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Expression alteration of long non-coding RNAs and their target genes in the intestinal mucosa of patients with Crohn's disease

Na Li[a,b], Ruihua Shi[a,b,*]

[a] Medical School of Southeast University, Ding Jia-qiao Road 87, Jiangsu Province, Nanjing 210009, China

[b] Department of Gastroenterology, Zhongda Hospital, Affiliated Hospital of Southeast University, Ding Jia-qiao Road 87, Jiangsu Province, Nanjing 210009, China

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ABSTRACT

Backgroud: CD(Crohn's Disease)is a chronic idiopathic inflammatory disorders of the GI tract. It is increasing worldwide and has become a global health problem. The key pathological mechanism for CD occurrence has not been identified, and present treatments are mostly anti-symptom therapy, which has limited efficacy. In this study, we investigate whether lncRNAs are involved in the pathogenesis of CD and how they may regulate the target genes in CD process.

Methods: CD patients were diagnosed in Zhongda Hospital of Southeast University between May 2017 and May 2018. Pathological and normal intestinal mucosa were collected and total RNA was extracted the expression of lncRNA and mRNA was profiled and analyzed by using lncRNA and mRNA gene chips. The lncRNAs and mRNAs with significant alternations (≥10 times and $P < 0.05$) were identified and verified. The co-expressed mRNAs with the differentially expressed lncRNAs were revealed by CNC analysis. The potential regulatory factors were determined by the Ce (cis/tans) mechanism analysis with the use of miRbase, Targetscan, and NCBI database. Finally, the lncRNA-miRNA/TF-mRNA expression network was predicted.

Results: Eight lncRNAs were found to be differentially expressed between the pathological mucosa and the normal mucosas in the ileal end. CNC analysis of the differentially expressed lncRNAs revealed fifty co-expressed mRNAs with positive or negative regulation. Base on the mRNAs KEGG pathway analysis, most of them appeared to be involved in cell signaling pathways. Six lncRNAs in the cytoplasm participated in the Ce mechanism, and the rest two lncRNAs in the nucleus participated in the cis/trans regulation mechanism. Finally, ternary relationship of lncRNAs-miRNAs and TFs-mRNAs was obtained by CNC,KEGG enrichment analysis and Ce (trans/cis) analysis.

Conclusion: The differential expression of lncRNAs in CD mucosa indicated that lncRNAs were involved in immune reaction. These lncRNAs might contribute to the regulation of intestinal mucosa function through the genetic network of lncRNAs -miRNAs/TFs-mRNAs.

1. Introduction

Crohn's disease (CD) is characterized by chronic , segmental, asymmetric distribution of granulomatous inflammation involving any part of the gastrointestinal tract, predominantly the terminal ileum and adjacent colon [1]· Statistics shows that while the incidence of CD in China is lower than that in European and American areas, there is a clear trend of increase in the incidence in China in recent years [2], CD represents a global health problem and a research hotpot [3,4]. Although the use of inflammatory transmitter inhibitors such as 5-amino salicylic acid (5-ASA) and salazosulfylpyridine can alleviate the symptoms of CD to some extent, the treatment efficacy is low, and such anti-symptom treatment does not stop CD pathogenic pathway [5,6]. Better understanding on the mechanism of CD pathogenesis may help the development of new treatment modality.

Based on clinical and experimental studies on genetic susceptibility, immune function defects, intestinal microflora disorders, and food antigen stimulation, various hypotheses for CD pathogenesis have been raised by different investigators [7,8]. CD is generally thought to be a result of interative actions by genetic backgrounds d and environmental factors. Multiple risk factors for CD, including immune dysfunction, delayed hypersensitivity, alterations of intestinal flora, and activation induced by some special food [9] or bacterial antigen [10–13], have been identified. However, each mechanism ultimately involves the gene

* Corresponding author at: Department of Gastroenterology, Zhongda Hospital, Affiliated Hospital of Southeast University, Ding Jia-qiao Road 87, Jiangsu Province, Nanjing 210009, China.

E-mail address: ruihuashi@126.com (R. Shi).

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and broken immune tolerance. But the molecular mechanism and pathways remain unclear. Recent studies indicate that gene mutation and altered expression in non-coding RNAs-such as lncRNAs, miRNAs, circRNAs, sRNAs may contribute to intestinal mucosa inflammation [14–16]. However, few studies focused on systematic screenning for pathogenic genes in CD through sequencing and validation of pathological pathways in intestinal tissues. With the development of next generation sequencing technology, high-throughput sequencing is widely applied. This technology can provide important information on gene expression regulation. Several high-throughput studies analyzed serum samples. But blood composition is complicated by numerous physiological factors, and the finding could be useful for disease screening purpose but may not directly reveal the pathogenic changes. We sought to detect the expression changes of lncRNA and mRNA in ileal tissues of CD patients in comparison with the control group by using micro-array chips. Bioinformatic analyses will help us to determine the potential role of candidate lncRNA and the downstream genes. Combined analyses on lncRNAs/target mRNAs, with the gene expression profile involved in inflammatory activation, apoptosis and other signaling pathways may increase ouir knowledge on the CD pathogenesis.

2. Materials and methods

2.1. Study subjects

CD patients (n = 60) diagnosed in the Inpatient Department of Zhongda Hospital of Southeast University between May 2017 and May 2018 were enrolled in this study. All CD patients were screened strictly according to WHO (World Health Organization) diagnostic criteria. Each patient was diagnosed with CD for the first time, and the disease was in active phase. The pathological tissue at the end of ileum was taken out,and the normal tissue nearest to the end of ileum was taken out for control from healthy people in Physical examination. The WHO diagnostic criteria used were: 1) Discontinuous regional lesions (X-ray or endoscopy); 2) paving stone like manifestation or longitudinal ulcers (X-ray or endoscopy); 3) full wall inflammation (X-ray or endoscopy); 4) non-caseous epithelioid granuloma (pathological examination); 5) fissured ulcer (X-ray); and 6) anal lesions. Suspected cases were diagnosed, or two of them are diagnosed. The first three requirements for suspected diagnosis, plus any of criteria 4–6 can be used to definitively diagnose CD. Alternatively, patients can also be diagnosed if the symptoms meet criterion 4, plus any two of criteria 1–3.

To control the interference by other diseases and drug treatment, patients with any of the following conditions were excluded: 1) various immune diseases; 2) acute or chronic inflammation in non-intestinal systems; 3) hematological diseases; 4) hyperthermia and the use of inflammatory inhibitors, such as non-steroidal anti-inflammatory drugs, steroids and opiates.40 patients were screened for experimental analysis. Written consent was received from each study subject. Pathological ileal terminal tissues(n = 40) and normal ileal mucosa tissues(n = 40)from fourty healthy people were collected and divided into two groups named normal groups and pathological group. There were no significant differences in age and gender between CD patients and healthy people($\chi^2 = 0.354$,p = 0.607; $t = 1.784$, p = 0.087). Three tissues from each group were selected randomly(Normal tissues vs Pathological tissues = 3:3) for screening with lncRNA and mRNA chip analysis.

2.2. Collection of diseased and normal ileal tissues

Transanal enteroscopy was performed and pathological ileal tissues and normal ileal mucosa tissues were collected. Distal ileal specimens of 100 mg and normal ileal mucosa of 100 mg were obtained with biopsy forceps. The tissue was cut into pieces with sterile scissors and placed in TRIzol reagent. Samples were fully dissolved in TRIzol to form a clear and non-sticky liquid, then stored in liquid nitrogen or at −80 °C. Three tissues from each group were randomly selected (Normal tissues vs Pathological tissues = 3:3) and used for screening with lncRNA and mRNA chip analyses.

2.3. RNA extraction from tissues

Total RNA was extracted from lesions and normal tissues with the use of Trizol reagent Kit. DNA digestion was performed to eliminate genomic DNA contamination. Nanodrop ND-1000 instrument was used to determine the values of OD260, OD280 and OD230, and the concentration and purity of RNA were calculated.

2.4. Library construction and sequencing

Reagents and kits used were: the NEBNext® Poly(A) mRNA Magnetic Isolation Module (New England Biolabs), RiboZero Magnetic Gold Kit (Human/Mouse/Rat) (Epicentre, an Illumina Company), and the KAPA Stranded RNA-Seq Library Prep Kit (Illumina) and used the NanoDrop ND-1000 instrument. The constructed libraries were examined with an Agilent 2100 Bioanalyzer to determine library concentration and fragment size (400–600 bp) and finally verified by real time-PCR. The sequencing libraries for different samples were denatured by 0.1 M NaOH to produce single strand DNA. Single strand DNA was amplified with a TruSeq SR Cluster Kit v3-cBot-HS (#GD-401-3001, Illumina) in situ. The resulting fragments were sequenced for 150 cycles using an Illumina HiSeq 4000 and other sequencers.

The 10 most up and down-regulated lncRNAs was verified by real-time PCR to verify The trend was same as that found in the chip test, and eight lncRNAs were significantly differentially expressed ($P < 0.05$). We further examined the differentially expressed genes by FISH and found where the lncRNAs were located inside cells. At present, it was reported that lncRNAs can regulate genes at the transcriptional level in both cytoplasm and nucleus. Target mRNAs of the top lncRNAs were obtained by CNC analysis. Total target mRNAs function of the top lncRNAs were obtained by KEGG enrichment and we selected mRNAs related to inflammation. In addition, we determined ultimate mRMAs' regulatory factor miRNAs/TFs from the Ce(cis/trans) mechanism's reverse tracking, which is based on miRbase,targetscan,NCBI. Finally,we obtained the lncRNAs-miRNAs/TFs-mRNAs expression networks. NCBI and Esemble websites were used to proved non-protein-coding RNAs or protein-coding RNAs.

2.5. Data analysis

Quality control was used to assess whether the sequencing data could be used for post-sequencing data analysis. The trimmed data generated by the pre-processing filtering step after quality control (removal of the connector sequence in the read and the overly short segment) were aligned to the reference genome. If the comparison results were good, quantitative analysis of gene and transcript expression, based on the level of gene expression, was performed.

3. Analysis of sample and RNA-seq data quality control

3.1. Initial quality control of sample RNA degradation

A Qiagen RNeasy Mini Kit was used to extract RNA from the 3 lesion tissues and 3 normal tissues. The standard of RNA quality was RIN ≥ 7.0, 28S/18S > 0.7 and 260/280 > 1.6. In agarose denaturing gel electrophoresis, as shown in Fig. 1 and Table 1, the 28S and 18S bands in RNA samples were clear, and almost no 5S degradation bands appeared, indicating that the samples were not degraded.

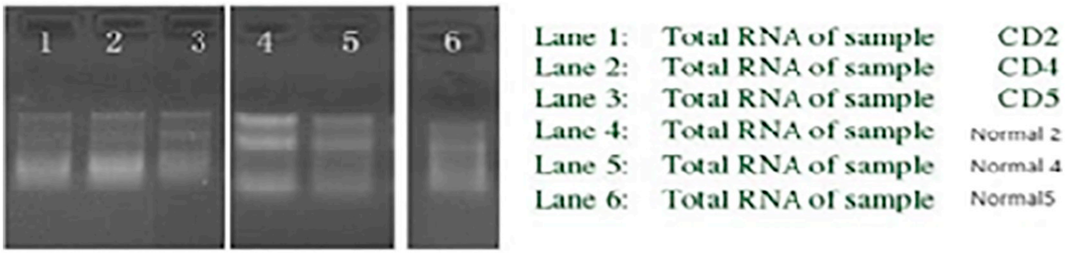


Fig. 1. RNA Integrity and cDNA contamination test by denaturing agarose gel electrophoresis.
*The 28S and 18S ribosomal RNA bands should be fairly sharp, intense bands. The intensity of the upper band should be about twice that of the lower band. Smaller, more diffuse bands representing low molecular weight RNAs (tRNA and 5S ribosomal RNA) may be present. It is normal to see a diffuse smear of ethidium bromide staining material migrating between the 18S and 28S ribosomal bands, probably comprised of mRNA and other heterogeneous RNA species. DNA contamination of the RNA preparation will be evident as a high molecular weight smear or band migrating above the 28S ribosomal RNA band. Degradation of the RNA will be reflected by smearing of ribosomal RNA bands.

Table 1
RNA quality control: total RNA quantification and quality assurance assessed by spectrophotometry.

Sample name	OD260/280 ratio[a]	OD260/230 ratio[b]	Conc.(ng/ul)	Volume(ul)	Total amount(ng)
CD2	1.90	2.41	894.57	40	35,782.80
CD4	1.94	2.18	1097.40	15	16,461.00
CD5	1.95	2.28	894.12	80	71,529.60
Normal2	1.86	2.37	531.53	40	21,261.20
Normal4	1.88	2.23	462.85	50	23,142.50
Normal5	1.88	1.98	324.79	50	9743.70

[a] For spectrophotometry, the O.D. A260/A280 ratio should be close to 2.0 for pure RNA (ratios between 1.8 and 2.1 are acceptable).
[b] The O.D. A260/A230 ratio should be > 1.8.

3.2. Quality assessment of the sequencing library

Sequencing library quality was assessed with an Agilent 2100 Bioanalyzer using an Agilent DNA 1000 chip kit (Agilent, part #5067-1504).

3.3. Reliability and quality control of transcript data obtained from sequencing

Original sequence data in FAST Ca format were generated with an Illumina sequencer. To valuate the quality of sequencing, the original sequence of each sample was counted as Q30: $Q = -10\log_{10}$ (P), and the results are shown in Table 2.

3.4. Quality control for transcript coverage data

Transcription Data Coverage: A transcription coverage map was used to assess the 5′ or 3′ preference of the sample library fragments, as shown in Fig. 2. The fragment distribution of samples was generally balanced across the transcripts, indicating good quality of file construction.

Table 2
Q statistics of transcripts obtained from sequencing.

Sample	Number of reads	Total number of bases	Number of base(Q ≥ 30)	Q30(%)[a]
CD2	96,541,236	144,811,192,900	13,515,018,932	93.33%
CD4	118,716,854	17,807,528,100	16,644,900,872	93.47%
CD5	105,627,738	15,844,160,700	14,819,429,523	93.53%
Normal3	162,877,484	244,316,226,600	22,849,439,705	93.52%
Normal6	116,064,992	17,409,748,800	16,295,309,077	93.60%
Normal8	130,789,710	19,618,456,500	18,394,056,926	93.76%

[a] Q = 30 means that the probability of false identification is 0.1%; that is, the error rate is 0.1%, and the accuracy rate is 99.900. Generally, Q30 ≥ 80% means that the quality of sequencing is extremely high.

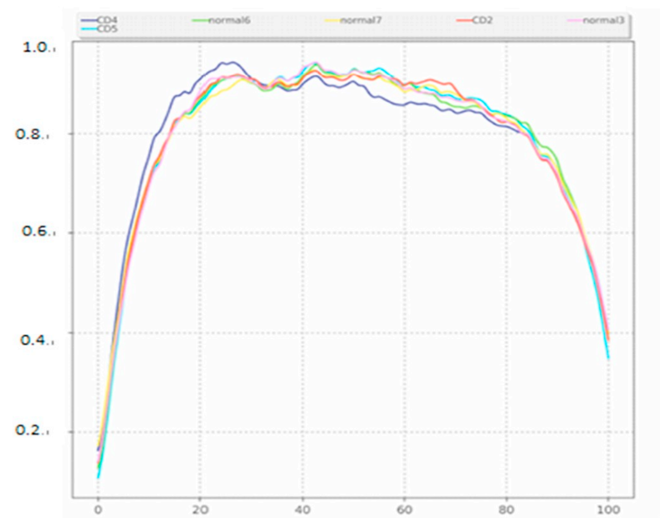


Fig. 2. Coverage of all transcripts in six tissues by chip library genes.
*Where the X-axis represents the length of the transcript (standardized to 100 bp), and the Y-axis represents the number of reads covered.

4. Results

4.1. Prediction and screening of different lncRNAs

4.1.1. Screening of differentially expressed lncRNAs in CD versus normal tissues

A total of 51,388 lncRNAs were detected by microarray screening. A total of 400 significantly differentially expressed lncRNAs were found in the lesion tissues. Of those, 243 were significantly overexpressed, and 157 were significantly reduced in the lesion tissues compared with the control group ($P < 0.01$). The cluster thermograms of up- and down-regulation of all lncRNAs in CD vs. normal tissues are shown in Fig. 3.

4.1.2. RT-PCR verification of differential expression of lncRNAs in the ileum tissues

The top 10 most significantly up-regulated and down-regulated lncRNAs were screened and clustered by bioinformatics analyses. The differentially expressed lncRNAs were verified in clinical tissues. RT-PCR was performed for 40 cases of CD ileum lesions from CDpatients and and normal controls, respectively. A total of eight differentially expressed lncRNAs were found. The results are as follows: ENST00000487539.1_1, ENST00000409569.2_1, ENST00000491430.1_1, ENST00000392442.6_1, ENST00000524613.5_1 ENST00000472375.5_1, ENST00000465606.5_1,

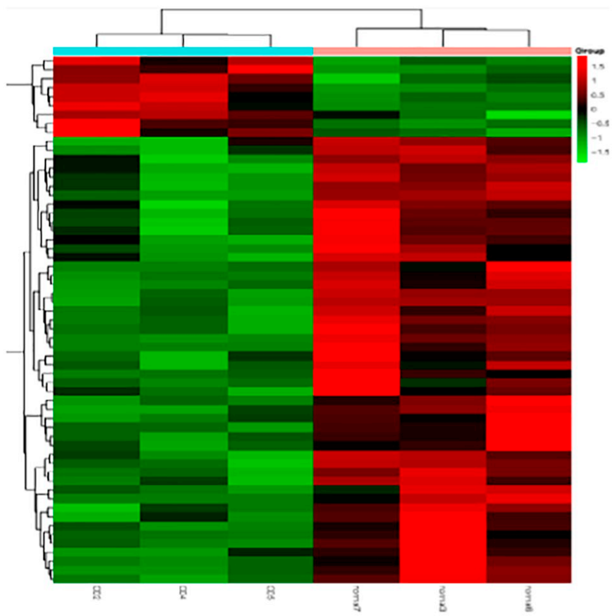


Fig. 3. Differential expression clustering thermograms of lncRNAs upregulated and downregulated in CD vs. normal tissues.
*The X-axis shows the fluorescence signal intensity in the 60 μg/mL^{-1} CVB-D group, and the Y-axis shows the fluorescence signal intensity in the control group. Each row represents a gene, and each column represents a sample. Red represents significantly upregulated genes, and green represents significantly downregulated genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

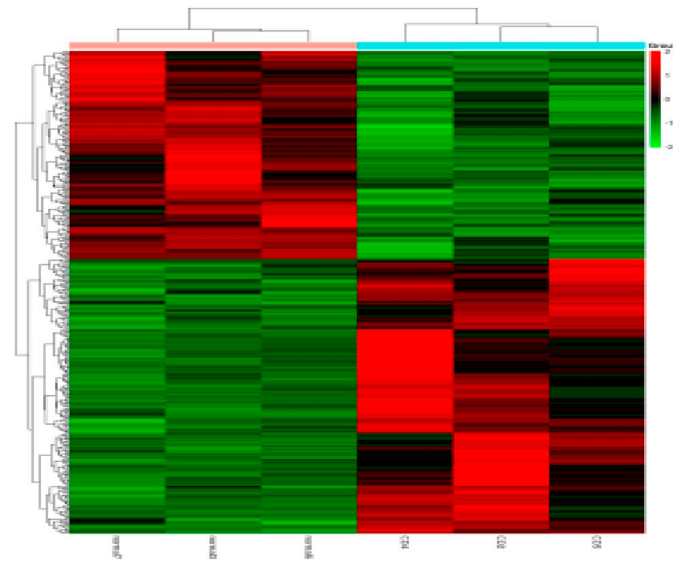


Fig. 4. mRNAs differential expression clustering thermography for CD vs. normal tissues.
The X-axis shows the intensity of the fluorescence signal in CDs of the 60 μg/mL CVB-D group; the Y-axis shows the intensity of the fluorescence signal in different gene control groups; green indicates low expression, and red indicates high expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and ENST00000558814.1_1. The first four transcripts were overly expressed in the CD tissue, and the remaining four have decreased expression. These transcripts, which are longer than 200 nt in length, were searched on NCBI and Esemble Websites and proved to be non-protein-coding RNAs.

4.2. Prediction and screening of co-expression of target genes with lncRNAs

4.2.1. Screening of differential expression of mRNAs in CD vs. normal control

Bioinformatic analyses of mRNAs obtained from high-throughput sequencing of the two groups of samples yielded significantly differentially expressed mRNAs. The cluster thermograms of up- and down-regulated mRNAs in CD tissues are shown in Fig. 4.

A total of 4500 differentially expressed genes were identified from the high-density hybridization dot matrix of the gene chip, analyzed quantitatively, and standardized using a computer program. Base to the standard for significant difference (ten times difference), there were 392 differentially expressed genes in the diseased intestinal mucosa compared with the normal mucosa. There were 223 up-regulated genes with 169 down-regulated genes.

4.2.2. Prediction and screening of target genes of RT-PCR validated lncRNAs

(1) CNC analysis was used to identify the mRNAs co-expressed with differentially expressed lncRNAs (Pearson's correlation analysis). After tissue validation, a list of differentially expressed lncRNAs were obtained, and the normalized data were used to calculate the correlation coefficients with all genes. The genes with PCC ≥ 0.9, P value and FDR < 0.05 were selected, and Cytoscape tools were used to draw Fig. 5.

CNC analysis of the differently expressed lncRNAs revealed 50 co-expressed genes with positive or negative regulation and adjacent location. Base on the mRNAs' KEGG pathway analysis database, most of them appeared to be involved in cell signaling pathways, such as interaction with neuroactive ligand receptors, stimulation signals and complement systems, endocytosis, Th1/Th2 transformation, apoptosis, and Th17 cell differentiation, suggesting that these lncRNAs and their co-expressed mRNA may play an important role(s) in autoimmune activity, a mechanism directly related to inflammatory bowel disease.

(2) The inflammatory response mRNA was obtained by KEEG pathway enrichment analysis.

Top ten analysis of the GO functions of target genes showed that they were mainly involved in infection, antigen processing, and inflammatory bowel disease, as shown in Table 3. Studies have shown that alteration of lncRNAs expression may contribute to the development of autoimmune diseases [12]. These findings is consistent with the previous observation, and this consistency also provides support to the reliability of the data.

4.3. The localization of lncRNAs and different lncRNA-miRNA/TF-target gene relationships

We constructed fluorescent single-stranded nucleic acid probes for eight differentially expressed lncRNAs verified by PCR, and performed hybridization with intestinal mucosa tissues. The localization of lncRNAs in cells was observed under a high-power fluorescence microscope. Then the location of lncRNAs in cells was observed by FISH, lncRNAs in the cytoplasm were analyzed by the Ce mechanism, and lncRNAs in the nucleus were analyzed by the cis/trans regulation mechanism (Targetscan Human/Pictar software). We found that ENST00000409569.2_1 ENST00000392442.6_1, ENST00000524613.5_1, ENST00000487539.1_1, ENST00000491430.1_1, and ENST00000558814_1 mainly located in the cytoplasm, while ENST000465606.5_1 and ENST00000525216.1_1 localized in the nucleus (Fig. 6). Six lncRNAs in the cytoplasm were analyzed by the Ce mechanism, and two lncRNAs in the nucleus were analyzed by the cis/trans regulation mechanism.

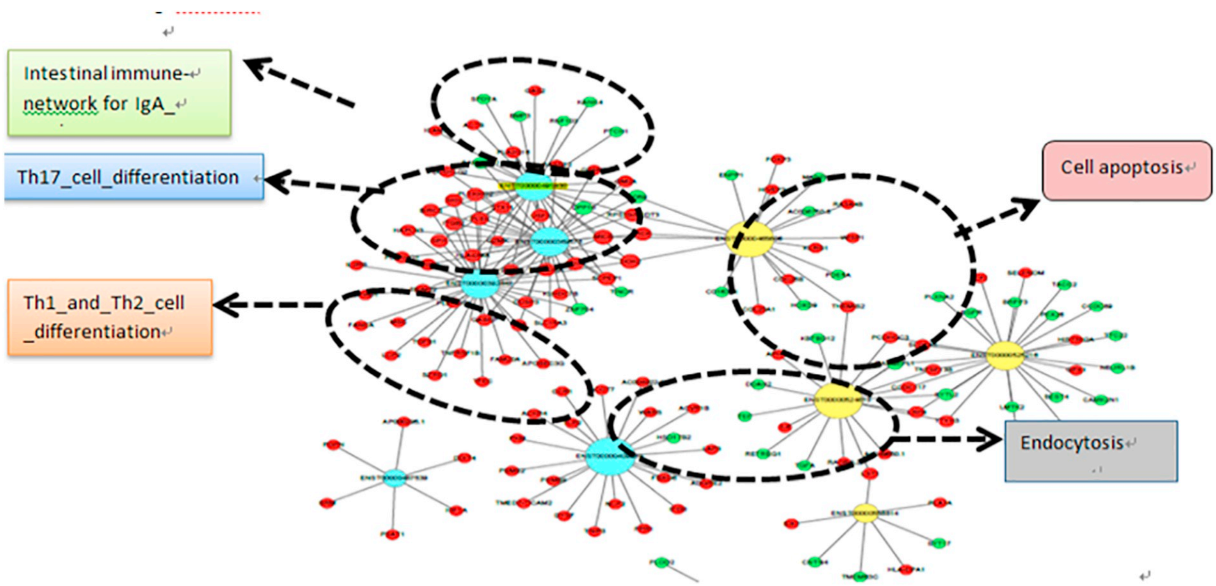


Fig. 5. LncRNA co-expression with mRNAs obtained from CNC analysis, and related biological function.
*The green circle node indicates downregulated mRNA; the yellow circle node indicates down regulated lncRNA; the red circle node indicates up regulated mRNA; and the blue circle node indicates up regulated lncRNA. Positive correlation is the realizationline, and the negative correlation is shown by a dashed line. All information is summarized in Fig. 5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Totally 50 genes (from sequencing data) were analyzed by CNC, and 100 genes (from sequencing data) were obtained through KEGG enrichment analysis. We predicted that 50 overlapping genes, such as *FOXP3*, *TGF-beta*, *MHC-II*, *IL-22*, and *ATG5*, could be obtained by combining and intersecting the two groups of genes. These genes were related to the expression of lncRNAs and involved in inflammatory activation and apoptosis signaling pathways, and they may be the key target gene pool (target gene library 1) for lncRNA-activated CD. We established a gene library based on a ternary relationship of lncRNA-miRNA/TF-mRNA (target gene library 2, predicted by Target scan software/ Pictar software). Target gene library 1 and target gene library 2 were combined and intersected to obtain the target genes (Fig. 7) and regulatory factor miRNAs/TFs, which are closely related to the expression of lncRNAs and are involved in inflammatory activation, apoptosis and other signaling pathways. Finally, a relatively reliable ternary relationship of lncRNAs-miRNAs/transcription factors-mRNAs was obtained (see Table 4 for details).

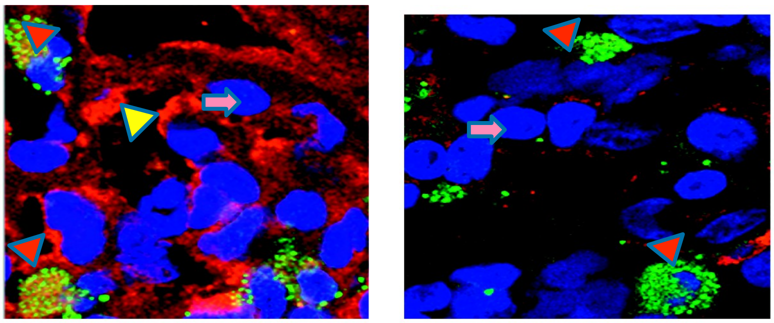
5. Discussion

Previous studies have indicated that the occurrence of CD is related to gene mutation. Recently, Genome Wide Association Study (GWAS) has identified a number of genes and DNA methylation regions associated with susceptibility to CD. Aberrant methylation of HLA and MIR21 regions were related to the pathogenesis of CD [17,18]. Murthy et al. have found that lncRNA ATG induces stress and apoptotic protease activation in intestinal epithelial cells in the inflammatory environment of CD. They also observed an increased caspase-3-mediated ATG16L1 cleavage, which led to the intestinal epithelial cell autophagy abnormalities [19]. An in-depth study has found that an increased activity of DDX5 in Th17 cells results a large amount of lncRNA Rmrp unlinking, which would bind to ROR γ -t, thus causing the latter to undergo nuclear transport, acting on the corresponding promoter, and promoting the development of Th17. Th17 maturation has a protective effect on CD [20–22]· As mentioned above, many lncRNAs have been found to be related to the pathogenesis of CD. The current study using high-throughput sequencing constructs a list of lncRNAs that may be deeply involved in the pathogenesis of CD.

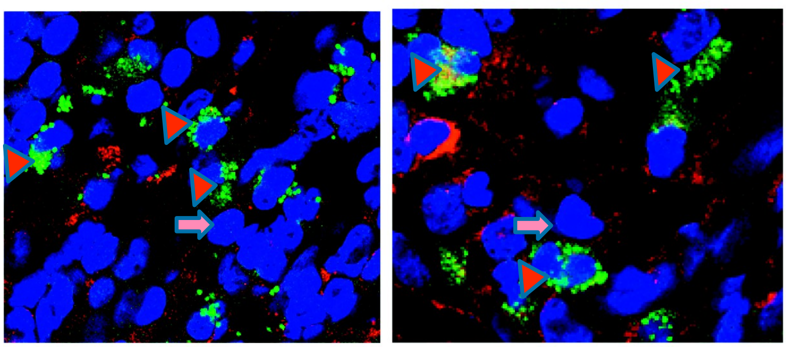
Only limited research has been performed for high-throughput lncRNA sequencing screening on CD patients' specimens. Chen et al. had used another micro array to identify a total of 1988 and 2993 dysregulated lncRNAs and mRNAs between the serum sample from CD and control groups. Our study found a larger number of lncRNAs and mRNAs than previous report [23,24]. We used the OE Biotech Human lncRNA chip V2.0 and simultaneously found 51,388 lncRNA and 4500 mRNA differently expressed between the pathological and control

Table 3
KEGG analysis outcome for highest frequency top ten signaling pathways in CD vs. normal tissues.

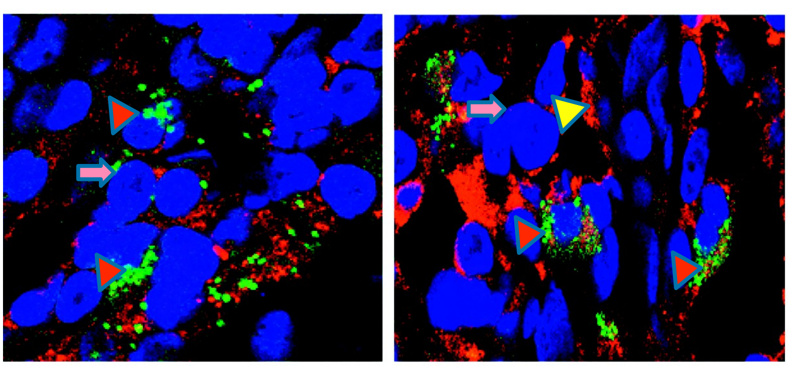
ID	Term	P_value	Gene
Hsa5150	Staphylococcus_aureus_infection	1.71209E-13	C2//C4A//C4B//CFB//CFI//FPR1//HLA-DMA//HLA-DPA1//HLA-DPB1/HLA-DRA//ICAM1//ITGB2//PTAFR//SELPLG
hsa04612	Antigen_processing_and_presentation	3.56251E-09	CD74//HLA-DMA//HLA-DPA1//HLA-DPB1//HLA-DRA//HLA-E//IFI30//PSME1//PSME2//RFX5//TAP2//TNF-α
hsa05140	Leishmaniasis	2.76561E-08	CYBB// //HLA-DPA1//HLA-DPB1//HLA-DRA//ITGB2//NCF1
hsa05323	Rheumatoid_arthritis	2.20366E-07	EGFR//AKT//HLA-DPB1//HLA-DRA//ICAM1//IL6//ITGB2//TGFB1//TLR2//TNF//TNFSF13B/IL-1R
hsa04672	Intestinal_immune_network_for_IgA_production	9.60132E-07	ACTB//CD40//TGF-β/HLA-DPA1//HLA-DPB1//LC3II //HLA-E//ICAM1//IL-22
hsa05416	Viral_myocarditis	4.29007E-07	ACTB//CD40//HLA-DMA//HLA-DPA1//HLA-DPB1//HLA-DRA//HLA-E//ICAM1//ITGB2
hsa05321	Inflammatory_bowel_disease_(IBD)	1.00429E-06	CD40//HLA-DMA//IL10 //HLA-DPB1//IL8 //IL6//TGFB1//TNFSF13B/IL-4
hsa05330	Allograft_rejection	2.34044E-06	FOXP3//HLA-DMA//HLA-DPA1//HLA-DPB1//HLA-DRA//IL6//TGFB1//TLR2//TNF
hsa05332	Graft-versus-host_disease	4.00161E-06	CD40//HLA-DMA//HLA-DPA1//HLA-DPB1//HLA-DRA//HLA-E//TNF
hsa04145	Phagosome	6.94773E-06	HLA-DMA//HLA-DPA1//HLA-DPB1//FOXP3//HLA-E//IL6//TGF-β



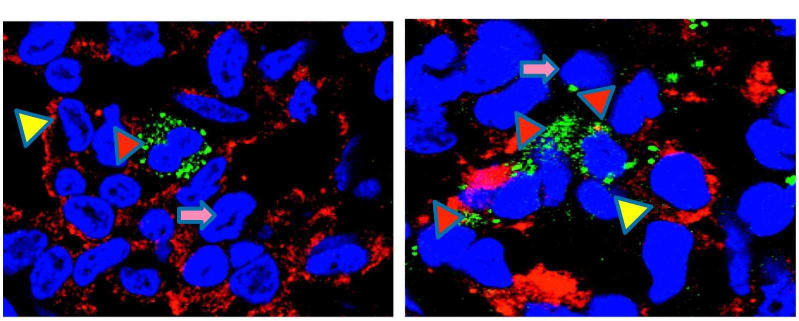
ENST00000465606.5_1（located in nucleus）ENST00000525216.1_1（located in nucleus



ENST00000409569.2_1（localized in cytoplasm） ENST00000392442.6_1（localized in cytoplasm）



ENST00000524613.5_1（localized in cytoplasm）ENST00000487539.1_1（localized in cytoplasm）



ENST00000491430.1_1（localized in cytoplasm）ENST00000558814.1_1（localized in cytoplasm）

Fig. 6. Localization of differentially expressed lncRNA cells, as determined by FISH assays.
*Under microscope magnification 400 times, 8 lncRNAs were observed in cell localization. : The nuclei stained by DAPI were blue under ultraviolet excitation as the pink arrows show. : LncRNAs probes was fluorescence labeled by fluorescein showing green as the red triangles show. : The intestinal epithelial cell membrane labeled with CK20 antibody showed red under ultraviolet excitation as the yellow triangles show. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tissues. Interestingly, there was no overlap of under-regulated lncRNAs between our results and the top 10 under-regulated lncRNAs extracted from CHEN's paper. Two reasons could explain the discrenpancy between the two studies. First, serum composition changes may not fully represent the local changes in CD lessions. Secondly, OE Biotech Human lncRNA chip V2.0 contains more lncRNAs and mRNA probes.

An obvious advantage of the chip assay use in this study is its simultaneous detection the expression of both lncRNA and mRNA, which

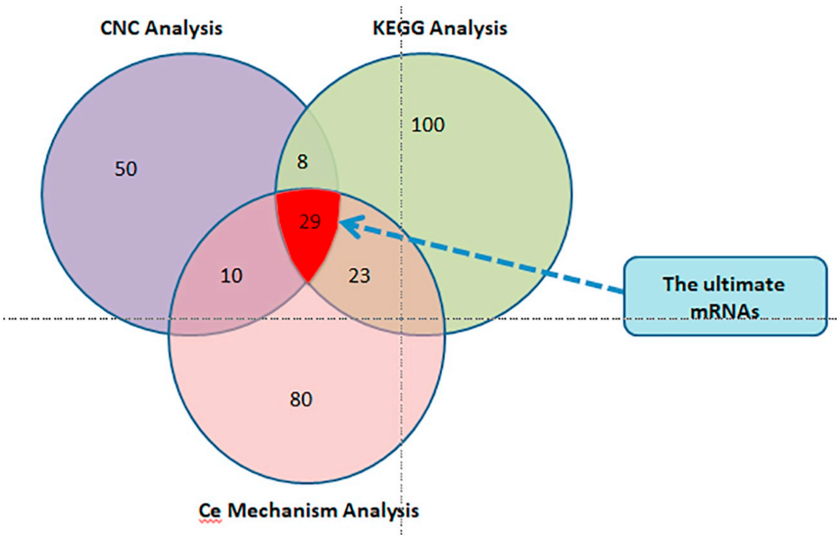


Fig. 7. Determination of the final target gene pool by information analysis.
*Total 50 target mRNAs of the top lncRNAs were obtained by CNC analysis as shown in the purple circle.Total 100 target mRNAs of the top lncRNAs were obtained by KEGG enrichment and found to have differential expression related to inflammation as shown in the green circle. By analyzing the Ce and cis/trans mechanisms, total 80 target mRNA were obtained for Top 10 lncRNAs as shown in the pin circle. Then we crossed them then obtained ultimate 29 targeted mRMAs as shown in the red circle. These 29 target genes were identified to be co-expressed with the lncRNAs by CNC analysis. KEGG analysis confirmed that they were associated with inflammatory response. In addition, microRNAs/TF, an intermediate regulatory factor, could be obtained by Ce analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
LncRNA-miRNA/TF-mRNA gene pool composed of three elements.

lncRNA	MicRNA/TF	mRNA	Signal pathway
ENST00000465605.5_1	miR-128, miR-132, miR-135a,miR-141, miR-181	IL17//RORγ-t//TGF-β//MHC-II //IL-22	Th17_cell_differentiation Cell migration
ENST00000409569.2_1	miR-3165,miR-338,miR-362, miR-366	MCC//EGFR//AKT//TGF-β//IL-6	Cell proliferation/fibrosis
ENST00000392442.6_1	miR-456, miR-462 miR-479, miR-577	Beclin1//ENPP1//ENTPD5 //IL-22//LC3	Cellular immunity
ENST00000524613.5_1	miR-121, miR-197, miR-200a	ANK3//EGFR//ERBB3//TRL5	Cellular immunity
ENST00000525216.1_1	XBP1s , EBP	APC//ITGB2/MYLK	Cell proliferation
ENST00000487539.1_1	miR-67, miR-123, miR-636, miR-133 miR-128, miR-212,	EGFR//FOXP3//TGF-β/CASPASE9//ATG5//CASPASE10 IL17/EGFR//FOXP3//GATA-3//HLA-DPB1	Cell apoptosis Endocytosis
ENST00000558814.1_1	NF-Kb, NKX2-1		Autoimmune inflammation

leads to a reduced experimental inaccuracy, and therefore, an increased dataquality. This is the first time to detect both lncRNA and RNA level in CD patients in the same chip experiment. By CNC analysis of the data obtained from chip assay, we established correlation between lncRNAs and mRNAs, which may help us to elucidate the regulatory network underlying the development of CD.

This research provided direction for future analysis on how lncRNAs regulates inflammation pathways, and revealed that the intestinal gene expression changes can initiate a persistent immune response. We found that ENST00000487539.1_1 may promote intestinal epithelial cells apoptosis or phagocytosis by regulating some related target genes, such as EGFR//FOXP3//TGF-β/Casepase9//ATG5//Casepase10. Qiao et al. reported that the serum level of ENST0000487539_1.1 was increased in CD patients, and ENST0000487539_1.1 could regulate Treg cell function by modulating the level of Foxp3 [25]. Thwe increased ENST0000487539_1.1 in serum as well as in CD lesions raised a possibility that suggesting that ENST0000487539_1.1 may be released into circulation by the intestinal epithelial cells. For further study, We need to verify whether lncRNA ENST0000487539_1.1 can induce intestinal epithelial cells apoptosis by regulating target genes such as Foxp3, ATG5 and Casepase10. Moreover, biological function of aerum ENST0000487539_1.1 need to be addressed in cell experiments as well as animal models.

In conclusion, as summarized in Table 4, this study identified numerous is study identified numerous candidate lncRNA differentially expressed in CD lesions, determined their potential targets, and constructed a premliminary lncRNA-miRNA/TF-mRNA network that may be related to CD pathogenesis. Findings from the study help us to narrow the scope of CD gene search, and laid a foundation for follow-up studies of gene functions and regulatory mechanisms.

Author contributions

Na Li and Ruihua Shi designed the study; Na Li completed all data analysis; Na Li performed colonoscopies and collected ileal mucosa tissue from crohn's disease patients. Na Li performed experimental operations; Na Li summed up the experimental results and wrote the manuscript; Ruihua Shi provided the financial support for this work.

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Appendix A. Supplementary data

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