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Circular RNA expression alterations in colon tissues of Crohn's disease patients

YU QI QIAO, CHEN WEN CAI, JUN SHEN, QING ZHENG and ZHI HUA RAN

Division of Gastroenterology and Hepatology, Key Laboratory of Gastroenterology and Hepatology, Ministry of Health, Inflammatory Bowel Disease Research Centre, Shanghai Institute of Digestive Disease, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200001, P.R. China

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Abstract. Genetic factors are crucial in the development of Crohn's disease (CD). Circular RNAs (circRNAs) are known to function as microRNA (miRNA) sponges and regulate a number of signalling pathways via circRNA-miRNA interactions. As competing endogenous RNAs, the functions of circRNAs in CD should be investigated. In the present study, colon biopsy tissues were collected from ileocolon (L3)-active CD patients and healthy controls. circRNA microarrays were performed with colon tissues from 3 CD patients and 3 controls. Subsequently, the candidate circRNAs were verified via reverse transcription-quantitative polymerase chain reaction using colon tissues from a further 10 CD patients and 10 controls. Targeted miRNAs, genes and pathways of candidate circRNAs were predicted and analysed. Arraystar circRNA microarrays demonstrated that there were 163 upregulated circRNAs targeting 435 miRNAs and 55 downregulated circRNAs targeting 207 miRNAs (fold-change >2 and P<0.01) in CD patients. As a candidate circRNA, hsa-circRNA-102685 was observed to putatively target hsa-miR-146b-5p, hsa-miR-182-5p and hsa-miR-146a-5p. Furthermore, Kyoto Encyclopaedia of Genes and Genomes pathway analysis predicted that hsa-circRNA-102685 potentially participated in apoptosis, and in the Toll-like receptor and p53 signalling pathways. Overall, the current study suggested that circRNA alterations serve an important role in the pathogenesis of CD. circRNAs, such as hsa-circRNA-102685, are involved in certain important signalling pathways of CD, and may be novel targets for diagnosis or treatment in this disease.

Correspondence to: Dr Zhi Hua Ran, Division of Gastroenterology and Hepatology, Key Laboratory of Gastroenterology and Hepatology, Ministry of Health, Inflammatory Bowel Disease Research Centre, Shanghai Institute of Digestive Disease, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, 145 Middle Shandong Road, Shanghai 200001, P.R. China
E-mail: zhihuaran@vip.126.com

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Introduction

Inflammatory bowel disease (IBD) refers to a group of conditions characterised by chronic inflammation of the bowel, including Crohn's disease (CD) and ulcerative colitis. A number of factors are associated with the development of IBD, including genetic, environmental, microbial and immune response factors (1,2). It has been reported that microRNAs (miRNAs) serve important roles in IBD (3-7). For instance, several studies have demonstrated an association between miR-21 and IBD in patients and animal models (8,9). In addition, Brain *et al* (10) provided a profile of how nucleotide-binding oligomerization domain-containing protein 2 (NOD2) induced miR-29 to limit interleukin-23 release in human dendritic cells (DCs). NOD2 mutations have also been reported to be associated with CD (11). Brest *et al* (12) identified that a variant in immunity-related GTPase family M protein (IRGM) gene altered a binding site for miR-196 and caused the deregulation of IRGM-dependent xenophagy in CD. In a murine model of IBD, miR-146 regulated NOD2-derived gut inflammation and served an important role in amplifying inflammatory responses (13). Other functions of miR-146 were also confirmed to be associated with IBD, including the regulation of the Toll-like receptor (TLR) signalling pathways, T cell differentiation and regulator T (Treg) cell function (7,14).

Circular RNA (circRNA) is a type of RNA that can form a covalently closed loop. The 5' cap or 3' poly(A) tail are not found in circRNA, and this characteristic results in the difference of circRNAs from other linear RNAs. In the 1970s, circRNAs were detected in viruses and extracted from certain eukaryotic cells; however, their functions were not fully understood at that time (15,16). In 2010, Poliseno *et al* (17) demonstrated that expressed pseudogenes were able to regulate the expression of coding genes. Salmena *et al* (18) further hypothesised that circRNAs are competing endogenous RNAs (ceRNAs). This hypothesis suggested that miRNAs do not interact with messenger RNAs (mRNAs) unidirectionally, but they interact with the pool of mRNAs, transcribed pseudogenes and long noncoding RNAs (18). In 2013, Memczak *et al* (19) described circRNAs as a large class of RNAs with regulatory potency, with thousands of circRNAs potentially existing stably in eukaryotic cells. These authors further revealed that the human circRNA CDR1as was able to be densely bound

by miRNA (19). circRNAs reportedly function as miRNA sponges, while they have not been found to function as coding proteins to date. Currently, the most likely role of circRNAs is considered to be as ceRNAs.

Although miRNAs are known to serve important roles in the pathogenesis of IBD, the roles of circRNAs in the development of IBD remain unclear. The present study investigated the expression levels of circRNAs, considered as miRNA sponges, in the colonic tissues of CD patients. In addition, one of the potential circRNA-miRNA-gene networks was predicted by further examining a circRNA candidate.

Materials and methods

Patients and samples. Between April 2015 and March 2017, 13 patients with active CD (8 males and 5 females, mean age 30.69±6.25, from 18 to 38, without any other known diseases) who were treated at the Renji Hospital (Shanghai, China) and 13 healthy controls (7 males and 6 females, mean age 32.77±5.89, from 24 to 43, without any other known diseases), who were physicians at this hospital, were enrolled into the present study. The diagnosis of CD was established based on the medical history, endoscopy, biopsy, imaging and serological studies. The diagnosis of CD was confirmed by two different gastroenterologists. All patients were classified into the L3 (ileocolon) type of CD according to the Montreal Classification, and exhibited a Harvey-Bradshaw index score of ≥5 (20,21). All colonic samples were collected under colonoscopy prior to treatment. Samples were stored in RNAlater stabilization reagent (Qiagen, Valencia, CA, USA) following the protocol provided by the manufacturer. Samples from 3 random patients with active CD and 3 random healthy controls were initially selected for circRNA microarray analysis. The remaining samples from 10 CD patients and 10 healthy controls were used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) verification. All patients and healthy donors were well informed of the study details, and provided written informed consent. The experiments were approved by the Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University (Shanghai, China).

RNA isolation. Total RNA was extracted from the colonic samples using TRIzol™ reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the protocol provided by the manufacturer. Next, RNA was purified with the RNeasy Mini kit (Qiagen) according to the manufacturer's guidelines. A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.) was used to measure the concentration of RNA based on the OD_{260}/OD_{280} ratio.

circRNA microarray analysis. Arraystar Human Circular RNA Array (Arraystar, Inc., Rockville, MD, USA) analysis was respectively performed in 3 samples from patients and controls to determine the expression profile of circRNAs. The sample preparation and microarray hybridization were performed based on the standard protocols provided by Arraystar. Briefly, total RNA from each sample was amplified and transcribed into fluorescent complementary RNA utilizing random primers, according to the Arraystar Super RNA Labelling protocol (Arraystar, Inc.). The labelled circRNAs were hybridised onto the Arraystar Human circRNA Array (6x7K; Arraystar, Inc.). Subsequent to washing the slides, the arrays were scanned by the Axon GenePix 4000B microarray scanner (Molecular Devices, LLC, Sunnyvale, CA, USA). The scanned images were then imported into the Axon GenePix Pro 6.0 software (Molecular Devices, LLC) for grid alignment and data extraction. The R package (https://www.r-project.org/) was used for quantile normalization and subsequent expression matrix processing. Differentially expressed circRNAs between the two groups that presented a statistically significant variance were confirmed by filtering with a volcano plot. For the selection of circRNA candidates, differentially expressed circRNAs were defined as having a fold-change of >2 and $P<0.01$. Hierarchical clustering was then performed to identify distinguishable patterns of circRNA expression among the samples. Since miR-146 has already been demonstrated to be involved in the pathogenesis of CD (7), one of the circRNAs that putatively targeted both miR-146a and miR-146b was selected as the candidate.

RT-qPCR. To validate the circRNA expression profile detected using the Arraystar Human circRNA Array, the expression level of hsa-circRNA-102685 (circRNA-102685) was further validated by RT-qPCR in the remaining samples from 10 CD patients and 10 healthy controls. Briefly, total RNA was incubated for 1 h at 37˚C with 0 units (serving as the mock treatment) or 20 units of RNase R (Epicentre; Illumina, Inc., San Diego, CA, USA) (22). RNA was subsequently purified with the RNeasy Mini kit (Qiagen), and reverse transcribed to synthesise cDNA with ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). The process of reverse transcription was performed according with the manufacturer's instructions: Incubation of RNA solution at 65˚C for 5 min, followed by incubation at 37˚C for 15 min, and lastly heating at 98˚C for 5 min. Next, the AFD9600 Real-time PCR Detection system (Hangzhou AGS MedTech Co., Ltd., Hangzhou, China) and the SYBR Green qPCR SuperMix (Invitrogen; Thermo Fisher Scientific, Inc.) were applied to perform qPCR assay. Primers were designed using Primer Premier 6.0 (Premier Biosoft International, Palo Alto, CA, USA), and were as follows: circRNA-102685, 5'-ACATTCTGCCAGTGCGTCAAC-3' (forward) and 5'-GAATCTTCAGGACAACGTGGAGAG-3' (reverse); GAPDH, 5'-ACTTTGGTATCGTGGAAGGAC TCAT-3' (forward) and 5'-GTTTTTCTAGACGGCAGGTCA GG-3' (reverse). GAPDH served as an internal standard control. The PCR conditions included a 95˚C denaturation for 5 min, followed by 40 cycles at 95˚C for 5 sec and 61˚C for 30 sec. Three independent samples were tested for each CD patient and control individual, and all reactions were performed in triplicate. The quantification cycle (Cq) was applied to define the expression of circRNA-102685, and the $2^{-\Delta\Delta Cq}$ method was used to calculate the relative expression levels (23).

Bioinformatics analysis and biological function prediction. Arraystar's homemade miRNA target prediction software based on TargetScan (24) and miRanda (25) was applied for the prediction of circRNA-miRNA interactions. The top 5 potential target miRNAs were selected from the analysis. IBD-associated miRNAs were selected based on the review published by Kalla *et al* (7), while TargetScan

(http://www.targetscan.org) was used to predict the putative target genes of miRNAs. A visualised circRNA-miRNA-gene network was subsequently generated using the Cytoscape software platform (https://cytoscape.org/). Gene Ontology (GO) enrichment analysis was conducted for annotating the functions of the target genes, while Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis was also performed to determine the involved pathways (26-28).

Statistical analysis. Student's t-test was used for continuous variables, and data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using R package (version 3.4.4) and GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA).

Results

circRNA expression profiling in colon tissues of CD. Overall, 218 circRNAs were identified to be differentially expressed in colon tissues between CD patients and controls. A total of 163 circRNAs were upregulated in the CD group, and these putatively targeted 435 miRNAs. In addition, 55 circRNAs were downregulated in the CD group and putatively targeted 207 miRNAs. Hierarchical clustering and patterns of circRNA expression are displayed in Fig. 1. Of these putative miR-146-targeting circRNAs, 4 were upregulated and 3 were downregulated (Table I). circRNA-102685 was the only upregulated circRNA that putatively targeted both miR-146a and miR-146b, and was thus selected for further investigation.

circRNA candidate selection and validation with RT-qPCR. According to the aforementioned results, circRNA-102685 was selected as a candidate for further verification. In the circRNA microarray analysis, the genomic locus of circRNA-102685 was detected to be on chromosome 2, and the predicted best transcript was uc002rpd.3. Furthermore, circRNA-102685 was upregulated by 3.02-fold in the CD group as compared with the control group (P<0.01). According to the results of RT-qPCR validation, circRNA-102685 was upregulated by 1.46-fold in the CD group as compared with the control group (P<0.01; Fig. 2).

Candidate circRNA-miRNA-gene network prediction. As circRNAs are potential upstream molecular sponges for their target miRNAs, the TargetScan and miRanda tools were used to predict the circRNA-miRNA-gene network. The top 3 putative miRNA targets of circRNA-102685 were hsa-miR-146b-5p, hsa-miR-182-5p and hsa-miR-146a-5p. In total, 47 target genes of these 3 miRNAs were identified by target gene prediction using TargetScan database. Next, the circRNA-102685-miRNA-gene network was constructed, and is displayed in Fig. 3. Subsequent prediction of biological pathways provided a profile of the potential functions of these target genes. The target genes were strongly associated with KEGG pathways that were associated with cancer (including pathways in cancer, prostate cancer, pancreatic cancer, melanoma, chronic myeloid leukaemia, colorectal cancer and small cell lung cancer), cell growth and death (including apoptosis, cell cycle, and p53 signalling pathways), immune system (including TLR, RIG-I-like receptor and chemokine signalling pathways), nervous system (namely neurotrophin signalling pathway), cellular community-eukaryotes (adherens junction), endocrine system (melanogenesis), transport and catabolism (endocytosis), and signalling molecules and interaction (cytokine-cytokine receptor interaction). The significant KEGG pathways are displayed in Fig. 4A. Furthermore, GO analysis revealed that these target genes were mainly associated with cell death, apoptosis and phosphorylation (Fig. 4B).

Table I. Differentially expressed circRNAs that putatively target microRNA-146 in Crohn's disease.

circRNA	Fold change	P-value
Upregulated		
hsa_circRNA_102774	8.564309	<0.001
hsa_circRNA_101753	7.143933	0.006
hsa_circRNA_102685	3.020609	0.004
hsa_circRNA_102261	4.532291	<0.001
Downregulated		
hsa_circRNA_101598	3.31592	0.008
hsa_circRNA_101863	2.424199	0.003
hsa_circRNA_100189	2.337042	0.002

circRNA, circular RNA.

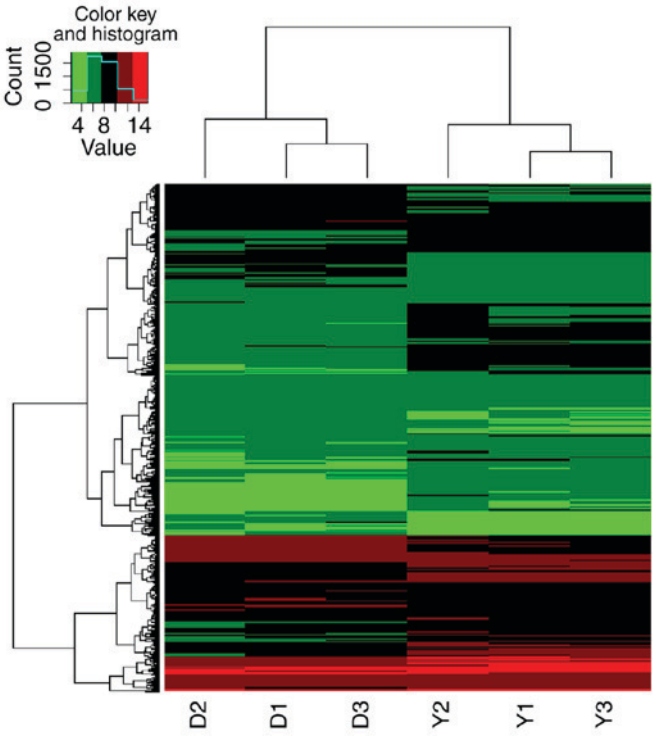

Figure 1. Hierarchical clustering patterns of circRNA expression among the samples. A total of 218 circRNAs were found to be differentially expressed in colon tissues between CD patients and controls (fold change of >2 and P<0.01). In the CD group, 163 circRNAs were upregulated and 55 circRNAs were downregulated. Y1-Y3 represent the CD patients, and D1-D3 represent the healthy controls. circRNA, circular RNA; CD, Crohn's disease.

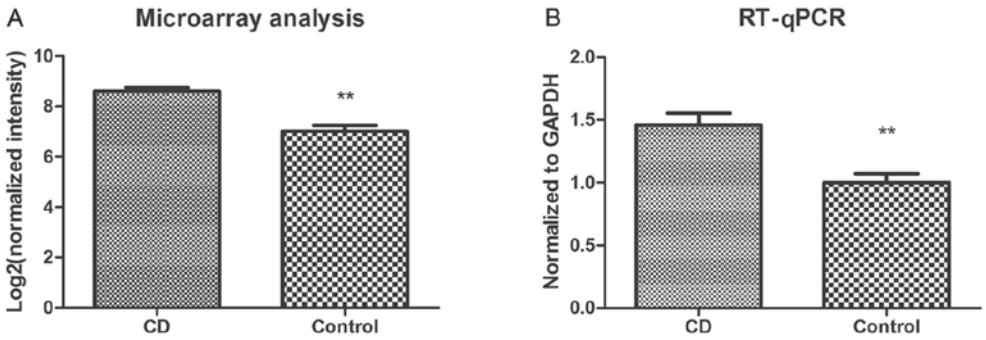

Figure 2. Expression of hsa-circ-RNA 102685 in CD group and control group. (A) The circRNA microarray indicated that hsa-circRNA-102685 is 3.02-fold upregulated in the CD group compared with the control group. The fold change is presented with a logarithm base 2 transformation. (B) The RT-qPCR assay indicated that hsa-circRNA-102685 is 1.46-fold upregulated in the CD group compared with the control group. **P<0.01 CD group vs. control group. circRNA, circular RNA; CD, Crohn's disease; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

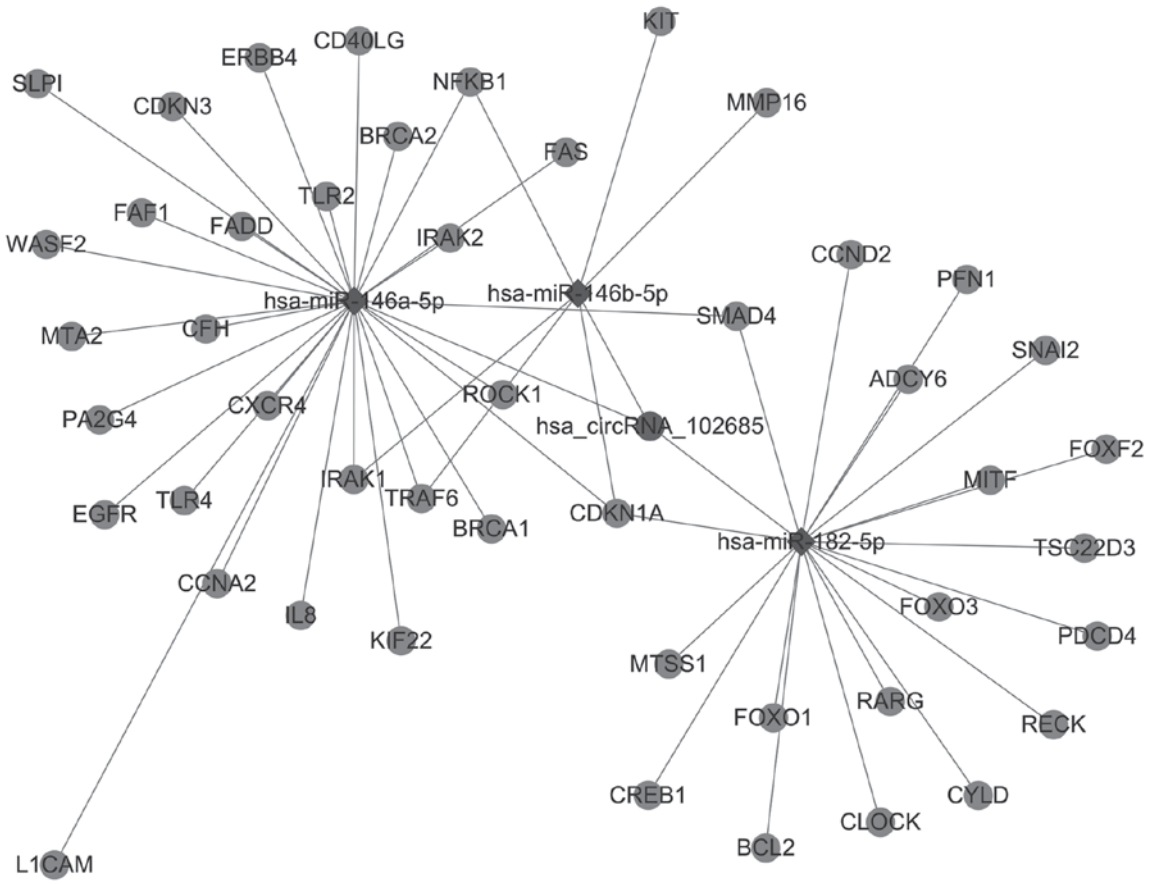

Figure 3. Predicted hsa-circRNA-102685-miRNA-gene network. The top 3 putative miRNA targets were hsa-miR-146b-5p, hsa-miR-182-5p and hsa-miR-146a-5p. The 3 miRNAs and 47 targeted genes are presented in circRNA-miRNA-gene network. circRNA, circular RNA; miR, microRNA.

Discussion

CD is a life-long disease involving chronic gastrointestinal inflammation; however, its causes are currently not fully understood. As the functions of miRNAs have been reported to be associated with CD (7), the current study aimed to examine the profile of circRNAs in this disease, which are another type of RNAs. circRNAs can function as miRNA sponges, and may work together with miRNAs to regulate the expression of target genes (29). In addition, circRNAs are very stable in eukaryotic cells, with a half-life that can reach 48 h, while the half-life of miRNAs is only 10 h (30). This characteristic makes circRNAs stable molecular markers in diseases.

circRNAs have been reported to be potential molecular markers in different types of cancer (31). However, a number of studies have demonstrated that circRNAs may also be involved in certain non-cancer diseases. For instance, Wang *et al* (32) observed that a heart-associated circRNA targeting miR-223 was able to protect the heart, and serve as a potential therapeutic target in the treatment of cardiac hypertrophy and heart failure. Furthermore, the circRNA CDR1as

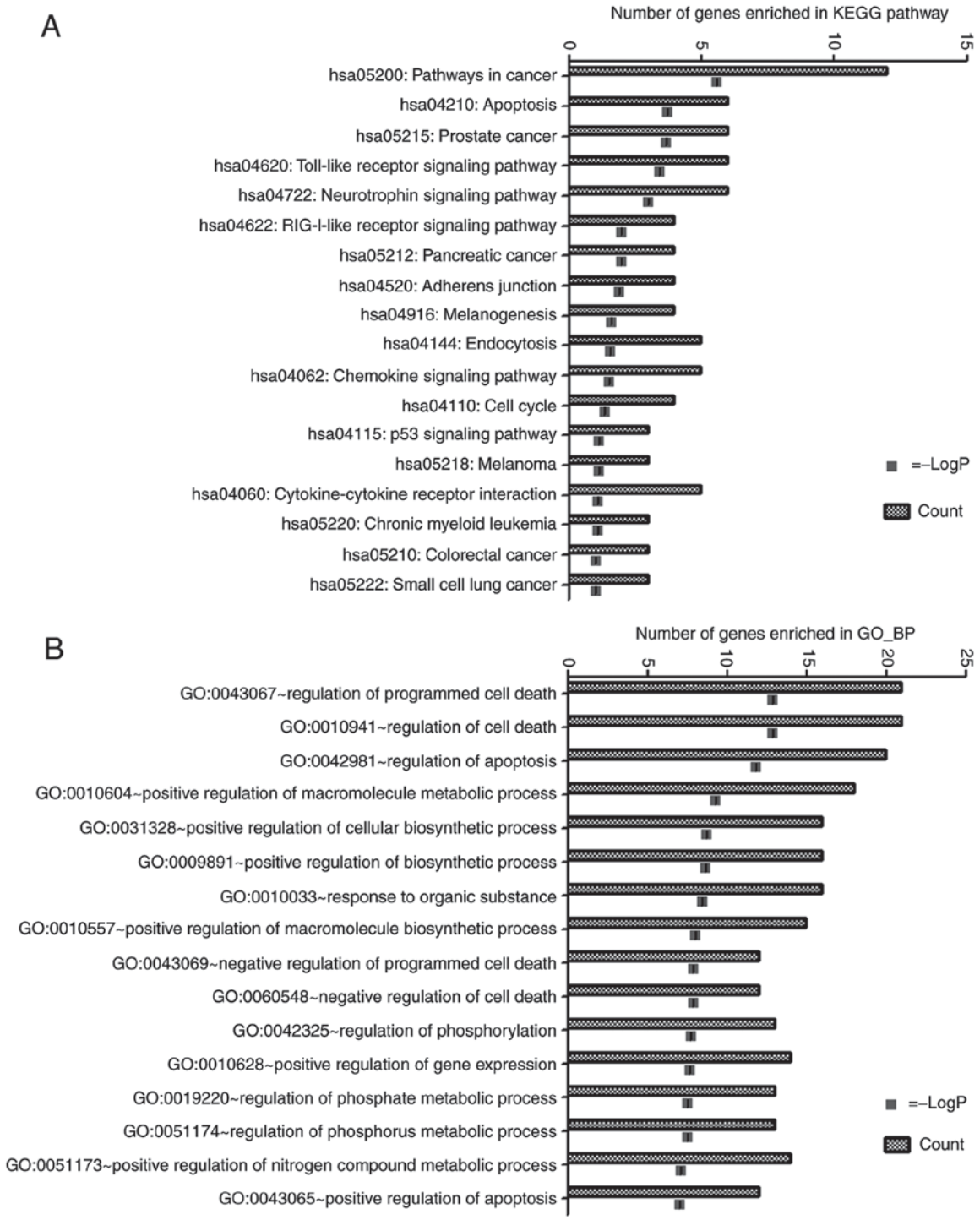

Figure 4. Annotation of hsa-circRNA-102685 putative targeted pathways. (A) KEGG pathway enrichment analysis. (B) GO pathway enrichment analysis. circRNA, circular RNA; KEGG, Kyoto Encyclopaedia of Genes and Genomes.

was reported to be associated with neurodegenerative conditions (33). In chronic autoimmune disease, circRNAs are differentially expressed. The study by Liu *et al* (34) indicated that circ-CER may be associated with degenerative changes in the joint cartilage, which can be a potential target in osteoarthritis.

As a type of chronic autoimmune disease, CD was investigated in the present study, and alterations in the expression of circRNAs in CD patients were examined. The results demonstrated that >200 circRNAs were differentially expressed between CD patients and healthy controls. These circRNAs may function as sponges of miRNAs and serve different roles in the pathogenesis of CD.

Among the differentially expressed circRNAs identified in the current study, 7 candidates putatively targeted miR-146. Previously, miR-146 has been reported to be involved in the TLR signalling pathway, and thus serves a role in the pathogenesis of CD (35). Taganov *et al* (35) identified that the expression of both miR-146a and miR-146b in monocytes was induced by lipopolysaccharide, flagellin, peptidoglycan

and exposure to TLR ligands. In animal experiments, Nata *et al* (36) demonstrated that miR-146b improved intestinal inflammation by activating nuclear factor-κB. Furthermore, in a small sample study, Fasseu *et al* (37) identified that miR-146 was upregulated in CD tissues compared with the controls. A review by Xu and Zhang (5) summarised that miR-146 may affect the differentiation, maturation and functions of several different immune cells, including DCs, helper T cells and Treg cells. The present study identified that circRNA-102685 was upregulated by performing a circRNA array analysis, and that it potentially targeted both miR-146a and miR-146b. Thus, circRNA-102685 was selected as a candidate circRNA for further validation by RT-qPCR. The RT-qPCR results demonstrated that circRNA-102685 was upregulated in colon tissues obtained from CD patients as compared with the healthy controls. Therefore, circRNA-102685 may serve a role in regulating target gene expression via miR-146 in the pathogenesis of CD.

Further investigation focused on the potential circRNA-miRNA-gene network. In the current study, it was predicted that circRNA-102685 potentially functioned as a sponge of miR-146. In the predicted circRNA-miRNA-gene network, a number of important genes were identified that are known to be associated with the pathogenesis of CD, such as IRAK1, IRAK2, BCL2, NFκB1, CREB1 and TRAF6 (38-42). Furthermore, the prediction of KEGG pathways presented several top pathways associated with circRNA-102685, including apoptosis, TLR and p53 signalling pathways. These pathways have been demonstrated to be associated with IBD (43-46). Other pathways, such as the RIG-I-like receptor signalling pathway, chemokine signalling pathway and cytokine-cytokine receptor interaction pathway, are potentially associated with IBD (47-49). The association between cancer and IBD has already been demonstrated (50), and the prediction conducted in the current study identified the involvement of certain cancer-associated pathways. GO enrichment analysis presented similar results. However, the role of circRNA-102685 in these pathways requires further research.

There are several limitations in the current study. As the size and number of biopsy tissues were limited in patients with active CD, it was not possible to verify the expression of other circRNAs. In addition, only 13 CD patients and 13 controls were enrolled in the study. More studies on the role of circRNAs in IBD should be performed in the future.

In conclusion, the present study revealed that circRNAs were differentially expressed in colon tissues of CD patients in comparison with the healthy controls. circRNA expression alterations may serve an important role in the pathogenesis of CD. The expression changes of hsa-circRNA-102685 may be involved in the process of CD. Certain associated pathways that may be involved in the process of CD were identified, and these may serve as novel targets in the diagnosis and treatment of this disease.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

YQQ and JS designed the study. YQQ worked on the bioinformatics analysis and drafted the manuscript. CWC collected the samples and performed the verification work. QZ and ZHR co-worked on the analysis and critically reviewed the interpretation of data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All patients and healthy donors were well informed of the study details, and provided written informed consent. The experiments were approved by the Ethics Committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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