Expression of miRNAs derived from plasma exosomes in patients with intestinal Behçet's syndrome

C.-C. Hou, H.-F. Bao, Y. Shen, J.-F. Ye, H.-F. Ma, J.-L. Guan

Department of Rheumatology and Immunology, Huadong Hospital affiliated to Fudan University, Shanghai, China.

Abstract

Objective

MicroRNAs (miRNAs) derived from plasma exosomes are potential diagnostic biomarkers. However, little is known about the expression of miRNAs derived from plasma exosomes in patients with intestinal Behçet's syndrome (BS). This study aimed to explore the difference of miRNAs derived from plasma exosomes between intestinal BS patients and healthy people, and further identify potential biomarkers that predict the disease activity of intestinal BS.

Methods

A total of 43 intestinal BS patients and 23 healthy volunteers were enrolled, among whom 23 were active intestinal BS and 20 were stable intestinal BS. The miRNAs expression profiles of plasma exosomes in 3 active intestinal BS patients and 3 healthy volunteers were determined using next-generation high throughput sequencing. Additionally, significantly differentially expressed miRNAs were further analysed by quantitative real-time polymerase chain reaction (qRT-PCR) in a validation cohort of 60 subjects.

Results

From the sequencing analysis, 15 miRNAs were identified to be differently expressed ($p<0.05$). Of these, 13 miRNAs were up-regulated, and 2 were down-regulated in intestinal BS patients compared with healthy volunteers. Furthermore, qRT-PCR analysis confirmed that miR-141-3p was down-regulated and miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p and miR-342-5p were up-regulated in intestinal BS patients' plasma exosomes. Additionally, the level of miR-141-3p was negatively correlated with disease activity indicators of intestinal BS, while miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p and miR-342-5p was positively correlated with disease activity indicators of intestinal BS.

Conclusion

Circulating miR-141-3p, miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p and miR-342-5p derived from plasma exosomes may serve as biomarkers of disease activity in intestinal BS.

Key words

Behçet's disease, exosomes, miRNA

Cheng-cheng Hou, MD
Hua-fang Bao, MD
Yan Shen, MD
Jing-fen Ye, MD
Hai-fen Ma, MD
Jian-long Guan, MD, PhD

Please address correspondence to:
Jian-long Guan,
Department of Rheumatology and Immunology,
Huadong Hospital affiliated to Fudan University,
221 Yan'an West Road,
Shanghai 200040, P.R. China.
E-mail: jianlong_guan@126.com

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Introduction

Behçet's syndrome (BS), also known as Behçet's disease, is a rare vasculitis disease characterised by recurrent oral and genital ulcerations, ocular inflammation, and skin lesions (1). The disease occasionally generates severe manifestations involving cardiovascular, haematology, neurological systems and gastrointestinal tract (2). When BS patients present with typical intestinal ulcers, intestinal BS can be diagnosed. Recently, our study found that intestinal involvements are the most common major organ involvements in a cohort of 860 BS patients with a prevalence of 20.7%, and nearly half of patients (43.6%) with intestinal BS without gastrointestinal symptoms were found under endoscopy (3). Moreover, some intestinal BS patients often undergo acute abdominal surgery due to sudden intestinal bleeding and perforation, and reperforation at the surgical incision site is common. However, there were few specific biomarkers of intestinal involvement in BS patients. It is necessary to identify novel biomarkers thar can be used for the diagnosis and prognostic evaluation of intestinal BS.

Exosomes are a type of endogenous extracellular vesicles ranging from 30 nm to 150 nm, and containing many bioactive substances, such as proteins, lipids, DNAs, mRNAs and miRNAs, and their surface is rich in CD9, CD63 and other transmembrane proteins which can be used for the detection of exosomes (4, 5). As one of the key substances in exosomes, miRNAs can regulate mRNA expression in living organisms, thereby being capable of further regulating various biological pathways in living organisms (6), and multiple studies have also shown that miRNAs can regulate the expression of nearly half of the genes *in vivo* (7-9). However, circulating miRNAs are not stable in plasma, and exosomes can protect miRNAs from degradation. Therefore, exosomal miRNAs may provide potential biomarkers for disease diagnosis and treatment. In recent years, accumulating evidences have reported the importance of miRNAs obtained from exosomes as potential clinical diagnostic markers and therapeutic strategy (10-12). At present, studies on miRNAs derived from plasma exosomes in BS patients have not been reported. Only one study reported BS patients having elevated numbers of plasma extracellular vesicles, and extracellular vesicles could be internalised by peripheral blood mononuclear cells triggering interleukin 6 (IL-6) secretion (13).

Previous studies have found that miR-21, miR-146b, miR-326 and miR-155 may be associated with BS (14-16), but the expression of miRNAs derived from exosomes has not been well explored. Thus, in the present study, we aimed to explore the difference of miRNAs derived from plasma exosomes between intestinal BS patients and healthy people using high throughput sequencing, and further validate the relative expression of these differential miRNAs in a large sample of intestinal BS patients using quantitative real-time PCR (qRT-PCR) technique, based on the results of high throughput sequencing.

Patients and methods

Patients and clinical samples

BS patients who were admitted to Huadong Hospital affiliated to Fudan University from September 2019 to March 2021 were enrolled in this study, and later underwent a series of related examinations, including endoscopy, routine blood and biochemical tests. The diagnosis of intestinal BS was confirmed with extraintestinal systemic manifestations, the characteristic endoscopic, histopathologic, and radiological features, which helped to distinguish intestinal BD from Crohn's disease (17). Intestinal BS patients were divided into those with active disease and inactive disease, using the simplified Behçet's Disease Current Activity Form (BDCAF) (18). Active disease was defined as a BDCAF value ≥2. A total of 23 active, 20 stable intestinal BS and 23 healthy volunteers as normal controls (NC) were enrolled in this study. The clinical and demographic characteristics of intestinal BS patients and NC were collected. The clinical parameters of intestinal BS patients included erythrocyte sedimentation rate (ESR), C-reaction protein (CRP),

BDCAF value and the actual disease activity index for intestinal Behcet's disease (DAIBD). DAIBD was able to assess the intestinal manifestations of intestinal BS, composed of the following eight variables: general well-being, fever, extraintestinal manifestations, abdominal pain, abdominal mass, intestinal complications, and number of liquid stools in one week (19). 10 mL peripheral blood was collected from these patients and volunteers. Blood samples were centrifuged at 1500 g for 20 min and then 3000 g for 15 min at 4°C. The plasma was carefully isolated and stored at -80°C in the refrigerator until use. The study was approved by the Research Ethics Committee of Huadong Hospital affiliated to Fudan University (2018K031 and 2020K034), and informed consent was obtained from all participants.

Inclusion and exclusion criteria

Inclusion criteria were as follows:

1. the patients were between 18 and 65 years old; 2. all patients met the new International Criteria for Behçet's Disease (ICBD) (20); 3. all patients were diagnosed as intestinal BS with extraintestinal systemic manifestations, the characteristic endoscopic, histopathologic, and radiological features (17); 4. all patients had complete clinical data and laboratory parameters.

The exclusion criteria were as follows:

1. patients with other disturbing diseases, such as infective diseases, other autoimmune diseases, endocrinal disorders, or malignancies; 2. patients who had used non-steroidal anti-inflammatory drugs (NSAIDs) in the past 3 months; 3. patients who were pregnant or who were breast feeding.

Isolation of exosomes

Plasma from intestinal BS patients and NC were processed by serial centrifugations (21, 22). Briefly, the plasma was centrifuged at 10000g for 30 min at 4°C (repeated twice). Then the supernatant was passed through a 0.22μm filters and diluted with PBS. After dilution, the diluted supernatant was ultracentrifuged at 110,000g for 70 min at 4°C with Beckman Optima L-100XP. The exosomes pellets were washed with pre-cooled PBS followed by the second ultracentrifugation at 110,000g for 70 min at 4°C and then resuspended in PBS.

Identification of exosomes by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and western blot

For transmission electron microscopic visualisation of exosomes, 10ul exosomes suspended in PBS were placed on copper grids for 10 min and negatively stained with 2% uranyl acetate solution for 1 min. The images were obtained using an H-7650 Electron Microscope (Hitachi, Tokyo, Japan) operated at 80 kV. The particle matrix ZetaView PMX 110 was used to measure the concentration of the isolated exosomes under 405nm emission light. Then the exosomes diluted with PBS to $1x10^7$ particles/ml ~ $1x10^9$ particles/ml, and their size and quality were measured. Particles of 30–150 nm were designated as exosomes. The exosome pellets were homogenised in lysis buffer containing proteinase inhibitors, and the protein concentration was determined using the BCA kit (KGP902, KeyGen, China). Protein samples were loaded to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membrane (Millipore, Massachusetts, USA). The membrane was incubated overnight at 4°C with anti-CD9 (1:1000 dilution, ab92726, Abcam), anti-TSG101 (1:1000 dilution, ab125011, Abcam) and anti-CD63 (1:500 dilution, D160973, Sangon Biotech), followed by incubation with the secondary antibody (1:5000 dilution, 3310ES60, Yeasen) at room temperature for 1 h. The images were acquired with the ChemiDoc MP System (Bio-Rad, USA).

RNA isolation and RNA analyses

The total RNA was extracted and purified from plasma exosomes using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Germany) according to the manufacture's instructions. RNA concentration and purity were evaluated using the RNA Nano 6000 assay kit of the Agilent Bioanalyzer 2100 System (Agilent Technologies, CA, USA).

Library preparation and high throughput sequencing

For small RNA libraries, a total amount of 1ng-500ng RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using QIAseq miRNA Library Kit (Qiagen, Germany) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Finally, library quality was assessed on the Agilent Bioanalyzer 2100 and qPCR. The clustering of the index-coded samples was performed on acBot Cluster Generation System using TruSeq PE Cluster Kitv3-cBot-HS (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and paired-end reads were generated.

Sequencing data analysis

Bowtie software was used to compare clean reads with Silva database, GtRNAdb database, Rfam database and Repbase database, and ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA) and other ncRNA and repetitive sequences were filtered out. The remaining reads were used to detect known miRNAs and new miRNAs predicted by comparing with known miRNAs from miRbase and Human Genome (GRCh38), respectively. The read count for each miRNA was obtained from the mapping results, and transcript per million (TPM) was calculated. The differential expression analysis of two groups was performed using the edgeR software. Considering that low-expressed miRNAs have little biological significance, and in order to obtain more meaningful functional annotation analysis results, we only retained miRNAs with an average expression level of TPM higher than 10 as the final differentially expressed miRNA. A heat map for cluster analysis was created based on the relevant |log2| value, featuring miRNA with remarkable differences. Prediction of target genes of the differential miRNA was conducted based on RNAhybrid (http://bibiserv.techfak.uni-bielefeld. de/rnahybrid/) and miRanda

(http://www.microrna.org/Microrna/home.do). Meanwhile, Gene ontology (GO) (http://www.geneontology.org) and Kyoto encyclopedia of genes and genomes (KEGG) (http://www.genome.jp/kegg) analysis were performed for an enrichment analysis of the obtained target genes (23).

Validation of miRNA using qRT-PCR

The total RNA from exosomes was extracted using miRNeasy Serum/Plasma Advanced kit (Qiagen, Germany) according to the manufacturer's protocol. Reverse transcription was performed using a Mir-X miRNA First-Strand Synthesis kit (TAKARA, Japan) in accordance with the manufacturer's instructions. The abundance of target gene expression was detected by SYBR® Premix Ex TaqTM II kit (TAKARA, Japan) using qRT-PCR. Based on the results of high throughput sequencing and RefFinder tool, miRNA-30e-5p, the most stable reference miRNA, was chosen to normalise the relative expression ratio of other differential miRNAs. MiR-141-3p, miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p and miR-342-5p were selected from all the differential miRNAs for further PCR validation process, based on the results of high throughput sequencing. All samples were analysed in triplicate. The relative expression of targeted miRNA was calculated based on the threshold cycle (Ct) values as ratio=$2^{-\Delta\Delta Ct}$. MiRNAs with Ct above 37 were considered as not expressed. PCR primers are shown in Table I.

Table I. Primers used in qRT-PCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
miR-30e-5p	GCGCGTGTAAACATCCTTGAC	AGTGCAGGGTCCGAGGTATT
miR-141-3p	GGTCCTAACACTGTCTGGTAAAGTGG	CCAGTGCAGGGTCCGAGGT
miR-122-5p	TATTCGCACTGGATACGACACAAAC	GCCCGTGGAGTGTGACAATGGT
miR-150-3p	CAAGGACAGGAACCCTTCAGCA	CCATGATGCCTGGAAGACATTTC
miR-183-5p	CGCGGTATGGCACTGGTAGA	AGTGCAGGGTCCGAGGTATTC
miR-224-5p	CTGGTAGGTAAGTCACTA	TCAACTGGTGTCGTGGAG
miR-342-5p	CGCGAGGGGTGCTATCTGT	AGTGCAGGGTCCGAGGTATT

Table II. Basic information of intestinal BS (IBS) patients and NC.

Parameters	Active IBS (n=23)	Stable IBS (n=20)	NC (n=23)
Demographics			
Age (mean±SD)	29.48 ± 5.61	32.37 ± 4.52	28.80± 6.84
Sex (male:female)	13:10	10:10	11:12
ESR (mm/H)	24.61 ± 18.03	5.34 ± 3.91	–
CRP (mg/L)	11.83 ± 9.74	2.37 ± 1.80	–
DAIBD	47.75 ± 17.73	4.75 ± 11.75	–
BDCAF	2.39 ± 0.72	0.15 ± 0.37	–
Clinical characteristics of BS patients			
Major symptoms			
Oral ulcer	23	3	–
Genital ulcer	3	0	–
Skin lesion	3	0	–
Ocular lesion	1	0	–
Minor symptoms			
Arthritis	2	0	–
Gastrointestinal involvement	23	0	–
Epididymitis	0	0	–
Cardiovascular	0	0	–
Central nervous system involvement	0	0	–
Medication			
Immunosuppressive agent	23	20	–
Glucocorticoid	23	18	–

Statistical analysis

SPSS software v. 23.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All graphs were generated using GraphPad Prism 7.0 software. Continuous variables were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was used for quantitative data analysis. Non-parametric tests were applied for data with non-normal distributions. The Pearson or Spearman correlation analysis was used for evaluating the correlation between miR-141-3p, miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p, miR-342-5p and clinical parameters of intestinal BS patients, including ESR, CRP, BDCAF value and DAIBD respectively. A Receiving Operating Characteristic (ROC) curve was used to demonstrate the sensitivity and specificity of the above miRNAs for predicting intestinal BS activity, and the area under the curve (AUC) was calculated to assess the diagnostic potential of these miRNAs. $p<0.05$ was considered statistically significant.

Results

Clinical and demographic data

In this study, 46 intestinal BS patients including 23 active and 20 stable intestinal BS patients who hospitalised in Huadong Hospital affiliated to Fudan University and 23 healthy volunteers as NC were included. Six plasma exosomes of three active intestinal BS patients and three NC were selected for high throughput sequencing of miRNAs. The other people's samples were used for validation of differential miRNAs. The clinical and demographic characteristics of intestinal BS patients and NC are shown in Table II.

Identification of exosomes

Plasma exosomes were characterised by TEM, NTA and western blot. As the TEM result showed, these vesicles presented a double membrane and cup shape, which is typical for exosomes, the majority of which ranged from 30 to 150 nm (Fig. 1A). TSG101, CD63, and CD9 were enriched in the purified plasma exosomes by western blot (Fig. 1B). Moreover, exosomes were measured using NTA, which revealed a size distribution of the exosomes (mean size: 85nm) (Fig. 1C).

Differential miRNAs of intestinal BS-exosomes by high throughput sequencing

Six plasma exosomes of three active

intestinal BS patients and three NC were selected for high throughput sequencing of miRNAs. The correlation between sequenced samples is shown in Figure 2A. Data analysis showed that R2 of six samples were with a range of 0.86~1, which indicated that miRNAs expression among the samples of the same group were accredited biological replicates. Meanwhile, the known lengths of miRNAs in the human body mostly fall within a range of 20 to 25 nt. Based on this principle, detection of the isolated miRNAs was conducted, and a miRNA length distribution chart was created accordingly to visually evaluate the purity of the isolated miRNAs. The results indicated that the lengths of the extracted miRNAs were mostly centred at around 22 nt, suggesting that the isolated non-coding RNAs in this experiment were miRNAs (Fig. 1B). As disclosed by a volcano plot (Fig. 2C), 15 out of all the miRNAs had significantly different expression levels. Of these, 13 were up-regulated and 2 were down-regulated. Meanwhile, a heat map/cluster analysis of the selected 15 differential genes was conducted. As shown in Figure 2D, red indicates higher expression levels while blue indicates lower expression levels.

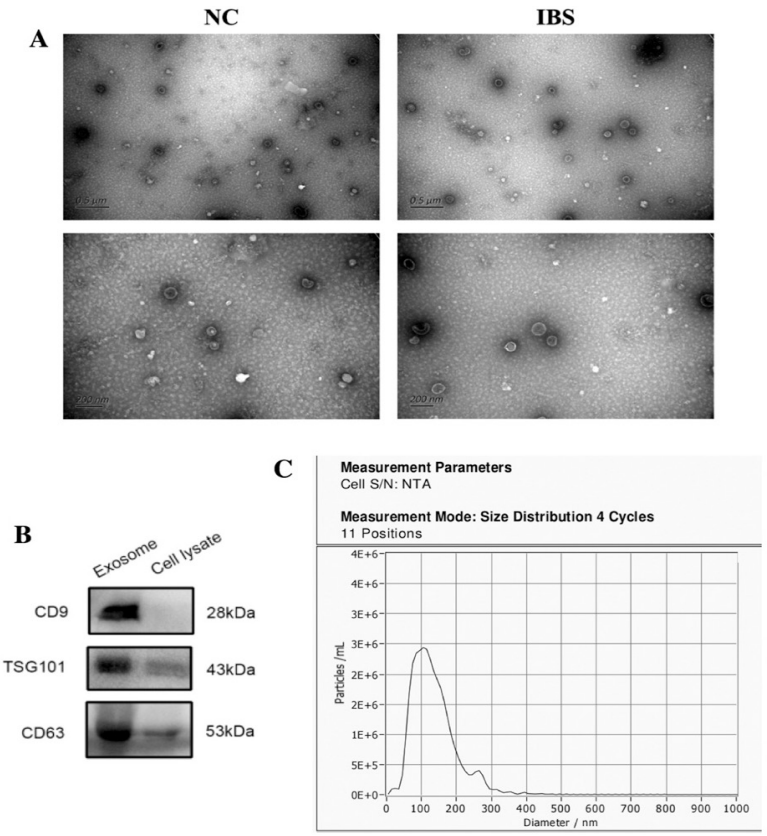

Fig. 1. Identification of exosomes.
A: Electron micrograph of negatively stained plasma exosomes. **B**: Western blot analysis of CD63, CD9, and TSG101 in plasma exosomes and lysates of blood cells. **C**: Representative NTA for plasma exosomes.

Table III. The correlation between differential miRNAs and disease activity indicators of BS.

Parameters	ESR	CRP	BDCAF	DAIBD
miR-141-3p (r/*P*)	-0.020/0.039	-0.050/0.012	-0.405/0.002	-0.019/0.002
miR-122-5p (r/*P*)	0.077/0.029	0.140/0.043	2.303/0.000	0.121/0.000
miR-150-3p (r/*P*)	0.135/0.000	0.139/0.020	1.495/0.000	0.071/0.000
miR-183-5p (r/*P*)	0.078/0.000	0.108/0.001	0.841/0.000	0.040/0.000
miR-224-5p (r/*P*)	0.204/0.000	0.173/0.035	1.815/0.000	0.095/0.000
miR-342-5p (r/*P*)	0.044/0.002	0.119/0.000	0.580/0.001	0.037/0.000

Results of KEGG-pathway classification and enrichment analysis of miRNA target genes

KEGG pathway analysis was performed to predict the pathways affected by the target genes of miRNAs. The results of KEGG-pathway classification suggested that a large proportion of target genes were enriched in multiple pathways of environmental information processing, organismal systems and cellular processes (Fig. 3A). As shown in Figure 3B, Ras signalling pathway, retinoic acid-inducible gene-I (RIG-I)-like receptor signalling pathway and phosphoinositide 3-kinase (PI3K)-Akt signalling pathway were highly enriched pathways.

Results of GO analysis of miRNA target genes

Functional enrichment analysis of all target genes was conducted using the GO database, which consisted of three parts: biological process (BP), cell component (CC) and molecular function (MF). GO analysis for the target genes of all miRNAs and differential miRNAs is presented in Figure 4A. GO-BP analysis showed that the target genes of all miRNAs and differential miRNAs were highly enriched in cellular process, single-organism process, biological regulation and metabolic process. GO-CC analysis showed that the target genes of all miRNAs and differential miRNAs were highly enriched in cell part, cell, organelle and membrane. GO-MF analysis showed that the target genes of all miRNAs and differential miRNAs were highly enriched in binding, catalytic activity, nucleic acid binding transcription factor activity and molecular transducer activity.

Validation of the sequencing data via qRT-PCR and correlation analysis

Six differential miRNAs derived from plasma exosomes including miR-141-3p, miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p and miR-342-5p were randomly selected for validation. Although the above miRNAs were randomly selected, the following criteria were used to enrol them: 1. the expression level of miRNA should be relatively high; 2. the expression level of selected miRNA should be relatively consistent in the samples of each group.

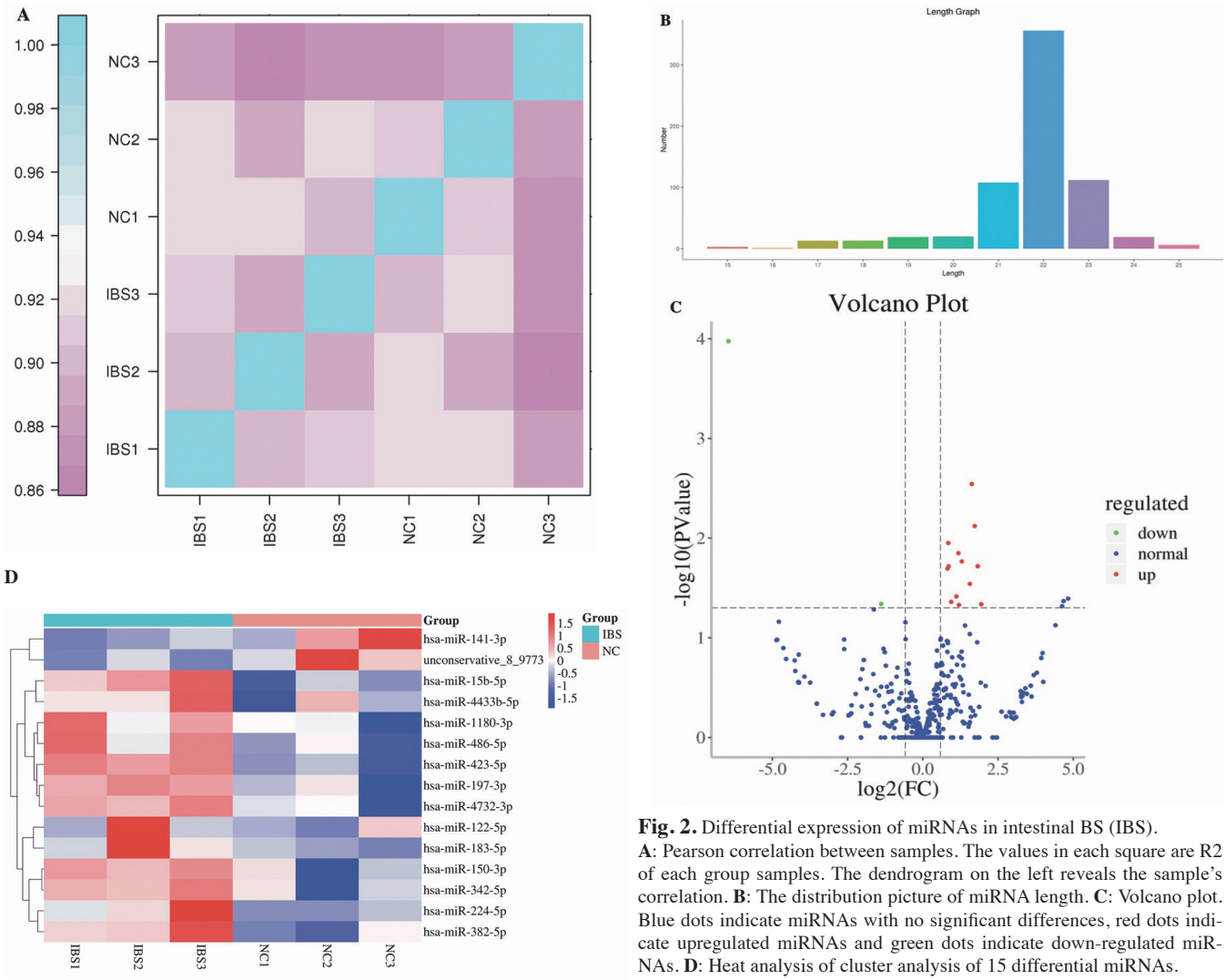

Fig. 2. Differential expression of miRNAs in intestinal BS (IBS). **A**: Pearson correlation between samples. The values in each square are R2 of each group samples. The dendrogram on the left reveals the sample's correlation. **B**: The distribution picture of miRNA length. **C**: Volcano plot. Blue dots indicate miRNAs with no significant differences, red dots indicate upregulated miRNAs and green dots indicate down-regulated miRNAs. **D**: Heat analysis of cluster analysis of 15 differential miRNAs.

The validation groups consisted of 20 NC, 20 stable intestinal BS and 20 active intestinal BS. The expression levels of miRNAs in NC, stable intestinal BS and active intestinal BS groups are shown in Figure 5. The results showed that compared with NC group, the expression level of miR-141-3p was significantly down-regulated in stable intestinal BS and active intestinal BS groups (Fig. 5A), while miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p and miR-342-5p were significantly up-regulated in active intestinal BS groups, which were consistent with those of high throughput sequencing (Fig. 5B-F). Additionally, there is a significant difference in the expression levels of miR-141-3p, miR-122-5p, miR-150-3p, miR-224-5p and miR-342-5p between stable intestinal BS and active intestinal BS groups (all $p<0.05$).

Then we analysed the correlation between the expression levels of the above miRNAs and the disease activity indicators of BS. The results suggested that miR-141-3p was negatively correlated with ESR, CRP, BDCAF and DAIBD while miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p and miR-342-5p were positively correlated with ESR, CRP, BDCAF and DAIBD (all $p<0.05$, Table III). ROC curve was used to demonstrate the sensitivity and specificity of the above miRNAs for predicting intestinal BS activity and stable. The AUC of miR-141-3p for predicting intestinal BS disease stable was 0.813 (95% CI, 0.683–0.942) (Fig. 6A). The AUC of miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p, miR-342-5p for predicting intestinal BS disease activity was 0.893 (95% CI, 0.788–0.997), 0.865 (95% CI, 0.745–0.985), 0.891 (95% CI, 0.794–0.989), 0.841 (95% CI, 0.719–0.964) and 0.749 (95% CI, 0.597–0.900), respectively (Fig. 6B-F).

Discussion

Although intestinal BS is a very rare disease, the disease prone to be active frequently, and patients often have no clinical symptoms. What is more, there is currently a lack of specific biomarkers for predicting disease activity in patients with intestinal BS. Recently, accumulating evidences have reported the importance of miRNAs derived from exosomes as potential clinical diagnostic markers and therapeutic strategy (10-12). However, studies on miRNAs derived from plasma exosomes in BS patients have not been reported.

Herein, we explored the difference of miRNAs derived from plasma ex-

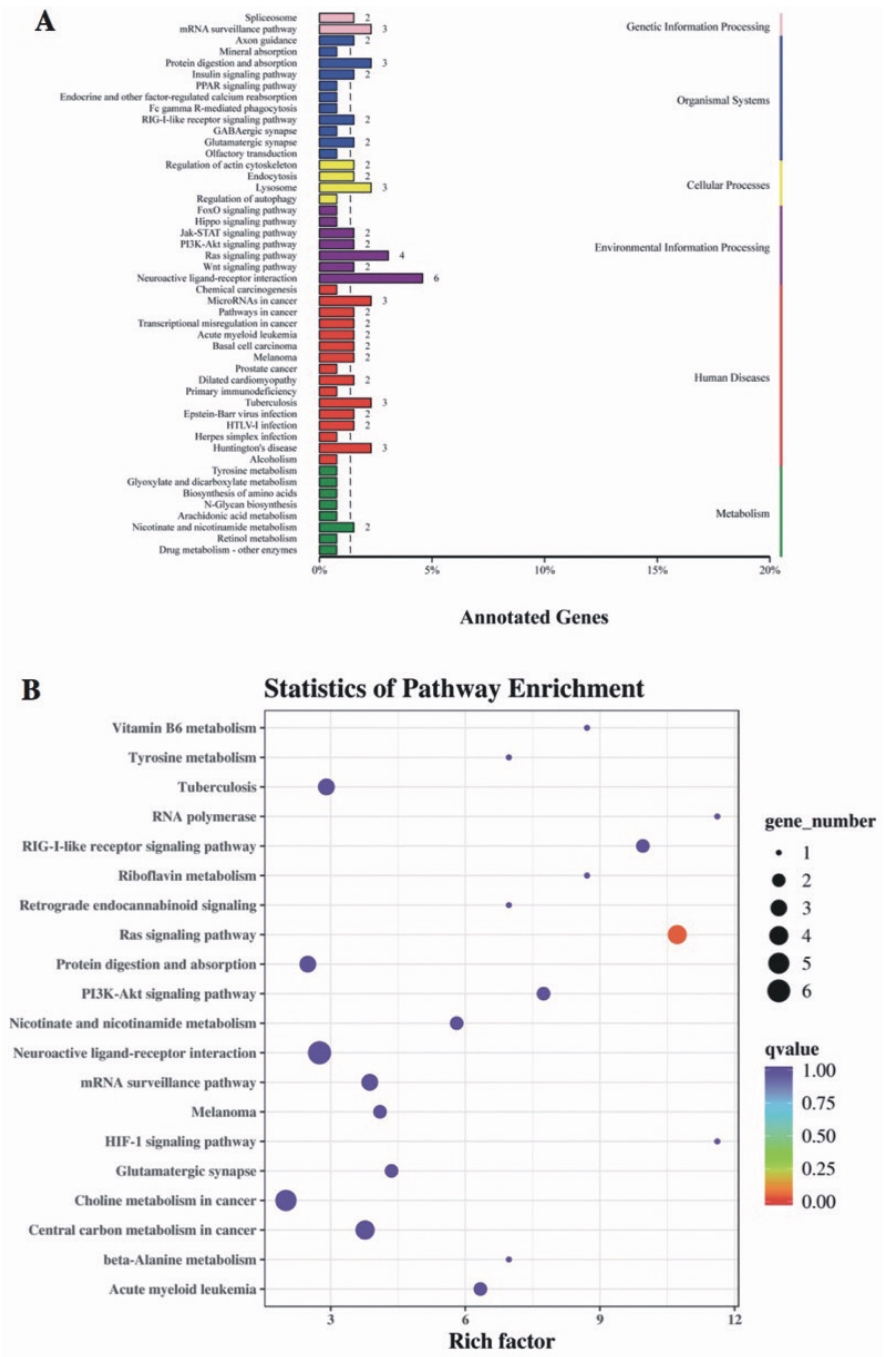

Fig. 3. KEGG pathway analysis for target genes of differential miRNAs.
A: Target gene KEGG-pathway classification chart. **B**: Enrichment bubble chart of KEGG enrichment analysis.

osomes between intestinal BS patients and healthy people using high throughput sequencing. As a result, 15 miRNAs (including 13 up-regulated and 2 down-regulated) had significantly different expression levels between the two groups. To further explore the potential role of these altered miRNAs in intestinal BS, bioinformatics analyses were performed. The results of the KEGG-pathway classification suggested that a large proportion of target genes were enriched in multiple pathways of environmental information processing, organismal systems and cellular processes. In our study, the enrichment analysis indicated that the target genes were highly enriched in multiple signalling pathways, including the RAS signalling pathway, RIG-I-like receptor signalling pathway and PI3K-Akt signalling pathway were highly enriched pathways. The RAS signalling pathway plays an important role in several aspects of normal cell growth and malignant transformation (24). RIG-I-like receptor signalling can mediate protective responses against experimental colitis and colitis-associated cancer and contribute to gastrointestinal homeostasis (25). The PI3K/AKT signalling pathway had been reported to be active in cells infiltrating inflamed human colon tissue (26). Furthermore, the activity of RAS signalling pathway promotes AKT phosphorylation in insulin-stimulated hepatocytes (27). Jiang *et al*. discovered that miR-223 can trigger cell apoptosis and inflammation in ulcerative colitis by up-regulating the PI3K/Akt-mTOR signalling pathway (28). However, these signalling pathways have not been reported in intestinal BS. Therefore, the results of this enrichment analysis can provide research directions for further exploring the specific mechanisms of miRNAs in intestinal BS.

On the other hand, the results of validation showed that compared with the NC group, the expression level of miR-141-3p was significantly down-regulated in stable intestinal BS and active intestinal BS groups, while miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p and miR-342-5p were significantly up-regulated in the active intestinal BS groups. Furthermore, miR-141-3p was negatively correlated with ESR, CRP, BDCAF and DAIBD, while miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p and miR-342-5p were positively correlated with ESR, CRP, BDCAF and DAIBD. The above results suggested that the higher the expression level of miR-141-3p, the more stable the disease, while the higher the level of miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p and miR-342-5p, the more active the disease. What is more, the results of ROC curve also indicated miR-141-3p was an indicator for predicting disease stable of intestinal BS, while miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p and miR-342-5p were indicators for predicting disease activity.

MiRNAs as small noncoding RNAs play an essential role in the regulation

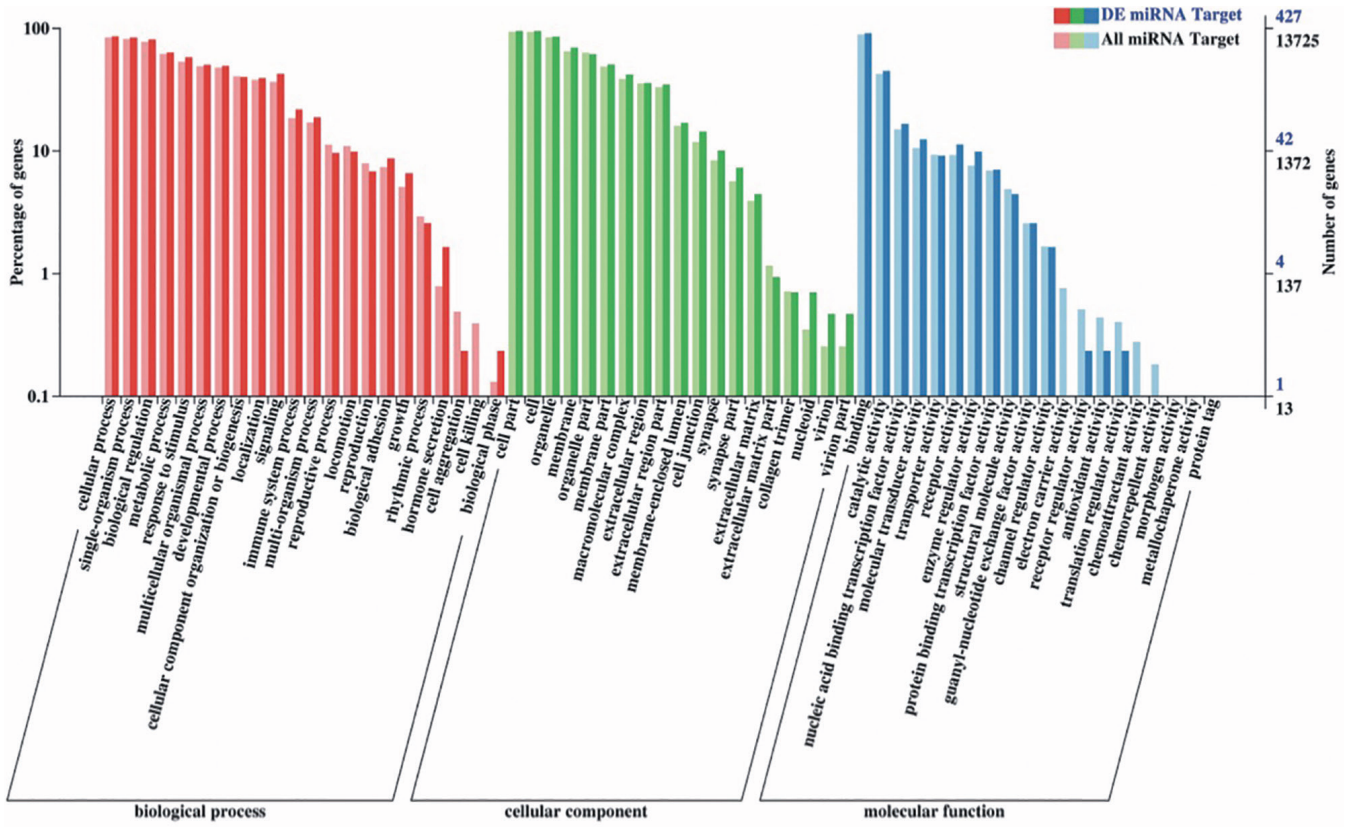

Fig. 4. GO enrichment analysis for target genes of differential miRNAs.

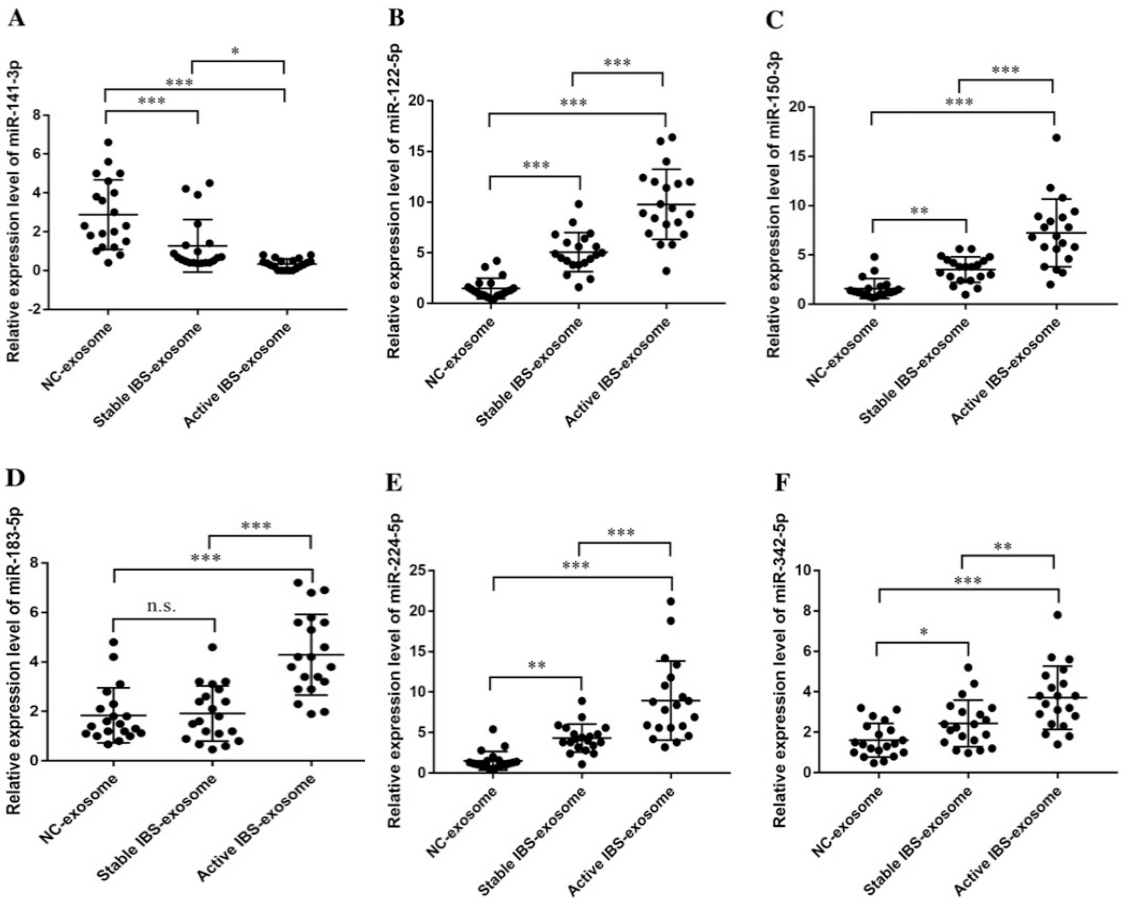

Fig. 5. Validation of the miRNAs sequencing data via qRT-PCR.
The relative expression levels of these miRNAs are calculated as $2^{-\Delta\Delta Ct}$.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

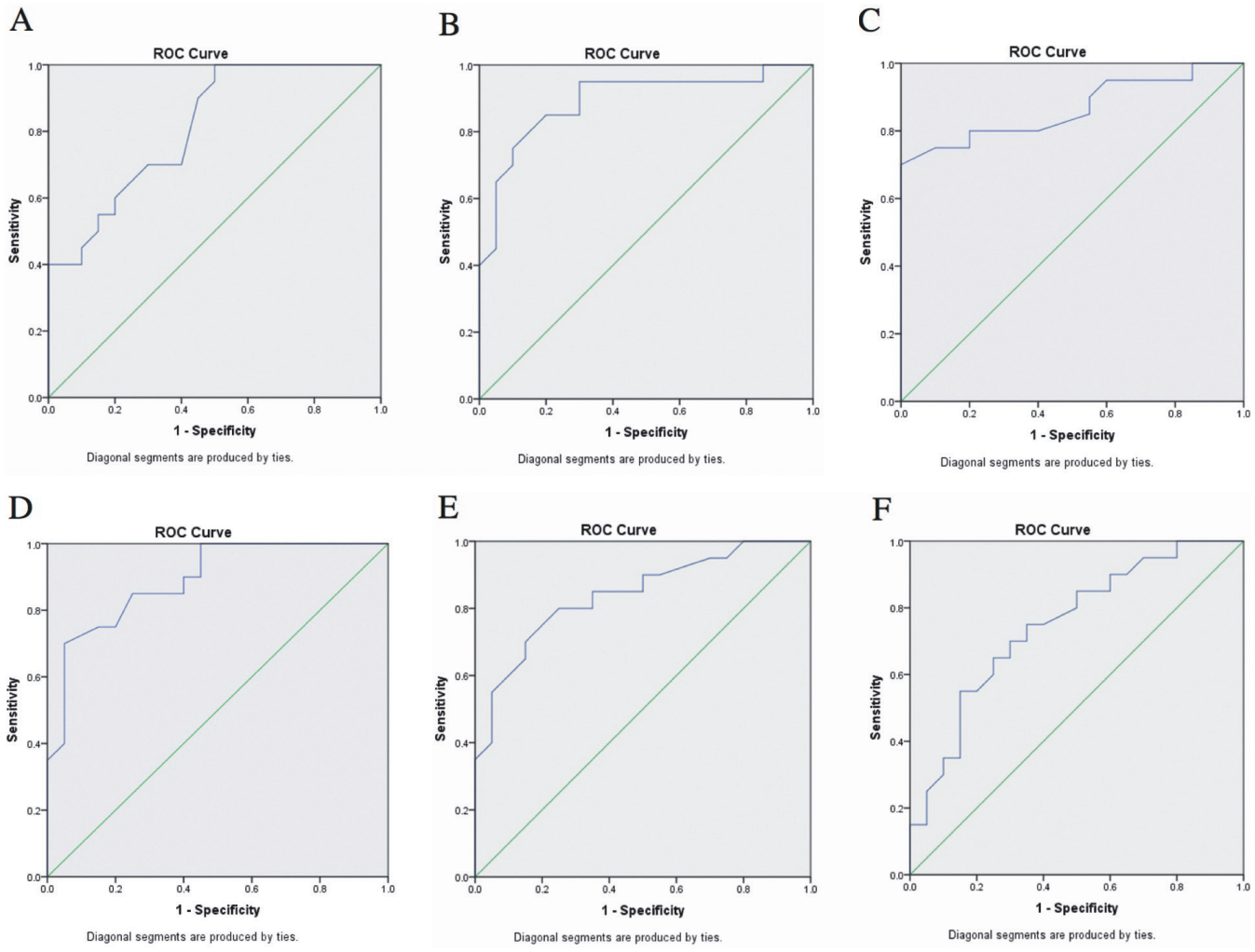

Fig. 6. Receiving Operating Characteristic (ROC) curve of differential miRNAs.
A: The AUC of miR-141-3p for prediction IBS disease stable was 0.813 (p=0.001).
B: The AUC of miR-122-5p for prediction IBS disease activity was 0.893 (p<0.001).
C: The AUC of miR-150-3p for prediction IBS disease activity was 0.865 (p<0.001).
D: The AUC of miR-183-5p for prediction IBS disease activity was 0.891 (p<0.001).
E: The AUC of miR-224-5p for prediction IBS disease activity was 0.841 (p<0.001).
F: The AUC of miR-342-5p for prediction IBS disease activity was 0.749 (p=0.007).

of several biological processes through their interaction with cellular messenger RNAs (29). Indeed, several studies have explored microRNA profiling of peripheral blood mononuclear cells (PBMCs) in patients with BS (30-32). Erre *et al*. (30) found that miR-139-3p and miR-720 may be biomarkers of BS. Puccetti *et al*. (31) performed a miRNA microarray analysis on PBMCs of BS patients and healthy controls, and they found 47 modulated miRNAs which included 45 down-modulated miRNAs, such as miR-143-3p, miR-199a-5p and miR-139-5p. Ahmadi *et al*. (32) reported that T-cell-associated miRNA expression levels, miR-25, miR-106b, miR-326, and miR-93 were significantly upregulated, while miR-146a and miR-155 levels were lower in the PBMCs of patients with BS when compared with the controls. However, studies on the expression of miRNAs derived from exosomes in patients with intestinal BS has not been well explored. In our study, we found that the expression level of miR-141-3p derived from plasma exosomes was significantly down-regulated in the stable intestinal BS and active intestinal BS groups, while miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p and miR-342-5p were significantly up-regulated in the active intestinal BS groups. A few studies on the functions and mechanisms of these dysregulated miRNAs in diseases especially in tumours had been performed. Recently, a study found that miR-141-3p was able to ameliorate receptor interacting protein kinase 1 (RIPK1)-mediated necroptosis of intestinal epithelial cells in necrotising enterocolitis (33), which indicated that down-regulated miR-141-3p in intestinal BS patients was positively correlated with disease activity. A negative correlation between miR-122-5p and the intestinal bacteria Bacteriodes in patients with type 2 diabetes mellitus was reported (34). Yugawa *et al*. (35) found that miR-150-3p was significantly reduced in cancer-associated fibroblasts-derived exosomes, and inhibited hepatocellular carcinoma

migration and invasiveness. Additionally, miR-150-3p derived from serum exosomes were found uniquely up-regulated in diabetic nephropathy patients compared with healthy volunteers (36). Shang *et al*. (37) reported that colorectal cancer cell-derived exosomes overexpressing miR-183-5p promoted angiogenesis in colorectal cancer by regulation of FOXO1. Zhou *et al*. (38) found that miR-224-5p-enriched exosomes promote tumourigenesis by directly targeting androgen receptor in non-small cell lung cancer. Besides, long-term exercise-derived exosomal miR-342-5p revealed a novel exerkine for cardioprotection (39). Furthermore, Han *et al*. found that circulating exosomal miR-342-5p have the potential to serve as novel diagnostic biomarkers for early-stage lung adenocarcinoma (40). In this study, we found that the AUC of miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p, miR-342-5p for predicting intestinal BS disease activity was 0.893, 0.865, 0.891, 0.841 and 0.749, respectively, while the AUC of miR-141-3p for predicting intestinal BS disease stable was 0.813. Therefore, miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p, miR-342-5p and miR-141-3p have the potential to serve as novel diagnostic biomarkers for predicting intestinal BS disease activity and stable disease. In the future, more research will be needed to explore the specific molecular mechanisms of the differentially expressed miRNAs involved in the pathogenesis of intestinal BS.

Conclusion

This study confirmed that patients with intestinal BS and healthy people have greater differences in expression of miRNA derived from plasma exosomes. The differential miRNAs including miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p, miR-342-5p and miR-141-3p may participate in the development of intestinal BS by affecting multiple biological pathways. Circulating miR-141-3p, miR-122-5p, miR-150-3p, miR-183-5p, miR-224-5p and miR-342-5p derived from plasma exosomes may serve as biomarkers of disease activity in intestinal BS.

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