

Exosomal miRNAs as novel potential biomarkers for endometriosis

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Research

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Abstract

Background: Endometriosis (EMS) is a chronic gynaecological disease. Exosomal miRNAs appear to be associated with the progression of EMS, which suggest potential circulating biomarkers in EMS.

Methods: Differential centrifugation, illumina small RNA sequencing, bioinformatics analysis and qRT-PCR are used to compare the pelvic fluid of patients with a clinical diagnosis of EMS and matched controls.

Results: Six miRNAs (miR-135a-5p, miR-196a-5p, miR-449b-5p, miR-4454, miR-4286, and miR-5100) showed significant differences in the EMS group in initial screening ($\log_2FC > 2$). Receiver-operator curve (ROC) analysis further indicated miR-4454 (area under the curve (AUC) = 0.956, $P < 0.05$) and miR-5100 (area under the curve (AUC) = 0.900, $P < 0.05$) were highly correlated with the pathogenesis of EMS. Validation on the samples from 10 patients and 10 healthy people.

Conclusion: Our miRNA expression data provide altered expression and potential prospects for biomarkers in EMS.

Introduction

Endometriosis (EMS), the presence of endometrial-like tissue outside the uterus, is a chronic gynaecological disease which affects approximately 10% of reproductive-aged women ¹. EMS morbidity has displayed a marked increasing trend in recent years ². It has been recognized as a precursor lesion of several types of malignancies and endometriosis-associated carcinoma, such as clear cell carcinoma, endometrioid carcinoma and seromucinous borderline tumor ³. Patients suffering from EMS typically experience chronic pelvic pain, infertility, or both, but can also be asymptomatic. Unfortunately, the pathogenesis of EMS remains unclear. Laparoscopic visualization of lesions confirmed by histology ⁴ is the go-to-method for EMS diagnosis, and clinically definitive diagnosis could take up to 11 years. Although CA125 and HE4 are widely used to monitor the occurrence of ovarian cancer, they are not specific for the detection of EMS ^{5,6}. There is a clear need for noninvasive and sensitive methods for early and specific diagnosis of EMS.

The recent discovery of exosomes presents a much-needed opportunity in this regard. Isolated from a variety of biological fluid, including blood, saliva, urine, nasal secretions, breast milk, and cerebrospinal fluid ⁷, these nanosized vesicles function as intercellular communicators with the inclusion of active proteins, mRNA, microRNA (miRNAs), and DNA ^{8,9}, of which miRNAs are of particular interest in this capacity as they regulate gene expression at the post-transcriptional level by binding to 3'-untranslated regions (3'-UTRs) of the target mRNAs ¹⁰. Recent studies have indicated that the level of exosomal miRNAs is strongly related to progression of certain diseases. For example, serum exosomal miR-126 and miR-125a-3p have been used as biomarkers for the diagnosis of non-small-cell lung cancer and early-stage colon cancer, respectively ^{11,12}.

In search for miRNAs as potential biomarkers of EMS, we are encouraged by the increasing amount of evidence that points to the important role of miRNAs in EMS progression¹³. For example, Zhang and coworkers reported that exosomal miRNA-138 could promote EMS through inflammation and apoptosis via the vascular endothelial growth factor (VEGF)/nuclear factor-kB signaling pathway¹⁴. Harp and coworkers demonstrated that exosomal miRNAs could contribute to EMS angiogenesis¹⁵, while Sun and coworkers revealed that EMS exosomes could promote neuroangiogenesis¹⁶. Most directly related to the development of biomarkers of EMS is the recent work by Zhang and coworkers. They found that the level of serum exosomal miR-22-3p and miR-320a is significantly higher in EMS patients than in the control group. Their potential uses as circulating biomarkers for EMS¹⁷ is thus suggested.

Progress notwithstanding, we note that in these studies the exosomes used were from serum, most of which being platelet- and/or megakaryocyte-derived¹⁸. As such, information so obtained does not directly reflect on the microenvironment in the endometriotic lesion. However, studies have shown that the local microenvironment plays a pivotal role in the onset and progression of an endometriotic lesion.

We believe that the state of EMS could be more directly correlated with certain miRNAs isolated from exosomes extracted from pelvic fluid (PF) due to its source. PF derive from macrophage secretions, ovarian exudate, refluxed tubal fluid, serum transudate, and refluxed endometrial material via retrograde menstruation, which can reflect better the changes in the pelvic environment¹³. EMS exosomes will appear in PF via some of the above fluid to promote pathological processes. Analyzing exosomes extracted from PF may be better to reveal the EMS pathological processes. The results presented here are from our initial efforts to identify exosomal miRNAs from the pelvic fluid or cyst fluid of EMS patients. We found that miR-4454 and miR-5100 are strongly expressed in the EMS patients studied, which portends their potential use as novel biomarkers of EMS.

Materials And Methods

Patient and sample design

Two EMS patients were recruited for the present study from the Department of Gynaecology of Shenzhen Maternity & Child Healthcare Hospital in the period from July to December 2018. They are respectively 30-50 years old, nonsmoker, and had not under any hormone therapy for at least 3 months prior to the present study. Their EMS state was detected by laparoscopy and further confirmed by histopathologic examination. Two infertile and EMS-free patients served as the negative control. Pelvic fluid and cyst fluid were collected in the surgical process, from which exosomes used in this study were extracted. The requirements of ethics of this study were approved by the Ethics Committee of Shenzhen Maternity & Child Healthcare Hospital. All participants were informed of the details of the project, signed and dated the Consent Form.

Isolation of exosomes from pelvic fluid (PF) and cyst fluid (CF)

The exosomes, including those (Ctr1 and Ctr 2) from the control group and those of the EMS patients (CF1 and CF2 from the cyst fluid; PF1 and PF2 from the pelvic fluid) were obtained in the following manner with the temperature maintained at 4 °C at all stages of the sample preparation and handling: 6 mL of pelvic fluid were collected from each of the four participants. The samples were stored in sterile centrifuge tube and subject to low-speed centrifugation. The resulting supernatant was collected, followed by centrifugation at 2000 × g (Eppendorf; 5418R) for 10 min. The supernatant thus obtained was collected and centrifuged at 10,000 × g for 20 min, and this last procedure was repeated once. The supernatant was then collected and diluted with an equal volume of 4% PEG6000 (Sangon biotech, China), and allowed to react for 5 min before centrifugation at 10,000 × g for 4 min.

Exosomes for the subsequent analyses were extracted by differential ultracentrifugation. Specifically, the supernatant was first thawed and then subject to sequential centrifugation at 13,000 g for 30 min, followed by centrifugation at 100,000 g for 70 min (Himac CS150GXII, HITACHI, Japan). The pellets were then suspended in 100 µL of phosphate-buffered saline and stored at - 80 °C for further analyses. Transmission electron micrographs (TEM) were obtained by using Hitachi Electron Microscope H-600 (Japan). The size of the serum exosomes was measured by using Nanosight analysis (Malver NanoSight 300 instrument, UK), while the protein markers of exosomes were detected by Western blotting.

Exosome RNA isolation and Small RNA sequencing

Isolation of fluid exosomal RNA was performed using QIAGEN exoRNeasy Serum/Serum Maxi Kit (Cat number: 77044). The quality and quantity of RNA were checked by using Agilent 2100 Bioanalyzer. The small RNA sequencing library was constructed with QIAseq miRNA Library Kit (Cat number: 331505) following Manufacture's instruction. Small RNA sequencing was performed by using SE75 on mode Illumina NextSeq 500 sequencing platform.

Bioinformatics analysis of miRNA-seq data

The miRNA-seq data were quantified and tested for differential expression with eRNA published in 2014¹⁹. After raw data cleaning, sequences with a length more than 18 nt were aligned against miRBase (v22). The miRNA profiling was normalized using reads per million (RPM) mappable miRNA sequences. Differential expression analysis of the miRNAs was performed for EMS patients and health controls using DESeq2 R-package. Only miRNAs with fold-change ≥ 2 ($p < 0.05$) were considered to be differentially expressed miRNAs. The prediction of miRNAs targets was performed by using the online bioinformatics tool. Miranda and RNA hybrids were used to carry out complementary pairs of miRNAs and target genes. Software parameters are as follows: Free energy of miranda ≤ -20 , score of miranda $>$

150, free energy of RNA hybrid ≤ -25 , p value of RNA hybrid < 0.05 . After obtaining the target genes of miRNAs, we performed GO function analysis (<http://www.geneontology.org/>) and KEGG (<http://www.genome.jp/kegg/>) pathway analysis. The GO terms with corrected p value ≤ 0.05 and the pathway with Q value ≤ 0.05 were considered as significant enrichment.

Validation of miRNA by quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

In order to validate the miRNA-sequencing data, transcript levels obtained from miRNA-seq for six selected different miRNAs (miR-135a-5p, miR-196a-5p, miR-449b-5p, miR-4454, miR-4286, and miR-5100) were confirmed by qRT-PCR. The expression levels of target miRNAs were normalized to that of U6. The primers used for qRT-PCR are provided in supporting Table S4. According to the Manufacturer's instructions, all reactions were carried out using a SYBR Green Master Mix (SYBR Premix EX Taq, TaKaRa). PCR amplification was conducted in a total volume of 20 μL with the following procedures: 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 30 s. All reactions were performed in triplicate. Relative transcription levels were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method. Each measurement was performed in three biological replicates.

Statistical analysis

Statistical analysis was performed using SPSS 18.0. All qRT-PCR amplifications were performed in triplicate. Data are expressed as the mean \pm standard deviation. Differences in the miRNA expression profile were analyzed using Student's t -test. $P < 0.05$ was considered statistically significant. We applied the area under the curve (AUC) to assess the diagnostic power of the predictors. AUC can be used as an accurate measurement of the diagnostic marker; the larger the AUC, the better the prediction model. AUC = 0.5 indicates no predictive power, whereas AUC = 1 represents a perfect predictive performance.

Results

Characterization of exosomes

The morphological feature of exosomes was observed by transmission electron microscope. As shown in Fig. 1A, most of the photo-captured round-shaped microvesicles are of a size of about 100 nm in diameter with the membrane bounded. The size distribution of the serum exosomes is shown in Fig. 1B. The protein markers of exosomes (TSG101) are shown in Fig. 1C. The results indicated that exosomes can be successfully isolated from serum using this protocol with quality adequate for subsequent experiments.

Sequence analysis of small RNAs

Thirteen sRNA libraries were generated from serum exosomes via high-throughput sequencing. We used Fast-QC software (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) for the overall evaluation of the quality of sequencing data and used BWA algorithm for small RNA mapping. There are 15548736, 13804825, 12383415, 16332234, 15327209, and 16970328 total reads in Ctr1, Ctr 2, CF1, CF2, PF1, and PF2 sRNA libraries, respectively (Table 2). The corresponding clean reads of 2654371(17.07%), 2911044(21.09%), 643916(5.2%), 5843973(35.8%), 2587012(16.9%) were respectively obtained (Table 2) after the removal of the low-quality reads, including 3'adapter null, insert null, 5'adapter contaminants, reads smaller than 18nt, and reads containing ploy A. The miRNA rates in total RNA were 16.03%, 2.01%, 1.66%, 2.17%, 7.17%, and 2.09%, respectively.

Table 1
Basic statistical information on the quality of the reads

Sample	Ctrl1	Ctrl2	CF1	CF2	PF1	PF2
Raw reads	15548736	13804825	12383415	16332234	15327209	16970328
Clean reads	2654371	2911044	643916	5843973	2587012	4870855
Clean Rate(%)	17.07	21.09	5.20	35.80	16.90	28.70
Q20(%)	95.65	94.25	93.21	94.72	94.15	96.4
Q30(%)	90.78	88.42	86.61	89.02	88.28	92.46
GC(%)	43.7	43.17	42.48	44.37	41.55	38.86
tRNA(%)	10.81	3.54	13.66	38.01	9.78	7.65
rRNA(%)	46.4	70.41	38.14	31.72	36.19	47.25
miRNA(%)	16.03	2.01	1.66	2.17	7.17	2.09

Differentially expressed miRNAs

We applied DESeq2 algorithm to filter the differentially expressed genes. An miRNA candidate is considered to be differentially expressed if the level between the two experiment groups shows a statistically significant difference ($p < 0.05$) of at least two folds. Compared with CF group, 54 types of miRNAs are down-regulated, and 154 are up-regulated in PF group. (Fig. 2a). The differently expressed miRNA profiles (Fig. 2B) were obtained from comparative analyses of the exosomes in the three groups (control, CF, and PF) according to the value of $\log_2FC > 2$ miRNA whose expression level was respectively highest or lowest. As shown in Table S3, the top five most down-regulated miRNAs in the patient group were miR-135a-5p, miR-196a-5p, miR-34b-3p, miR-100-5p, and miR-125b-5p, while the top five most up-regulated miRNAs were miR-4454, miR-4286, miR-5100, miR-7977, and miR-624-5p.

Table 2
Difference miRNA for down-regulation or up-regulation (Top 5)

down-regulation miRNA		up-regulation miRNA		
Number	miRNA	log2FC	miRNA	log2FC
1	miR-135a-5p	-7.73	miR-4454	7.72
2	miR-196a-5p	-6.18	miR-4286	7.43
3	miR-34b-3p	-6.01	miR-5100	5.95
4	miR-100-5p	-5.49	miR-7977	5.75
5	miR-449b-5p	-5.14	miR-624-5p	5.39

Validation miRNA expression by q-PCR

To further validate our miRNA profiling results, we screened 10 patients and 10 healthy people for validation of miRNA expression levels. We found that the expression trends of all miRNAs determined by q-PCR were identical to those obtained from the RNA sequencing (Fig. 3). Compared with the control group, the expression levels of miR-196a-5p and miR-449b-5p decreased, whereas those of miR-4454 and miR-5100 increased, both to a significant degree ($p < 0.05$). These observations suggest that miR-196a-5p, miR-449b-5p, miR-4454, and miR-5100 were involved in the pathogenesis of EMS and that they may serve as potential biomarkers of EMS.

Table 3
The miRNA primers sequence for q-PCR

miRNA Name	Forward primer	Reverse primer
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
miR-135a-5p	ACACTCGAGCTGGGTATGGCTGTTTATTCCT	TGGTGTCGTGGAGTCGGC
miR-449b-5p	ACACTCCAGCTGGGAGGCAGTGAATTGTTA	TGGTGTCGTGGAGTCGGC
miR-654-3p	ACACTCCAGCTGGGTATGTCTGCTGAGCAT	TGGTGTCGTGGAGTCGGC
miR-4449	ACACTCCAGCTGGGCGTCCCGGTGCTGCGC	TGGTGTCGTGGAGTCGGC
hsa-miR-5100	ACACTCCAGCTGGGTTTCAGATCGCAGCGGT	TGGTGTCGTGGAGTCGGC
hsa-miR-223-3p	ACACTCCAGCTGGGTGTCAGTTAGTCAAAT	TGGTGTCGTGGAGTCGGC

ROC curve analysis of exosomal miRNA as diagnostic biomarkers for EMS

We subsequently used ROC curve analysis to assess the potential of the 6 miRNAs (Table 3) as EMS biomarkers. The AUC of miR-135a-5p, miR-196a-5p, miR-449b-5p, miR-4454, miR-4286, and miR-5100 were 0.53 (95% CI, 0.400–0.800), 0.570 (95% CI, 0.700 – 0.600), 0.680 (95% CI 0.600-1.000), 0.956 (95% CI, 0.800-1.000), 0.520 (95% CI, 1.000-0.200), 0.900 (95% CI, 0.800–0.900), respectively, suggesting that miR-4454 and miR-5100 from serum exosomes hold a high potential as biomarkers of EMS.

Discussions

EMS has been described as a cancer-like process, involving cell migration and invasion. Its pathogenesis remains poorly understood, and related research findings and conclusions are controversial. The gold standard of EMS diagnosis is direct visualization of lesions at surgery, preferably coupled with histologic confirmation of endometrial glands and stroma in biopsies of suspected lesions. Compared with a minimally invasive diagnostic procedure, the surgical diagnosis has multiple drawbacks, including possible organ damage, hemorrhage, infection, adhesion formation, in addition to common anesthetic complications²⁰. EMS can be asymptomatic or misdiagnosed in the general population, and diagnosis is always delayed, leading to difficulties in medical and surgical treatments²¹. At the endometriosis deteriorating stages, the severity of pelvic pain may lead to hysterectomy that is often with oophorectomy²². Furthermore, increasing evidence exists for the malignant transformation of ovarian endometriomas to ovarian cancer, particularly the clear cell and endometrioid subtypes²³. Earlier detection and timely therapeutic follow-up with noninvasive biological markers with good specificity for endometriosis is thus considered to be ultimately necessary.

With the hope of identifying a feasible biomarker for early diagnosis of EMS, we extracted miRNAs from the pelvic fluid and cyst fluid of EMS patients and analyze their profiles using miRNA sequencing. To our knowledge, this is the first report of changes in pelvic fluid and cyst fluid miRNA expression in EMS. Moreover, we selected six (miR-135a-5p, miR-196a-5p, miR-449b-5, miR-4454,miR-4286, and miR-5100) of the above miRNAs for Q-PCR verification in another endometriosis samples. The results show that the levels of miR-196a-5p, and miR-449b-5p decreased significantly ($p < 0.05$),while miR-4454 and miR-5100 increased significantly ($p < 0.05$) in cyst fluid exosomes with endometriosis patients. ROC curve analysis showed that the AUC of miR-4454 and miR-5100 were 0.956 (95% CI, 0.800-1.000) and 0.900 (95% CI, 0.800–0.900). These results suggest that miR-4454 and miR-5100 from serum exosomes hold great potential as biomarkers for EMS diagnosis.

Our study first identified a number of miRNAs from the exosomes isolated from the cyst fluid of EMS patients and then verified them in the serum exosomes of a second group of EMS patients. Different from Zhang¹⁴ studies in serum, we believe that the miRNAs are more directly correlated with the development of EMS for the following two reasons: First is the the complexity of miRNA biogenesis, miRNA secretion, and variation in miRNA expression profiles according to variation epigenetic and environmental factors and the other the expression of lesion exosomal²⁴. RNA was at high and stable levels as compared with

that of the plasma samples, possibly due to RNA degradation associated with the ribonucleases present in plasma^{25,26}.

Furthermore, our study has several limitations. First, only a small number of miRNAs were verified by Q-PCR, limitations due to small sample size and suboptimal characterization of specimens. Second, this study lacked functional experiments at the cellular and molecular levels to identify the relationship between these miRNAs and endometriosis, further studies are needed to develop more clinical tests. Specifically, a using miRNAs identified as specific biomarkers will application in early detection of EMS

Conclusion

Our results demonstrated that miR-196a-5p, and miR-449b-5p were decreased significantly, miR-4454 and miR-5100 were increased significantly in endometriosis patients. Serum miR-4454 and miR-5100 may play a regulatory role in endometriosis procession and thus hold great potential as biomarkers for the early diagnosis of EMS.

Abbreviations

EMS

Endometriosis

ROC Curve Analysis

Receiver Operator Characteristic Curve Analysis

miRNA

MicroRNA

VEGF

Vascular Endothelial Growth Factor

NF- kB

Nuclear Factor-kB

PF

Pelvic Fluid

CF

Cyst Fluid

TEM

Transmission Electron Micrographs

RPM

Reads Per Million

qRT-PCR

Quantitative Reverse-transcription Polymerase Chain Reaction

AUC

Area Under Curve

Q-PCR

Declarations

Ethics approval and consent to participate

Approval for the study was given by the ethical committee of Shenzhen Institutes of Advanced Technology Chinese Academy of Sciences, SIAT-IRB-170315-H0157. Written consent for participation and data presentation was received from the patients who volunteered to take part in the study.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysis during this study are included in this published article and its Additional files.

Competing interests

All authors are affiliated to Affiliated Shenzhen Maternity & Child Healthcare Hospital, Southern Medical University.

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Authors' contributions

LZ, HL, PJ planned the project, selected patients, interpreted results, LZ, X-L C and L-T Z recruited patients for the study, HL performed differential centrifugation, illumina small RNA sequencing and qRT-PCR, LZ, HL and Q-X L performed bioinformatics analysis, HL, LZ, L-T Z and H-J L performed statistical analysis, HL, LZ and G-Y Y performed ROC curve analysis, LZ, HL, PJ, H-J L and L-T Z were the major contributors in writing the manuscript. All authors read and approved the final manuscript.

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Figures

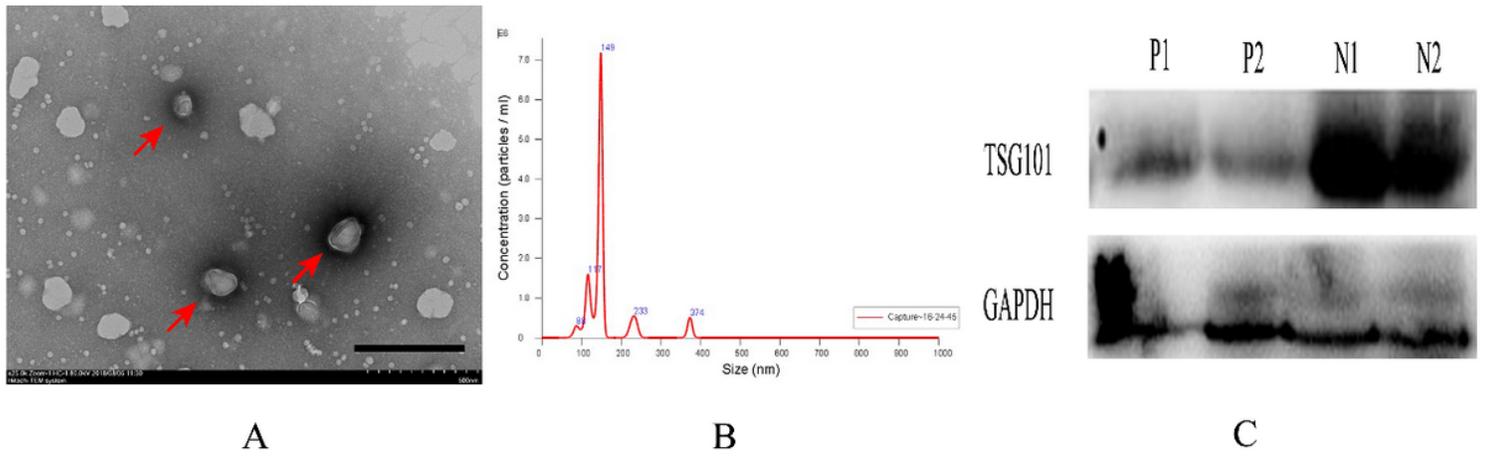
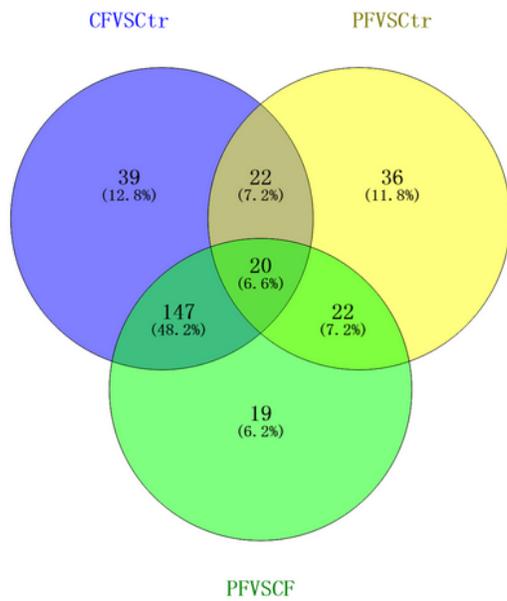
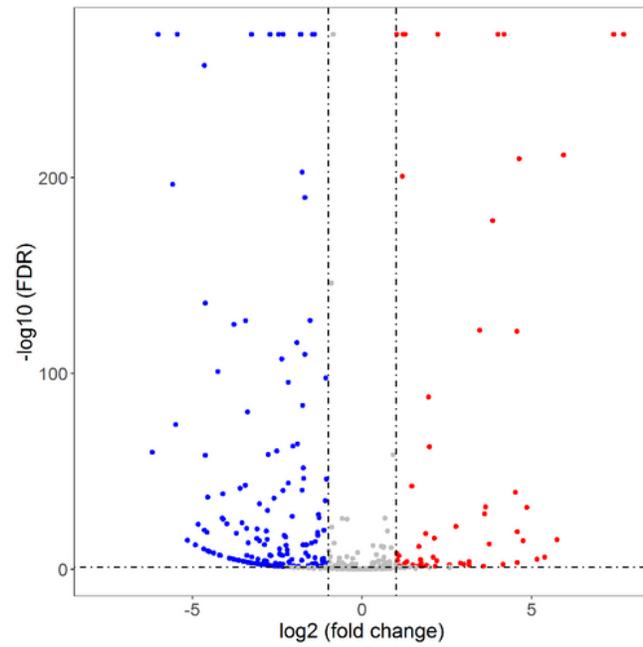


Figure 1

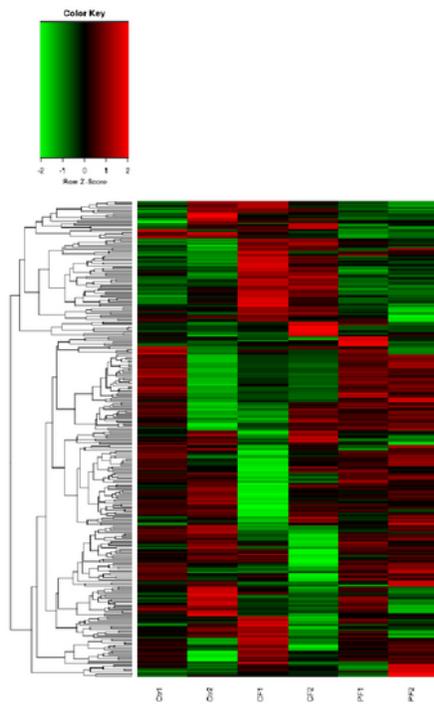
TEM images of serum exosomes (A) size distribution of exosomes (B); Western blotting analysis showing exosome-enriched medium with expression of the exosome marker of TSG101 (C).



A



B



C

Figure 2

Differential expression analysis: Venn diagrams showing miRNAs that are common in the three group (a); volcano for miRNA between CF and control group (b); Heat map showing hierarchical clustering analysis of difference miRNAs detected in individual patients and control group ($\log_2FC > 2$) (c).

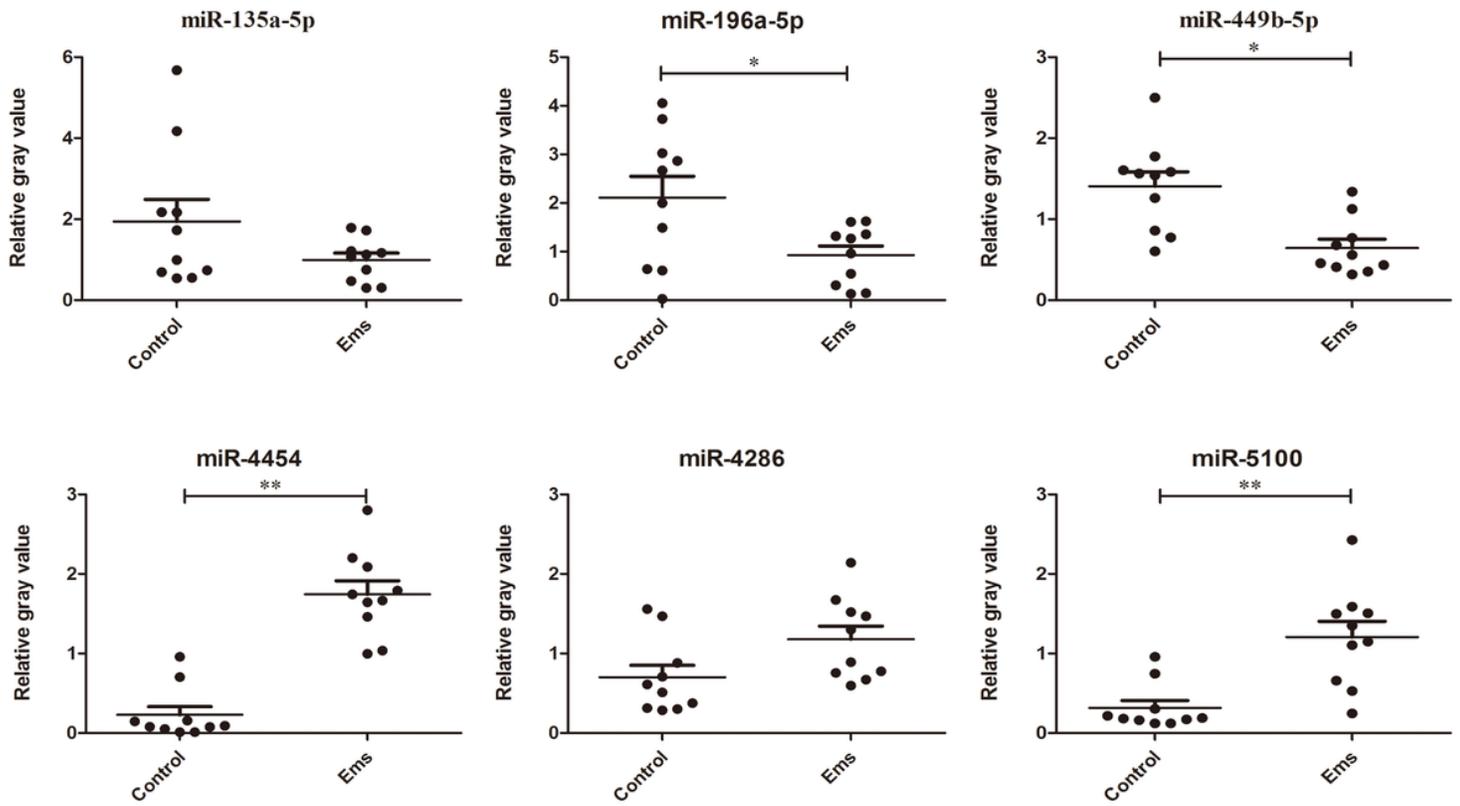


Figure 3

Validation of selected miRNAs by Q-PCR. * means $P < 0.05$, $n = 10$).

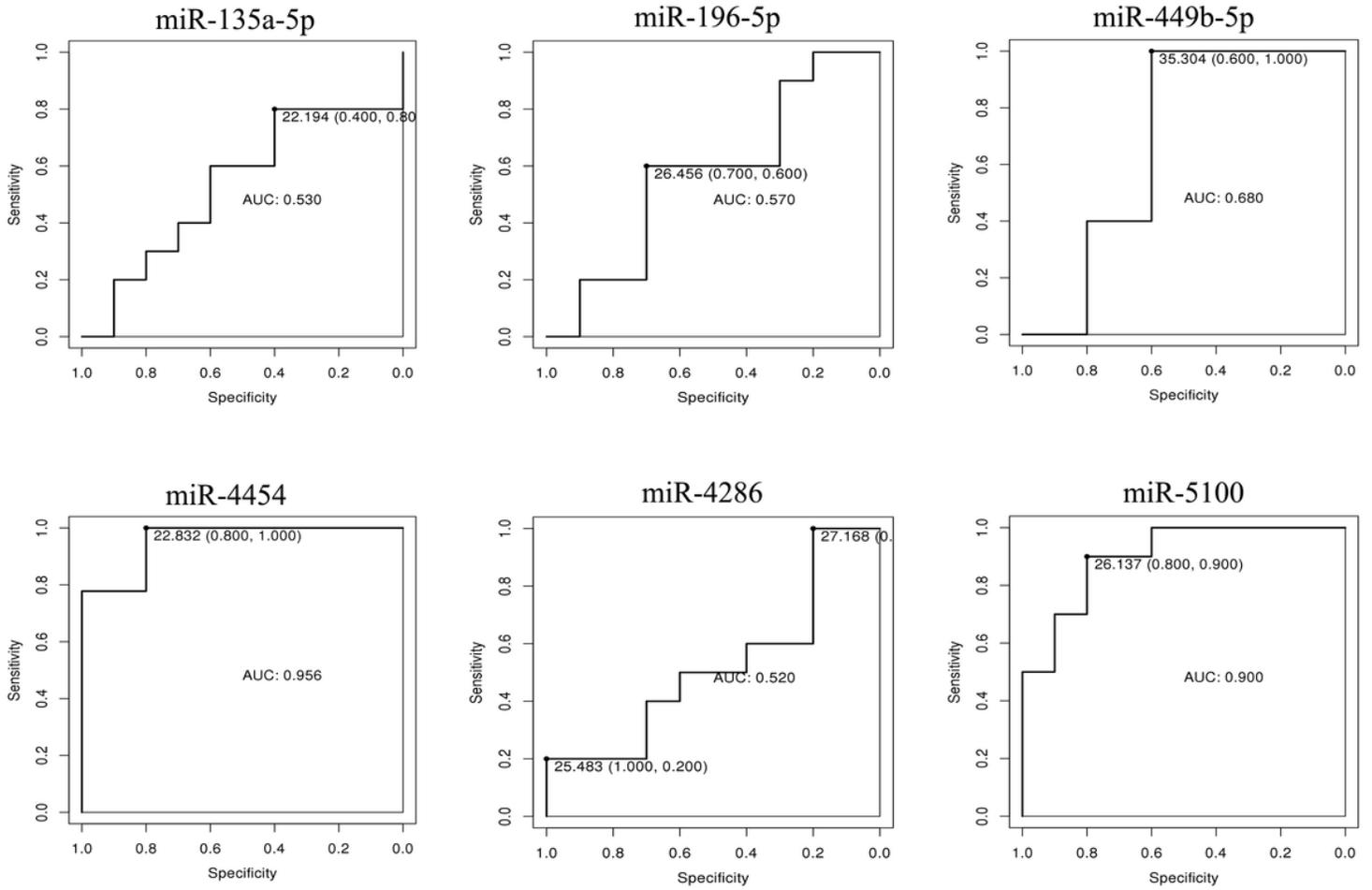


Figure 4

ROC curves of the analyses of different exosomal miRNAs obtained from 10 patients.