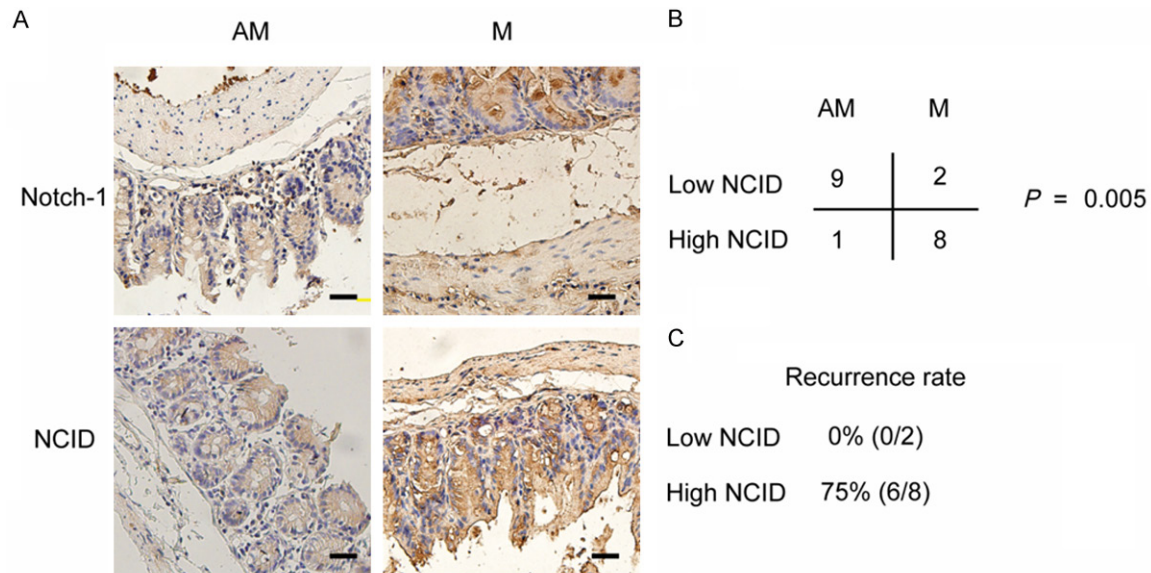


Figure 4. Up-regulated NCID predicts a poor outcome of CD. A. Representative photographs of Notch-1 and NCID expression in the mesenteric side (M) and anti-mesenteric side (AM) of CD tissues. Scale bar: 50 μ m. B. NICD expression was significantly up-regulated in the mesenteric side compared with the anti-mesenteric side of CD tissues. Fisher's exact test, $P = 0.005$. C. The recurrence rate of CD based on NCID expression.

TGF- β 1 in mesenteric side was significantly higher than in the anti-mesenteric side (**Figure 3B**). However, the expression of IGF-1 mRNA was also faintly increased, while no significant difference was found in NF- κ B expression (**Figure 3B**). Furthermore, the protein level of these alterations was also revealed by western blotting. In line with the observation in mRNA expression, Notch-1, TGF- β 1 and IGF-1 were also up-regulated at protein level in the mesenteric side compared with the anti-mesenteric side (**Figure 3C**). Lastly, we detected the expression and distribution of Notch-1 and its intracellular activation fragment NICD in the mesenteric side and anti-mesenteric side of CD sections by immunohistochemical analysis (**Figure 4A**). Expectedly, NICD expression was found significantly up-regulated in the mesenteric side compared with the anti-mesenteric side of CD tissues (**Figure 4B**). Excitingly, we also found that CD patients with higher NCID expression have a high recurrence rate (**Figure 4C**). Collectively, these data above suggest that Notch-1 signaling is specially activated in the anti-mesenteric side of ileum and might contribute to the recurrence of CD.

Discussion

Currently, the clinical data suggest that different types of surgical anastomosis (namely involving different histological sections of the

intestinal wall) have certain effect on the post-operative recurrence [8, 9]. In this study, we showed that the crucial factors involved in Notch signaling, including the intracellular activation fragment NICD and its downstream target genes Hes-1, were highly expressed in mesenteric side in relative to the anti-mesenteric side. Thus, our results indicate that Notch signaling might play an important regulatory role in progress and recurrence of CD.

By informatics analysis and sampling different wall tissues of the ileum from CD pathological specimens, including mesenteric side, left and right sides, anti-mesenteric side and normal control, we found that NICD and Hes-1 was significantly higher in the mesenteric side than anti-mesenteric side. This result demonstrated the diversity of time and space expression of Notch signaling in CD. When Notch-1 was knockdown in intestine epithelial cells, the expression of intercellular tight junction protein Claudin-5 was declined, which ultimately contributed to increased intercellular permeability, and decreased barrier function [16]. It has also been demonstrated that over-expressed Notch-1/Hes-1 signaling drastically reduce the number of goblet cells, which ultimately reduced the ability of intestinal epithelium against different pathogens [17]. And Notch-1 signaling is activated quickly in intestinal tissue damage

Implications of Notch signaling in Crohn's disease

including bowel resection and promotes the proliferation of intestine epithelial cells, suggesting that Notch-1/Hes-1 signaling plays an important role during the reconstruction of bowel barrier function [18]. These reports suggest the regulatory roles of Notch signaling under normal circumstance or repair process. In clinical, Dibenzazepine, the inhibitor of Notch signaling, can reduce the expression of IL-1 β and IL-6, maintain the number of goblet cells, and alleviate the symptoms of early CD patients [19]. In a mouse model of CD, Notch-1/Hes-1 signaling was also activated in the intestinal inflammation site and mediated cell proliferation [20]. Consistent with these notions, increased Notch-1 expression was found in CD and specially activated in the anti-mesenteric side of ileum.

Taken together, our result demonstrated that Notch signaling may play important regulatory role in the process of CD, and its important value as a guidance of operation method or as an important molecular marker in judging the prognosis of CD.

Disclosure of conflict of interest

None.

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