

A Novel Strategy to Identify mRNA 3' UTR and mRNA Level Difference in Crohn's Disease

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Abstract

Background and Aims: Crohn's disease (CD) is a chronic and refractory intestinal inflammatory disease. The 3' untranslated region (3' UTR) of mRNA transcript can regulate its own translational process post-transcriptionally, and its role in CD remains unclear. The aim of this study was to identify the critical genes influencing CD phenotype via the regulation of mRNA 3' UTR.

Methods: Isoform Structural Change Model (IsoSCM) was used to evaluate the length of 3' UTR of the whole transcriptome from a RNA-seq based online CD database. Correlations between 3' UTR length status and mRNA level were analyzed by Spearman coefficients. Correlated genes list was overlapped with published critical CD related gene list. Univariate and multivariate analysis were performed to identify the genes associated with CD phenotype.

Results: Compared with normal control, 3' UTR of 34192 genes were shortened and 57389 were lengthened among 432784 genes in CD patients. The 3' UTR changes of 255 genes were found significantly correlated with their mRNA level. Furthermore, 8 overlapped genes were shared by above 255 correlated genes and known CD related gene list (*ABCB1*, *FAF1*, *TUT2*, *IL27*, *JAK2*, *LTB*, *NAGLU* and *PTPN2*). The mRNA level of *ABCB1* and *JAK2*, and shortened 3' UTR length of *JAK2* transcript were found to be correlated with deep ulcer in CD patients by univariate and multivariate analysis.

Conclusions: The 3' UTR changes of CD related genes were confirmed to be related to the phenotype of CD. Our findings may provide further insight into the epigenetic mechanisms and therapeutic strategies for CD.

Keywords: 3' UTR; Crohn's disease; IsoSCM; Genetic; Phenotype

Abbreviations: 3' UTR: 3' untranslated region; CD: Crohn's Disease; IsoSCM: Isoform Structural Change Model; ApA: alternative cleavage and polyadenylation; miRNA: microRNA; GWAS: Genome wide association studies; NF kappa B: Nuclear factor kappa B; FID: Fas-interacting domain; *IFN-γ*: interferon gamma; Th17: T helper 17 cell; CDS: coding sequence; *ABCB1*: ATP binding cassette subfamily B member 1; *FAF1*: Fas-Associated Factor 1; *IL27*: interleukin 27; *JAK2*: Janus kinase 2; *PTPN2*: protein tyrosine phosphatase; Non-receptor type 2; *FUT2*: fucosyltransferase 2; *LTB*: lymphotoxin beta; *NAGLU*: N-acetyl-alpha-glucosaminidase.

Introduction

Crohn's disease (CD) is a chronic and relapsing intestinal inflammatory disease which influences millions of people in Europe, with an increasing prevalence in Asia and other developing countries [1,2]. It is generally accepted that CD is a genetically predisposed, environmentally triggered complex disease [3]. Nevertheless, the genetic background of CD remains unclear, especially the epigenetic mechanism. Understanding the underlying genetic mechanisms will be of great significance for early detection and therapeutic decisions.

The 3' untranslated region (3' UTR) is the untranslated sequence outside of the 3' terminal exon of mRNA. Over half of mammalian genes use alternative cleavage and polyadenylation (ApA) or splicing of terminal exons to produce various mRNA isoforms that distinguish in their length of 3' UTRs, whereas generating the same proteins [4]. In other words, mRNA with proximal ApA site or distal ApA site corresponds to short or long 3' UTR separately, while the exons of mRNA remain unaltered. The 3' UTR plays a significant role in post-transcriptional regulation such as regulating mRNA stability,

localization, rate of protein synthesis and localization of membrane proteins [5,6]. Dysregulation of 3' UTR has been proven to be related to a number of diseases, such as cancer, cardiovascular diseases and autoimmune diseases [7].

According to the mainstream view, functions of 3' UTR are mediated by micro-RNA, AU-rich-element and RNA-binding proteins (RBPs) etc [6]. In general, there are more microRNA target sites in the longer 3' UTR region, mRNA with a longer 3' UTR displays reduced translation, whereas mRNA with shorter one has a high level of translation [4].

Despite substantial progress in identifying the genetic spectrum of CD, our understanding is insufficient for the key mechanisms contributing to the disease. Over the past decade, specialized techniques have been developed to annotate RNA-seq data to reveal the transcriptome at the levels of RNA processing machineries, and their relationship with disease [8]. Isoform Structural Change Model (IsoSCM) is one of the tools that can analyze 3' UTR. The change-point analysis applied in IsoSCM is able to tremendously increase

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Received December 08, 2016; Accepted March 23, 2017; Published March 29, 2017

Citation: Hu T, Liu H, Zhou C, Wu X, Chen Y, et al. (2017) A Novel Strategy to Identify mRNA 3' UTR and mRNA Level Difference in Crohn's Disease. Chemotherapy 6: 226. doi:10.4172/2167-7700.1000226

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the 3' UTR annotation efficiency, by which we can integrate RNA-seq coverage to recognize polyadenylation sites and 3' UTR boundaries with higher sensitivity and specificity [7]. Hence, we applied a novel strategy (IsoSCM) to identify 3' UTR alternative splicing related to CD phenotype.

Materials and Methods

Microarray Data

Gene Expression Omnibus (GEO) database is a comprehensive public genomics database which stores individual gene expression profiles from curated datasets in the GEO repository. (<http://www.ncbi.nlm.nih.gov/geo/>). The following key words were used: ("Crohn's disease" [MeSH Terms] AND "Homo sapiens" [porgn] AND "gse" [Filter]). Database GSE62207 contains expression profile in a cohort of 310 treatment-naive pediatric CD patients and controls and all the expression profiles were measured using the platform of IlluminaHiSeq 2000 (Homo sapiens) 2.0 Array. Ileal biopsies were obtained during diagnostic colonoscopies of patients with IBD-like symptoms and biopsies were stored at -80 degrees. All patients received colonoscopy and histological score and normal controls were those with suspected CD, but with normal colonoscopic and histologic findings.

Encoding mRNA level and the corresponding 3' UTR length in the CD database

IsoSCM is a novel strategy which utilizes change-point analysis to increase the 3' UTR annotation efficiency [7]. With the help of IsoSCM, we integrated RNA-seq coverage of 432784 genes from 188 CD patients and 70 normal people in the GSE62207. And UTR differential usage between CD and their normal counterpart was calculated using the following equation:

$$\text{APA site usage} = 1 - \text{down coverage/up coverage}$$

$$\text{Differential usage} = \text{APA site usage (case)} - \text{APA site usage (control)}$$

The down coverage and up coverage are the UTR length in the downstream and upstream of the APA site. Moreover, the differential usage over 0 means the mRNA with short 3' UTR, whereas differential usage less than 0 corresponds the mRNA with long 3' UTR. Finally, the 3' UTR length change (namely, the differential usage) for above genes in CD patients were listed in Supplementary Table 1.

Finding shared CD-related genes

We downloaded 290 CD related genes based on data of LD analyses, Genome wide association studies (GWASs), meta-analyses and HuGE

Navigator [9-12]. And then, we compared 255 correlated genes with above 290 genes using the Microsoft Excel to find the overlapped genes shared by them.

Statistical Analysis

Descriptive statistics were computed for all variables including means and standard deviations (SD) or medians and interquartile ranges (IQR) for continuous factors, and frequencies for categorical factors. Comparisons of the distribution of clinical characteristics and mRNA level between the patients with or without deep ulcer were made by using the 2-tail t test or Wilcoxon rank sum test for continuous variables and chi-square test for categorical variables. Correlation analysis between 3' UTR length and expression level of 432784 genes were made by using Spearman correlation analysis. Multivariate analysis of the factors associated with the deep ulcer were constructed using the logistic regression analysis. All statistical analysis was performed with the SPSS software (version 16; SPSS, Chicago, IL). P value less than 0.05 was considered statistically significant.

Results

mRNA level and the corresponding 3' UTR length in the CD database

We obtained the lengthened or shortened status of 3' UTR length by IsoSCM and mRNA level of 432784 genes from 188 CD patients and 70 normal people in the GSE62207 database. There were 34192 genes with shortened 3' UTR and 57389 genes with lengthened one in CD patients (Supplementary Table 1).

Correlation between 3' UTR length and mRNA Level

RNA with longer 3' UTR length tends to have lower mRNA level, whereas that with shorter 3' UTR has higher one (Figure 1). Correlation analysis (Spearman correlation coefficients) between the 3' UTR length and mRNA level was performed across the whole transcriptome. The mRNA level of 255 genes were found significantly correlated with 3'UTR length status ($P < 0.05$, Spearman $r \geq 0.3$) (Supplementary Table 2). About half of the mRNA of the 255 genes was shortened while the other half were lengthened in CD patients (Figure 2).

The overlapped genes in CD database and published literatures

Sung et al reported that they found 290 genes associated with CD based on data from Genome wide association studies (GWAS), LD analyses and HuGE Navigator. Hence, we used the Microsoft excel to find the overlapped genes between the 290 CD related genes and the

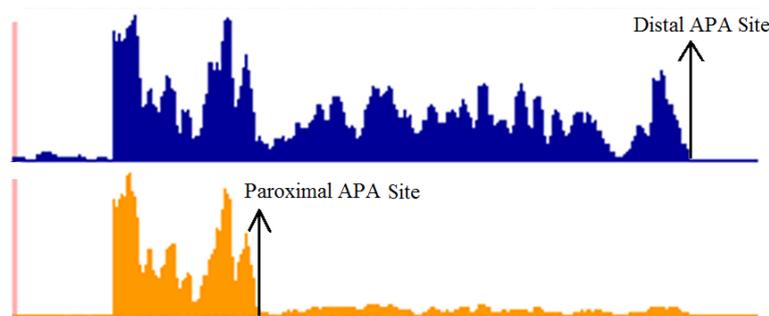


Figure 1: Model of genes with short or long 3' UTR length: Alternative cleavage and polyadenylation (ApA) leads to mRNA isoforms with different 3' UTR length. And mRNA with distal ApA site corresponds to a long 3' UTR length, while that with paroximal ApA site has a short 3' UTR length (Figure 1 was quoted from Zheng et al. *Nat Commun.* 2014 Nov 20;5:5274).

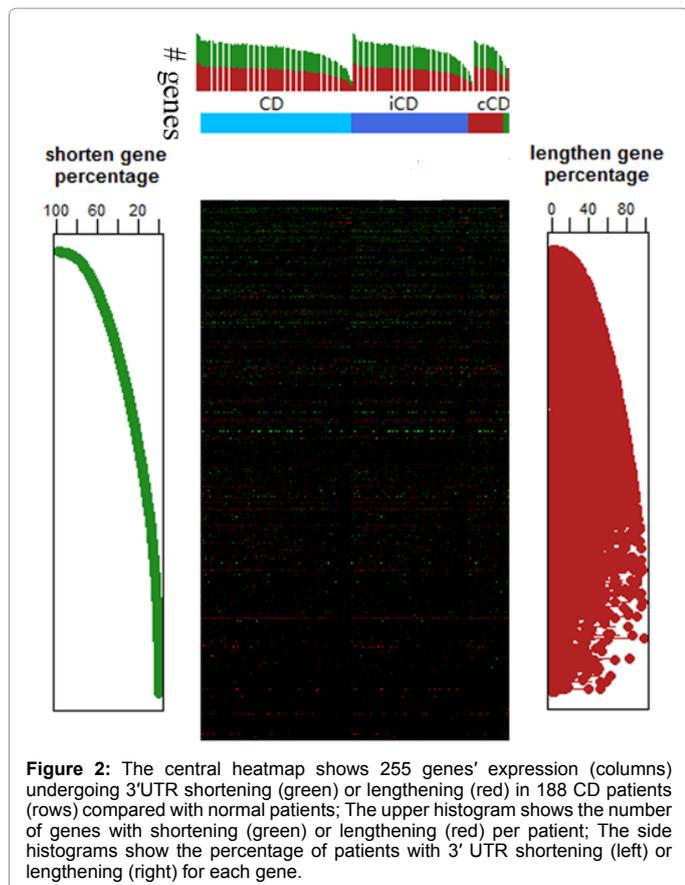


Figure 2: The central heatmap shows 255 genes' expression (columns) undergoing 3'UTR shortening (green) or lengthening (red) in 188 CD patients (rows) compared with normal patients; The upper histogram shows the number of genes with shortening (green) or lengthening (red) per patient; The side histograms show the percentage of patients with 3' UTR shortening (left) or lengthening (right) for each gene.

255 genes with 3' UTR and mRNA level correlation. In the end, 8 genes were found overlapped by the two gene list (*ABCB1*, *FAF1*, *FUT2*, *IL27*, *JAK2*, *LTB*, *NAGLU*, *PTPN2*). (Supplementary Table 3, Figures 3 and 4).

Genes related to deep ulcer in CD patients

Based on these findings, correlation between 3' UTR status and mRNA level of the 8 genes and clinical parameters (deep ulcer, age and gender) of the CD patients were analyzed by univariate and multivariate analysis. Univariate analysis showed that mRNA level of *ABCB1*, *FAF1*, *IL27*, *JAK2* and *PTPN2* were associated with deep ulcer status in CD patients. Multivariate analysis revealed that low mRNA level of *ABCB1* and high mRNA level of *JAK2* were independently associated with deep ulcer. Meanwhile, shortened 3' UTR of *JAK2* was associated with deep ulcer by univariate analysis (Tables 1,2 and Figure 5).

Discussion

Despite abnormal inflammatory response to gut microbiota were considered as the critical factors for CD pathogenesis, the epigenetic and genetic pathogenesis still remains unclear. Genome wide association studies (GWAS) and subsequent meta-analysis for CD have identified more than 140 susceptible genes contributing to CD, such as *NOD2*, *IL23R*, *ATG16L1*, *TNFSF15*, *IL17* etc. [13,14]. These researches focused on the functional relevance of genetic variations, the mechanisms of how these susceptible genes lead to the pathogenesis of CD has not been fully explained [15]. Since the 3' UTR plays significant role in post-transcriptional regulation, different 3' UTR lengths caused by alternative cleavage and polyadenylation (ApA) can lead to major difference in post-transcriptional binding site, such as binding sequence of miRNA. Several studies have demonstrated that shorter 3'

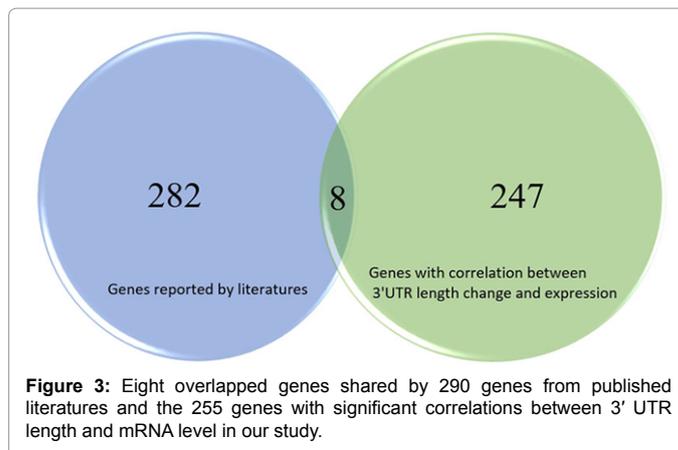


Figure 3: Eight overlapped genes shared by 290 genes from published literatures and the 255 genes with significant correlations between 3' UTR length and mRNA level in our study.

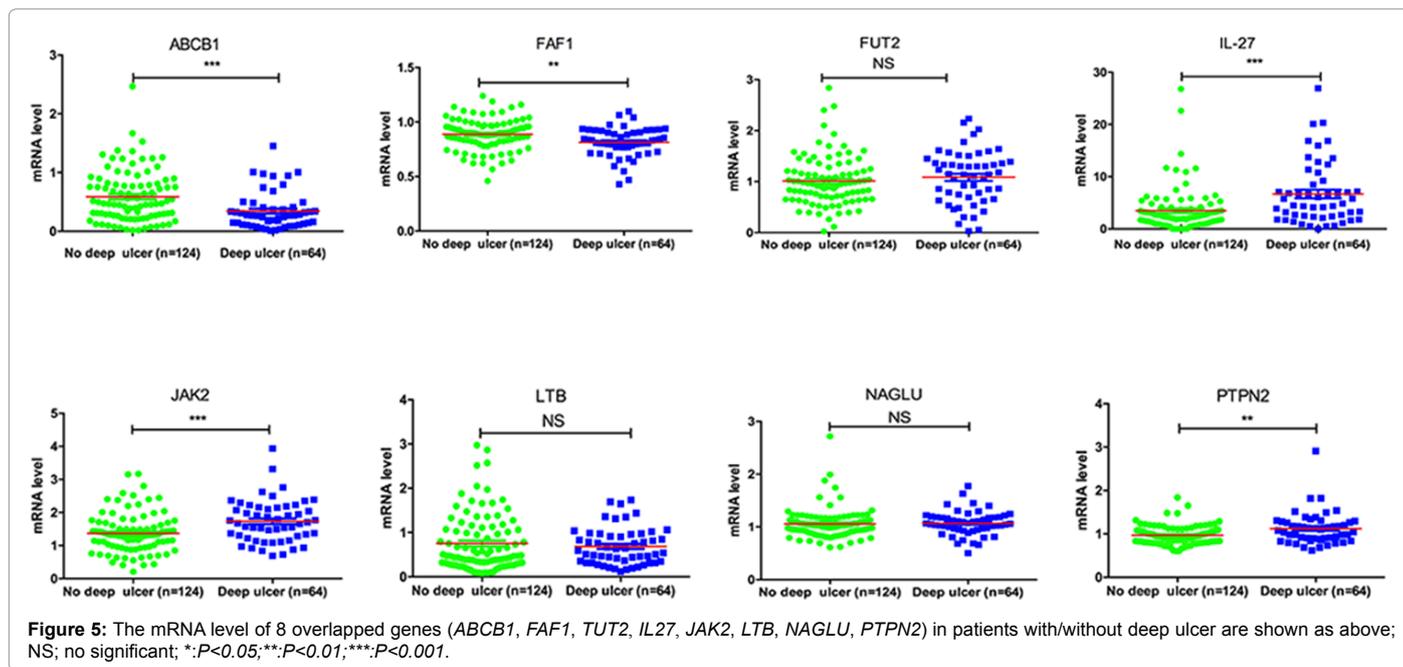
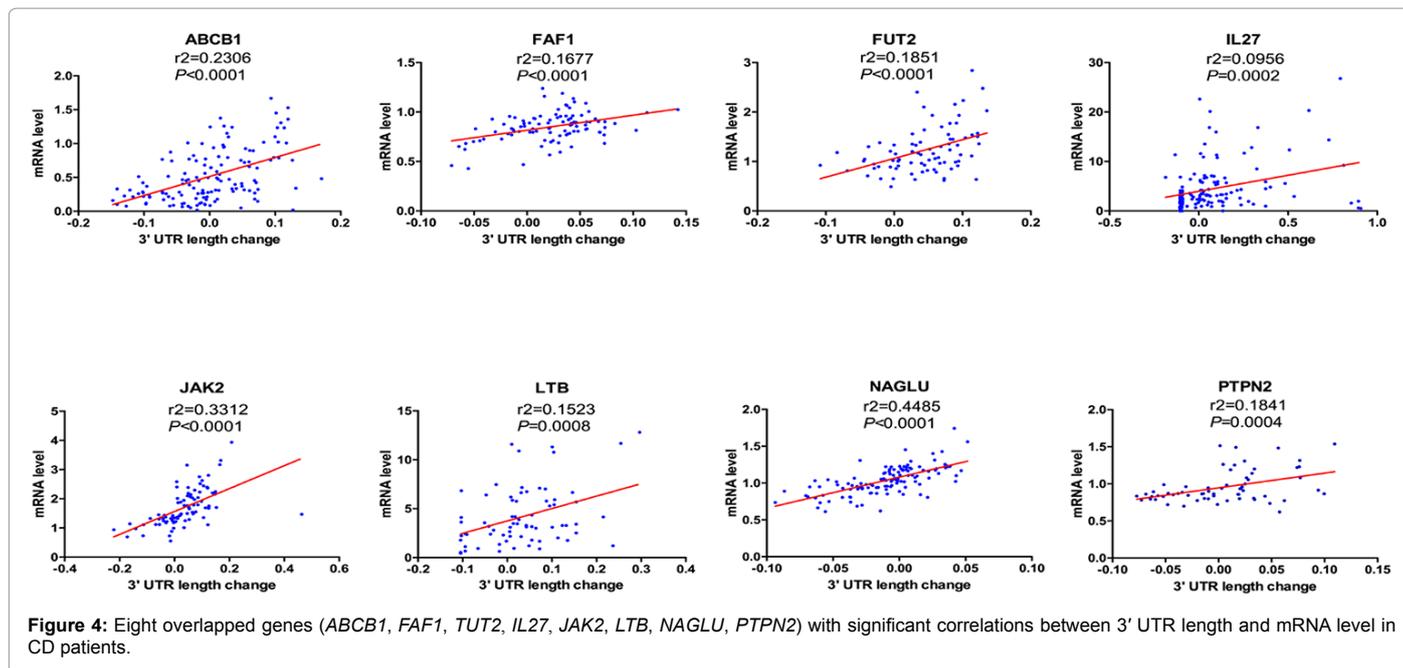
UTR of *IL23R* and *ATG16L1* displays elevated levels of both mRNA and protein production by the loss of binding capacity for the miRNAs (miRNA Let-7e and Let-7f for *IL23R* while miR-106b and miR142-3p for *ATG16L1*) [15-17].

From whole transcriptome data, we identified that the changes of 3' UTR length of 255 genes were correlated with mRNA level. Eight genes were shared by our 255 genes list and 290 CD related genes found by GWAS and deep sequencing. The relationship between mRNA level and the changes of 3' UTR length for the eight overlapped genes was confirmed by the Spearman correlation coefficients (Figure 4). Among them, low mRNA level of *ABCB1* and *FAF1* was connected with deep ulcers in CD patients while high mRNA level of *JAK2*, *IL-27* and *PTPN2* corresponded to that (Figure 5). Moreover, mRNA level of *ABCB1* and *JAK2* were independently associated with deep ulcers in CD patients by multivariate regression analysis. In addition, 3' UTR length of *JAK2* was related to deep ulcer by univariate regression analysis (Tables 1 and 2).

JAK2 (Janus kinase 2) is a protein tyrosine kinase related to multiple inflammatory signaling pathways [18]. *JAK2* is the juncture of two critical intracellular inflammatory pathways in Th17 cells (*IL17-JAK2-STAT3* and *IL23R-JAK2-STAT3* pathways), which plays an important role in the pathogenesis of CD [19,20]. Moreover, microRNA-375 was reported to activate the *JAK2-STAT3* pathway by binding to the 3' UTR of *JAK2* during inflammatory response [21]. We found that shortened 3' UTR and higher mRNA level of *JAK2* were mutually correlated and simultaneously associated with deep ulcer in CD patients (Figures 4 and 5). Furthermore, multivariate regression analysis and confirmed that high mRNA level of *JAK2* was an independent risk factor for deep ulcer in CD patients with *p* value as 0.042 OR (95% CI) as 1.89 (1.023-3.50). The underlying mechanism for shortened 3' UTR and higher mRNA level of *JAK2* in CD patients may be explained by the relative few microRNA binding sites in its 3' UTR region. Our findings provided a novel post-transcriptional regulation mechanism of *JAK2* in the pathogenesis of CD.

We also found the 3' UTR length change of seven other genes were correlated with their mRNA level (*ABCB1*, *FAF1*, *TUT2*, *IL27*, *LTB*, *NAGLU* and *PTPN2*) (Figure 4), but the length change of 3' UTR of these genes was not significantly associated with deep ulcer by univariate regression analysis (Table 2). These results suggested that the expression level of genes could be regulated by the length of 3' UTR, but this type of regulation might not have significant clinical influence in our dataset.

ATP binding cassette subfamily B member 1 (*ABCB1*) is an ATP-dependent drug efflux pump, which can decrease drug accumulation



involved in multidrug resistance [22,23]. Alfreda et al. found that 2 SNPs of *ABCB1* (rs10248420 and rs2032583) were significantly related to CD, and 5 SNPs (rs1128503, rs1202184, rs1202186, rs2091766 and rs2235046) were associated with non-inflammatory CD based on a genotype-phenotype analysis [24]. As an ATP-dependent drug efflux pump, *ABCB1* plays a crucial role in preventing the uptake of toxic compounds including bacterial toxins into the intestinal mucosa, which could account for its protective role in CD [25]. In line with these results, we also found that the mRNA level of *ABCB1* was lower in deep ulcer group of CD patients (Figure 5). And its protective factor was verified by univariate and multivariate regression analysis with p value of <0.001 and 0.014 respectively (Tables 1 and 2).

The 3' UTR of mRNA were found deeply involved in the signaling pathways in CD such as *NF κB*. Fas associated factor 1 (*FAF1*) was a protective factor for CD patients with lower mRNA levels in the deep ulcer group (Figure 5). *FAF1* could suppress *CD40*-induced *NF κB* activation by interacting with the *CD40* receptor [26]. And previous studies have demonstrated that *CD40* expression level was high in intestinal mucosa and dendritic cells in CD patients [27]. What's more, *IFN-γ/CD40L* induced cytokine secretion plays an important role in intestinal mucosal inflammation [28]. Therefore, we hypothesize that the loss of *FAF1* function in certain patients may contribute to pathogenesis and severity of CD through unchecked *NF κB* activation. And further investigation of this process is warranted, which may

Variations	Univariate Analysis				Multivariate Analysis	
	All cases (N=188)	Patients without deep ulcer(N=124)	Patients with deep ulcer(N=64)	P value	OR (95% CI)	P value
Age at diagnosis	12.2 ± 3.1	12.2 ± 3.3	12.2 ± 2.7	0.907	—	—
Male gender, n (%)	112(59.6%)	73 (65.2%)	39 (34.8%)	0.782	—	—
<i>ABCB1</i> , quantile	0.37(0.20-0.75)	0.47(0.26-0.80)	0.27(0.12-0.41)	<0.001	0.23(0.07-0.75)	0.014
<i>FAF1</i> , quantile	0.87(0.79-0.94)	0.90 (0.82-0.97)	0.80(0.74-0.91)	0.001	0.36(0.01-11.20)	0.563
<i>FUT2</i> , quantile	0.98(0.69-1.34)	0.95(0.69-1.20)	1.13(0.67-1.42)	0.204	—	—
<i>IL27</i> , quantile	2.94(1.62-5.87)	2.33(1.24-3.81)	4.82(2.34-9.97)	<0.001	1.07(0.96-1.19)	0.223
<i>JAK2</i> , quantile	1.41(1.11-1.87)	1.31(0.97-1.61)	1.67(1.26-2.15)	0.001	1.89(1.023-3.50)	0.042
<i>LTB</i> , quantile	0.51(0.31-1.01)	0.47(0.29-1.10)	0.59(0.33-0.97)	0.654	—	—
<i>NAGLU</i> , quantile	1.03(0.92-1.18)	1.01(0.89-1.67)	1.06(0.95-1.18)	0.246	—	—
<i>PTPN2</i> , quantile	0.98(0.83-1.16)	0.93(0.82-1.09)	1.10(0.89-1.27)	0.004	0.49(0.05-5.256)	0.55

Table1: Univariate and multivariate Analysis of the relationship between mRNA level and deep ulcer in CD patients.

Variations	All cases	Patients without deep ulcer	Patients with deep ulcer	P value
Number of patients	188	124	64	—
Age at CD diagnosis	12.2 ± 3.1	12.2 ± 3.3	12.2 ± 2.7	0.907
Male gender, n (%)	112(59.6%)	73 (65.2%)	39 (34.8%)	0.781
<i>ABCB1</i> , quantile	0.00(-0.04-0.049)	0.002(-0.03-0.06)	0.00(-0.04-0.027)	0.338
<i>FAF1</i> , quantile	0.02(0.00-0.04)	0.03 (0.00-0.045)	0.02(0.00-0.04)	0.538
<i>FUT2</i> , quantile	0.045(0.00-0.08)	0.04(0.00-0.08)	0.05(0.02-0.09)	0.240
<i>IL-27</i> , quantile	0.02(0.00-0.135)	0.01(0.00-0.13)	0.05(0.00-0.208)	0.133
<i>JAK2</i> , quantile	0.03(0.00-0.07)	0.01(0.00-0.06)	0.05(0.00-0.085)	0.009
<i>LTB</i> , quantile	-0.001(-0.002-0.043)	-0.001(-0.0029-0.039)	-0.001(-0.0018-0.035)	0.595
<i>NAGLU</i> , quantile	0.000(-0.02-0.008)	0.00(-0.03-0.01)	0.00(-0.02-0.006)	0.460
<i>PTPN2</i> , quantile	0.005(-0.02-0.071)	0.0035(-0.03-0.075)	0.006(-0.01-0.062)	0.773

Table 2: Univariate Analysis of the relationship between 3' UTR length change and deep ulcer in CD patients.

provide a novel theoretical basis for the molecule-targeted treatment of CD.

IL27 is a cytokine which belongs to interleukin 12 family, as well as a subunit of the *IL27/WSX-1* heterodimeric cytokine complex. We found that high mRNA level of *IL27* was related to deep ulcer in CD patients (Figure 5), which was in accordance with other studies [29-32]. And Honda et al revealed the inflammatory property of *IL27/WSX-1* signaling in intestinal inflammation, which may thus be a promising candidate for the therapeutic intervention in CD [33]. Bancroft and Takeda et al discovered that *IL27/WSX-1* signaling occupied a central position in triggering the production of *IFN-γ* by naive CD4+ T cells via *STAT1*-mediated T-bet activation [34,35]. Hence, *IL 27* may trigger excessive intestinal immune response by inducing *IFN-γ* production via the *IL27/WSX-1—STAT1* pathway, which could lead to the onset of CD.

PTPN2 is a member of the *PTP* family which is known to be signaling molecules regulating a variety of cellular processes including cell growth, differentiation, mitotic cycle and apoptosis [36]. Some studies showed that *PTPN2* was a susceptible gene for CD and *PTPN2* was reported to overexpress in the inflamed intestinal tissues of CD patients [37,38]. In accordance with them, we discovered that high mRNA level of *PTPN2* was significantly correlated with deep ulcer in CD patients (Figure 5). As a signaling molecule, *PTPN2* has a number of target genes including Janus kinases (*JAKs*), signal transducer and activator of transcription (*STAT*) 1 and 3, mitogen-activated protein kinase (*MAPK*) and epidermal growth factor receptor (*EGFR*) [39-41]. As mentioned above, *IL17—JAK2—STAT3* and *IL23R—JAK2 —STAT3* pathway were essential for the function of Th17 in inflammation. We suppose that *PTPN2* and *JAK2* may have overlapped effects on deep ulcer in CD since only *JAK2* was associated with deep ulcer in

multivariate analysis whereas both *PTPN2* and *JAK2* were significant in univariate analysis.

Among the 8 overlapped genes, there was no significant correlation found between deep ulcer phenotype and *FUT2*, *LTB* and *NAGLU*. Fucosyltransferase 2 (*FUT2*) is an enzyme engaged in the synthesis of the H antigen, while H antigen is both an attachment site and carbon source for gut bacteria. So we hypothesize that high mRNA level of *FUT2* may result in increased susceptibility to CD [42]. As for Lymphotoxin beta (*LTB*), a type II membrane protein of the *TNF* family, its role in the pathogenesis and phenotype of CD remains controversial. Krause et al and Spahn et al found that *LTB* could modulate the innate immune response and accelerate the recovery of intestinal mucus [43,44], while Friedrich et al and Wang et al hold an opposite opinion that *LTB* might exacerbate immune response of intestinal mucus [45]. When it comes to N-acetylglucosaminidase (*NAGLU*), the association of *NAGLU* with CD was not found in several databases such as PubMed. Despite there was no correlation between above three genes and deep ulcer phenotype, we have confirmed their relationship with CD by comparing our result with GWAS data.

This study was based on bioinformatic analysis for the data from GWAS and CD database. Up to now, we have been making every effort to collect clinical specimens of different CD phenotype for further investigation. Our study identified a novel epigenetic regulation mechanism for the mRNA level of CD related genes. Based on these findings, we can explore the potential mechanisms of how these genes are connected with CD phenotype. Furthermore, we can construct a clinically useful genetic profile that can aid in treatment decisions.

Acknowledgements

Grant Support: This work was supported by National Natural Science

Foundation of China (No. 81400603), National Natural Science Foundation of China (No. 81402019), Guangdong Natural Science Foundation (No.2015A030310190), Guangdong Natural Science Foundation (No.2015A030313108) and Science and Technology Planning Project of Guangdong Province (No.2015B020229001).

Author Contributions

TH, HSL and JK contributed to study concept and design, acquisition, analysis, interpretation of data and drafting of the manuscript. XRW, CZ, HSL, YFC, XHL, XWH, YFZ and XSH contributed to data collections and manuscript review. XJW and PL contributed to study concept and design, analysis and interpretation of data and critical revision of the manuscript for important intellectual content. XJW and PL supervised the study. All authors read and approved the final manuscript.

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