

## Original Article

# Circulating microRNAs from serum exosomes as potential biomarkers in patients with spontaneous abortion

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**Abstract:** Background: Spontaneous abortion (SA) is a common complication in early pregnancy. Nevertheless, SA's etiology is complex, and the underlying molecular mechanisms of the pathogenesis behind SA remains unclear. The present study aims to find the feasibility of using serum exosomal miRNAs as novel biomarkers for SA. Methods: In our study, we isolated the serum exosomes from the peripheral blood of the subjects. Then transmission electron microscopy (TEM), WB, and in vitro exosome tracing experiments were used. Comprehensive exosomal miRNA sequencing was performed to profile the differentially expressed miRNAs between the SA and normal pregnancy groups. Furthermore, genes targeted by miRNAs were further predicted and verified by TargetScan, miRDB, miRTarBase, miRWalk and HMDD V3.2. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and pathway category were performed by the DIANA-miRPath v3.0 online tool. We then validated the expression levels of selected miRNAs by qRT-PCR. ROC analysis was performed to explore the clinical utility of the two miRNA as biomarkers for SA. Results: TEM, NTA measurements and WB analysis showed the successful isolation of exosomes. Exosome labeling by PKH26 proved that exosomes could be efficiently taken up by primary decidual cells. Sequencing data found that with a total of 2,588, there were 189 significantly expressed exosomal miRNAs between the two groups. The most significantly expressed miRNA (miR-371a-5p, miR-206, miR-147b, miR-6859-5p, miR-410-3p, miR-1270 and miR-524-5p) were selected for further analysis. Through KEGG pathway analysis and pathway category, nine risk pathways were revealed. Among them, the Wnt signaling pathway, the Hippo signaling pathway, and the FoxO signaling pathway were pinpointed as major high-risk pathways. As a single marker, miR-371a-5p and miR-206 had a specificity of 83.3% and 70.8% at the sensitivity of 62.5% and 66.7%, respectively. The combined two markers achieved a specificity of 75% at the sensitivity of 79.2%. Conclusions: Our results suggest that the circulating miRNAs from exosomes are altered in patients with SA. Findings of this exploratory study may provide potential biomarkers for SA.

**Keywords:** Spontaneous abortion (SA), serum exosomal miRNAs, biomarkers

## Introduction

Spontaneous abortion (SA) is defined as the loss of pregnancy before a fetus reach viability. As reported, the average occurrence of SA once is 11% [1]. Then, if two or more SA occur, SA can develop into recurrent spontaneous abortion (RSA). As reported, around 5% of couples desiring a child were effected by RSA [2]. Though pregnancy loss is the most common complication in early pregnancy, it can substantially

impact the couples' physiology and psychology.

As a heterogeneous disease, many reasons can contribute to SA: gene factors, anatomical factors, microbiological factors, endocrine factors, psychological factors, immunological factors, hereditary thrombophilic factors, idiopathic factors, and so on. The percentage of idiopathic SA can amount to 50% [3]. Despite the rapid advances in assisted reproductive technology,

gies, SA is still a big problem globally, so uncovering the causes and the molecular mechanism of SA is quite urgent and demanding. Extracellular vesicles (EVs) are broadly divided into two categories based on size: ectosomes and exosomes. Ectosomes, including microvesicles, microparticles, and large vesicles, have a wide diameter range of 50 nm to 1 μm. Exosomes have a relatively small size of 50 to 150 nm with an endosomal origin [4]. The exosomes contain various functional molecules, including microRNA (miRNA), long non-coding RNA (lncRNA), circRNA, DNA, lipids, transfer RNA (tRNA), messenger RNA (mRNA), ribosomal RNA (rRNA), and so on [5]. Among them, mounting studies have demonstrated that exosomes miRNAs can mediate cell to cell communication in many physiological and pathological processes and then initiate functional responses and subsequent phenotypic changes because of the ability to protect the structure and function of miRNAs against degradation by an RNase [6]. Therefore, specific exosomal miRNAs may act as biomarkers in numerous diseases. However, serum exosomal miRNAs' role in SA has not yet been reported.

In this study, we examined serum-derived EVs from SA and normal pregnant participants to compare the expression of miRNA by conducting miRNA sequencing. Then the functions, pathways, and hub genes were predicted by bioinformatics analysis. The feasibility of using serum exosomal miRNAs as novel biomarkers for SA was evaluated by RT-PCR and ROC analysis at last.

### Materials and methods

#### *Study group and samples*

We included 68 women who presented to the Department of Gynecology of the First Affiliated Hospital of Guangzhou University of Chinese Medicine. Serum exosomes from 10 SA and 10 normal intrauterine pregnancy patients were subjected to miRNA sequencing. Another cohort of 24 SA and 24 normal cohorts were subjected to further qRT-PCR verification. This study was authorized by the Ethics Committee of Guangzhou University of Chinese Medicine (No. ZYYECK 2017-060-ND-02) and performed according to the guidelines of the Declaration of Helsinki. Each participant signed written

informed consent before recruitment. Baseline characteristics, such as age, days of pregnancy, number of pregnancies, number of deliveries and abortions were extracted by Excel.

#### *Exosome isolation and identification*

Blood specimens were collected using tubes not containing anticoagulant EDTA, centrifuged at 3000×g for 15 min at 4°C. The serum was transferred and stored at -80°C for further analysis.

Total exosome isolation reagent (from Serum, Cat.4478360, Invitrogen, CA, USA) was used to isolate exosomes following the manufacturer's instruction. Firstly, serum samples were centrifuged at 2000×g for 30 minutes to remove possible cell debris. Secondly, 1/5 volumes of exosome isolation reagent were mixed with serum and incubated at 4°C for 30 min. Finally, the exosome pellets were precipitated by centrifugation at 10,000×g for 10 minutes and resuspended in 1× phosphate-buffered saline (PBS) for further experiments. Transmission electron microscopy (TEM), Nanoparticle-tracking analysis (NTA), and Western Blotting (WB) analysis was used to confirm the successful isolation of exosomes.

#### *Exosomal RNA extraction, high-throughput sequencing*

The miRNeasy Mini Kit (Cat.217004, QIAGEN, Germany) was used to extract exosomal total RNAs. The extracted RNA's quality and purity were checked using Qubit (Life Technologies) and Agilent 2200 TapeStation (Agilent Technologies). The sequencing analysis of exosomal small RNA was conducted on Illumina HiSeq 2500 platform.  $|\log_2(\text{fold change})| \geq 1$  and  $P < 0.05$  were considered as differentially expressed. Raw sequence reads are available if required.

#### *In vitro exosome tracing*

The membrane of isolated exosomes was stained by PKH26 Fluorescent Cell Linker Mini Kit (Sigma-Aldrich, Mini26-1KT), and the nucleus of primary decidual cell was stained by DAPI according to the manufacturer's instructions with minor modifications. After extracting again, the exosomes with fluorescence signals were incubated with primary decidual cells for 24 h.

**Table 1.** Demographic and clinical characteristics of patients in the sequencing (left) and validation (right) cohorts

Characteristics	Sequencing cohorts			validation cohorts		
	NC (n = 10)	SA (n = 10)	<i>P</i> value	NC (n = 24)	SA (n = 24)	<i>P</i> value
Age (years)	29.10±6.15 (21-40)	27.20±4.78 (19-35)	0.451	28.92±5.21 (21-38)	31.33±6.51 (19-44)	0.162
Days of pregnancy	47.80±5.88 (41-57)	49.30±8.73 (40-61)	0.658	6.63±0.71 (6-8)	6.88±0.99 (6-9)	0.321
Number of pregnancies	2.30±1.25 (1-5)	3.30±1.70 (1-6)	0.152	2.67±1.61 (1-7)	2.67±1.27 (1-6)	1.000
Number of deliveries	0.80±0.79 (0-2)	0.80±0.63 (0-2)	1.000	1.04±1.00 (0-3)	0.96±0.95 (0-3)	0.769
Number of abortions	0.50±0.71 (0-2)	1.50±1.35 (0-4)	0.053	0.63±1.13 (0-4)	0.71±0.75 (0-2)	0.766

Values are the mean ± SD.

The exosomes were then observed by confocal microscopy (Leica Microsystems, Germany) to determine their interaction with cells.

#### Functional analysis and risk modules identification

TargetScan (<http://www.targetscan.org>), miR-DB (<http://mirdb.org>), miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw>), and miRWalk (<http://zmf.umm.uni-heidelberg.de>) were used to predict target genes of candidate miRNAs. Only genes recorded in four databases were considered as ultimate target genes. The HMDD v3.2 (<http://www.cuilab.cn/>) was also used for further confirmation [7].

The target genes regulated by candidate miRNAs were mapped to the miRPath v3.0 tool (<http://snf-515788.vm.okeanos.grnet.gr/>) to identify KEGG pathway and pathway target genes [8]. *P*-values < 0.05 were set as a threshold for pathways. The KEGG PATHWAY Database (<https://www.kegg.jp/kegg/pathway.html>) was also used to learn about and category identified pathways [9].

#### Validation of exosomal miRNA expression by qRT-PCR

The selected candidate exosomal miRNAs were further validated by qRT-PCR. All-in-one™ miRNA qRT-PCR Detection Kit (GeneCopoeia, Guangzhou, China) were used to reverse transcribe and quantify following the instructions, and the results were analyzed with a BIO-RAD CFX96 system. The relative expression values were normalized to U6 and calculated by  $2^{-\Delta\Delta Ct}$  method. The detailed primer sequences of hsa-miR-371a-5p and hsa-miR-206 (from 5' to 3') is ACUCAAACUGUGGGGGCACU, and UGGAAGUAAGGAAGUGUGUGG, and were purchased from GeneCopoeia.

## Results

### Patient cohort data

There were 20 patients with SA and normal pregnancy in the sequencing step and 48 patients with SA and normal pregnancy in the validating step. Statistical results showed that age, days of pregnancy, number of pregnancies and deliveries, and the number of abortions were not significantly different (**Table 1**).

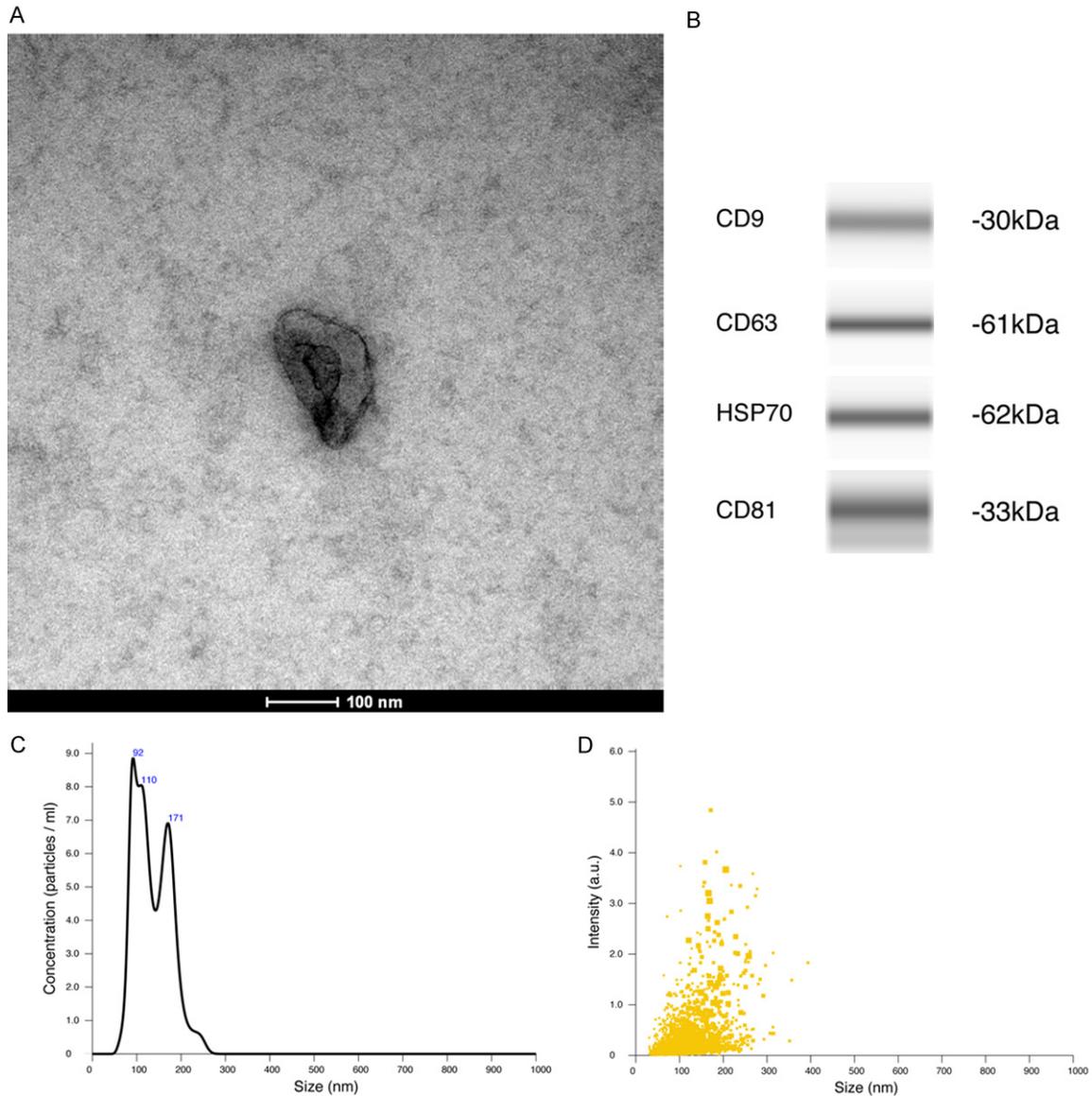
### EVs isolation, characterization and exosome uptake experiments

TEM identified vesicles with a cup shape morphology (**Figure 1A**). The NTA measurements showed that our serum exosome's average size was 135.5 nm, ranging from 86.1 to 187.1 nm (**Figure 1C**). Seeing from **Figure 1D**, the concentration of exosomes was  $8.09 \times 10^9$ /ml. Moreover, WB analysis proved the presence of exosomal protein markers, including CD9, CD63, HSP70, and CD81 (**Figure 1B**). These results proved that we were able to obtain exosomes from the serum.

We further checked the effect of serum exosomes in vitro culture. SA and control patients' serum exosomes were labeled with PKH26 and added to the primary decidual cells. After 24 h, exosomes' fluorescence signal could be detected obviously under the confocal microscope around the nucleus. This proved that the exosomes could be efficiently taken up by primary decidual cells, thus playing a role in SA (**Figure 2**).

### Differential expression of miRNAs in serum exosomes

Expression levels of miRNAs in the two groups were compared. A scatterplot was used to eval-

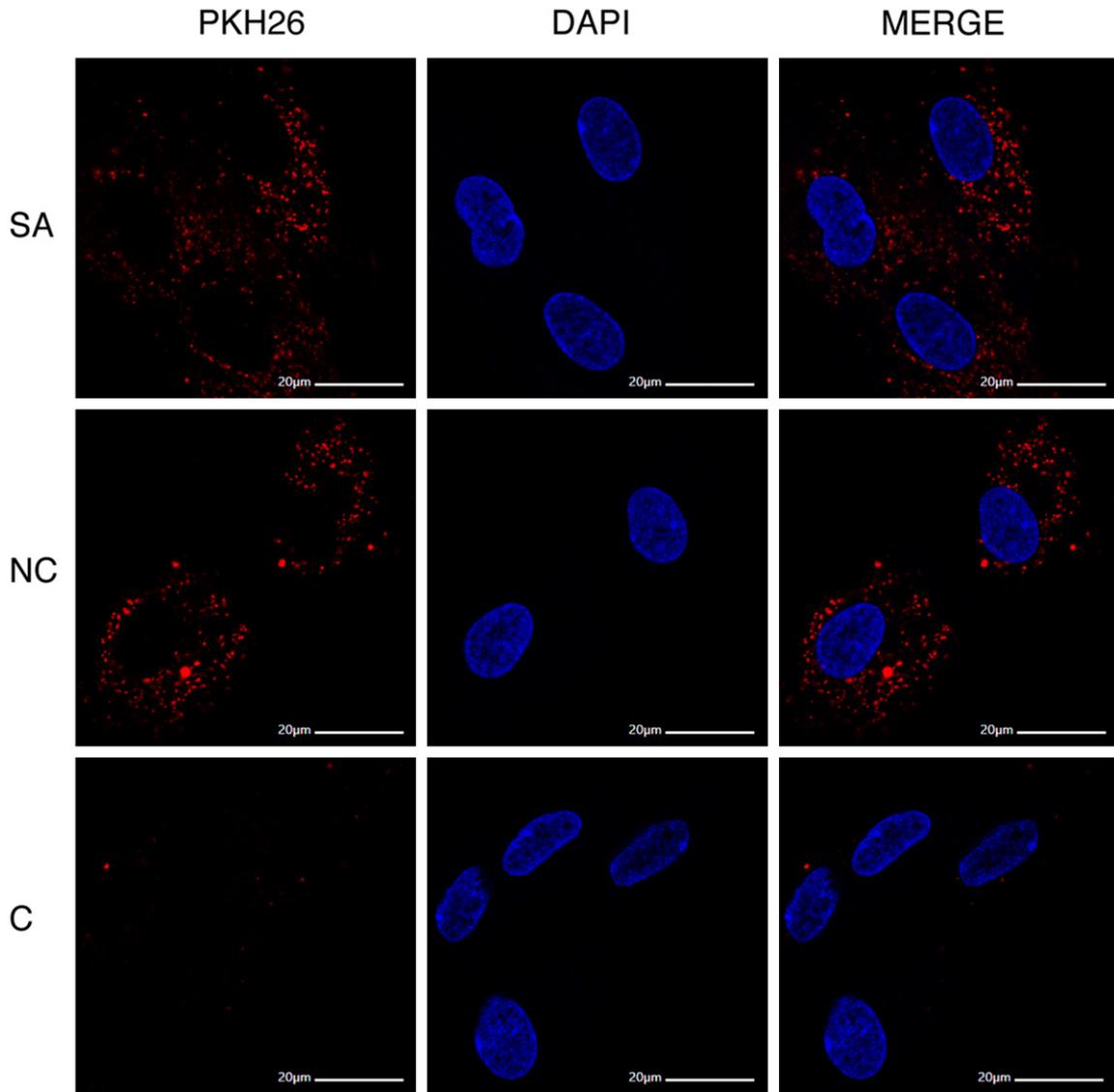


**Figure 1.** Characteristics of isolated exosomes from serum samples. A. Morphology of serum-derived exosome was visualized by TEM. B. The exosome-specific proteins CD9, CD63, HSP70, and CD81 were detected in the serum exosomes by WB analysis. C. Size distribution of serum-derived exosome was analyzed using NTA, indicating the diameter of isolated exosome in 86.1-187.1 nm. D. The exosomes' concentration was  $8.09 \times 10^8$ /ml.

uate the variation in miRNA expression between the patients with SA and controls. We identified a total of 2,588 miRNAs in human serum exosomes between patients with SA and controls, with up-regulated displayed in red and down-regulated displayed in green (**Figure 3A**).

Based on  $|\log_2(\text{fold change})| \geq 1$ , we obtained 63 miRNAs from 5p-arm, 75 miRNAs from 3p-arm, and 51 miRNAs from other arms; among them, 90 were up-regulated, 99 were

down-regulated. The hierarchical clustering analysis of the 189 miRNAs was depicted with the Heatmap package of R software (version 3.6.1). The color scale, ranging from green to red, indicated high to low expression of each miRNA transcript (**Figure 3B-D**). Then we also tried to explore the association of different miRNAs using the spearman' correlation test. As shown in **Figure 4A**, the red block showed a positive correlation, and the blue block showed a negative correlation. The result proved that



**Figure 2.** Exosome uptake experiments (100× magnification). SA: spontaneous abortion. NC: normal pregnancy controls. C: negative controls.

some miRNAs were interrelated. They may connect by some common genes or pathways.

According to the threshold the expression of miRNA  $\geq 1$ -fold changes and  $P < 0.05$ , we obtained 7 up-regulated (miR-3607-3p, miR-373-3p, miR-410-3p, miR-382-3p, miR-371a-3p, miR-34b-3p, miR-372-3p) and 3 down-regulated miRNAs (miR-548j-3p, miR-3677-3p, miR-374a-3p) from the 3p-arm, and 5 up-regulated (miR-371a-5p, miR-6859-5p, miR-200a-5p, miR-3124-5p, miR-1292-5p) and 7 down-regulated miRNAs (miR-542-5p, miR-4638-5p, miR-520c-5p, miR-518d-5p, miR-518f-5p, miR-

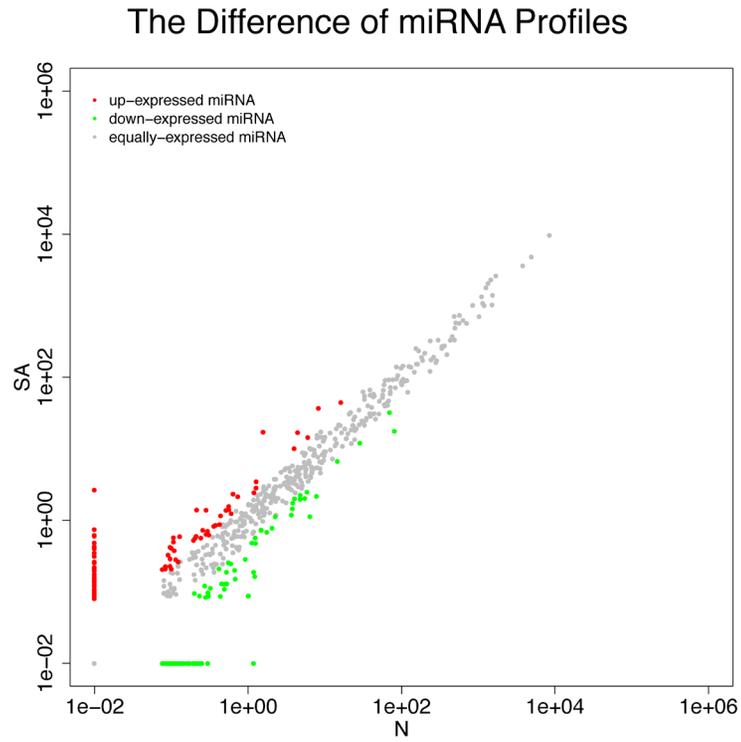
215-5p, miR-125a-5p) from the 5p-arm (**Figure 4B**).

#### *Identification of target microRNAs relating to spontaneous abortion*

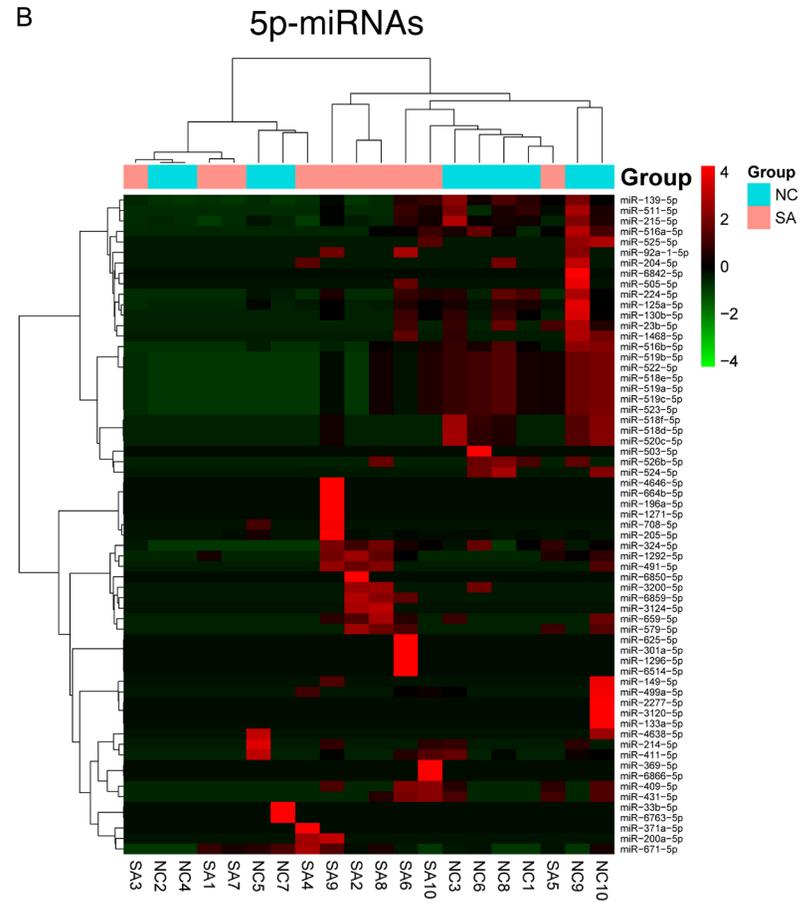
In this part, we selected the most differentially expressed miRNAs based on  $|\log_2(FC)| \geq 1$  and  $P < 0.01$ , that is, miR-371a-5p, miR-206, miR-147b, miR-6859-5p, miR-410-3p, miR-1270, and miR-524-5p. These miRNAs were all differentially expressed in serum exosomes between two groups, with five up-regulated (miR-371a-5p, miR-147b, miR-6859-5p, miR-

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A



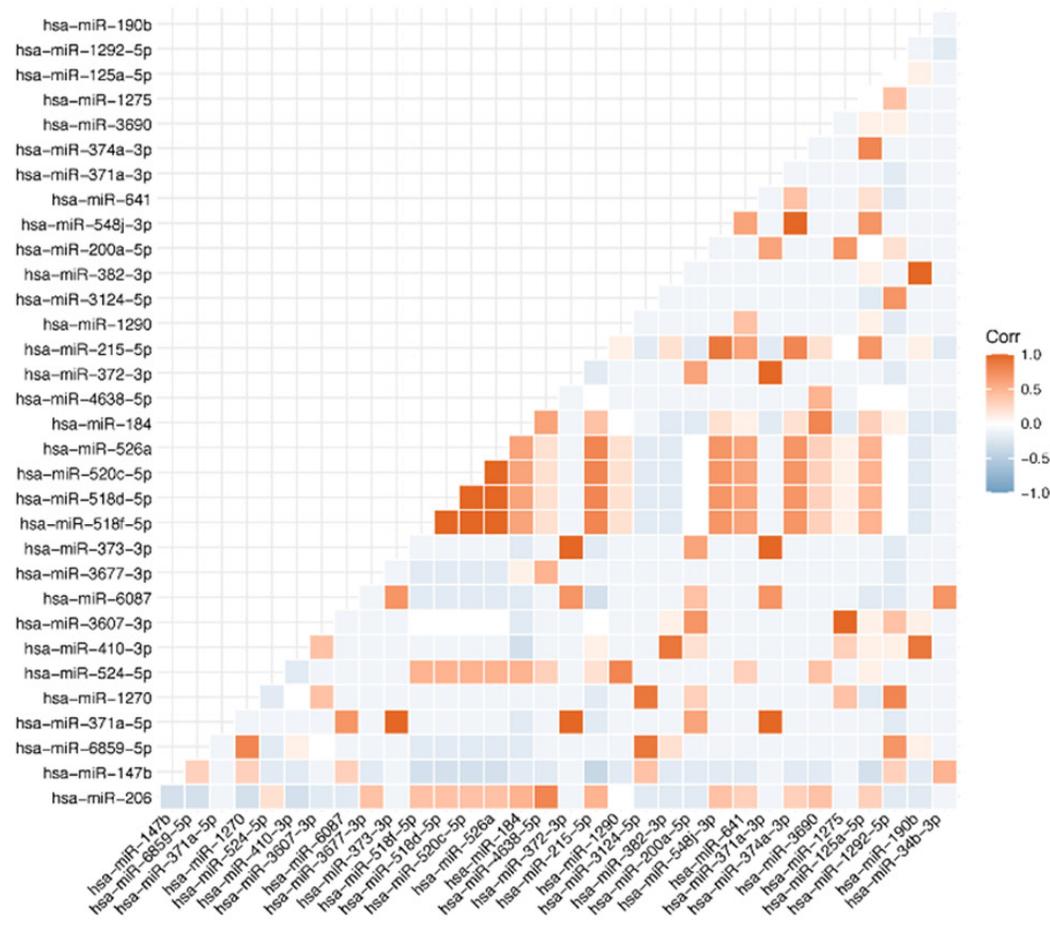
B



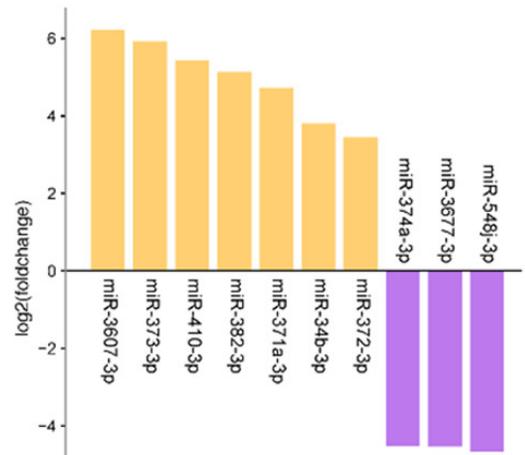


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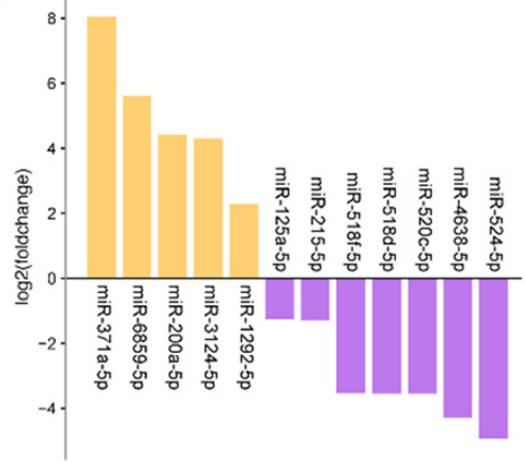
A



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C



**Figure 4.** A. Correlation matrix showing the Spearman's correlation scores between different miRNAs in SA patients compared to normal pregnancy controls. B. The expression profiles of miRNAs based on the threshold of FC  $\geq 1$  and  $P < 0.05$  from 3p-arm. C. The expression profiles of miRNAs based on the threshold of FC  $\geq 1$  and  $P < 0.05$  from 5p-arm.

410-3p and miR-1270) and two down-regulated (miR-206 and miR-524-5p), which may imply the related pathogenesis in patients with SA.

### *Identification of hub genes and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analysis and pathway category*

Every miRNA can interact with multiple genes. The target genes were further predicted and verified by TargetScan, miRDB, miRTarBase, miRWalk and HMDD V3.2. However, we found miR-147b had no target genes based on the integrated analysis, so it's excluded in the subsequent analysis.

Then we performed the KEGG analysis and pathway category to learn about the miRNA-mRNA network. Detected by the miRPath v3.0 online tool, there were 29 KEGG pathways and pathway target genes in the up-regulated miRNAs, 24 pathways and pathway target genes in the down-regulated miRNAs. The pathway functional category was determined using KEGG PATHWAY Database, and then supplemented by a manual search in PubMed literature.

From the above analysis, we could see that these candidate miRNAs could regulate gene expression and were involved in many pathophysiological pathways (**Table 2**). Next, we found out the critical target genes regulated by multiple miRNAs ( $\geq 2$  miRNAs) as hub genes to find relating pathways in patients with SA. As shown in **Figure 5**, the identified hub genes included WNT8B, PPP3R1, CSNK2A1, SGK3, SMAD3, HS3ST3B1, and NFATC2. Therefore, nine risk pathways were revealed, including Wnt signaling pathway, Hippo signaling pathway, FoxO signaling pathway, Hedgehog signaling pathway, Oocyte meiosis, PI3K-Akt signaling pathway, TGF-beta signaling pathway, Endocytosis, and Glycosaminoglycan biosynthesis - heparan sulfate/heparin.

### *Serum exosomal miR-371a-5p as diagnostic biomarkers for SA*

The most significantly up-regulated and down-regulated miRNAs, miR-371a-5p and miR-206, were selected for qRT-PCR validation using an independent cohort of serum-derived exosomes obtained from 24 SA patients and 24 normal early pregnancy subjects. The results showed that exosomal miR-371a-5p and miR-

206 were overexpressed between the two group patients, while the latter was contrary to the sequencing result (**Figure 6**).

The classification efficiency by serum exosomal miRNAs was then assessed by ROC curve analysis. As shown in **Figure 7**, the area under the receiver operating characteristic curve (AUC) of serum exosomal miR-371a-5p was 0.790 [standard error (SE) = 0.066; 95% confidence interval (CI) = 0.661-0.919;  $P < 0.0001$ ]. The AUC of serum exosomal miR-206 was 0.696 (SE = 0.078; 95% CI = 0.542-0.850;  $P = 0.0124$ ). We next conducted a multivariate logistic regression to explore the multi-marker diagnostic performance of the two miRNAs. The combination panel of exosomal miR-371a-5p and miR-206 achieved the AUC of 0.809 (SE = 0.065; 95% CI = 0.682-0.936;  $P < 0.0001$ ).

## Discussion

SA is a common problem in women of child-bearing age. The diagnosis is complicated due to SA's heterogeneity and a lack of reliable clinical and molecular markers. In this study, we aimed to explore potential biomarkers of SA using small RNA sequencing.

Exosomes are small secreted extracellular vesicles that can be found in almost all kinds of biological fluids. Importantly, they are considered critical mediators of cell-cell communication due to the ability to carry and deliver multiple messages to distal and surrounding cells. Besides, exosomes can be obtained in a non-invasive way, making them available to use in various clinical settings. Therefore, exosomal miRNAs, which were non-coding RNAs abundant in exosomes, might be useful for early detection of diseases.

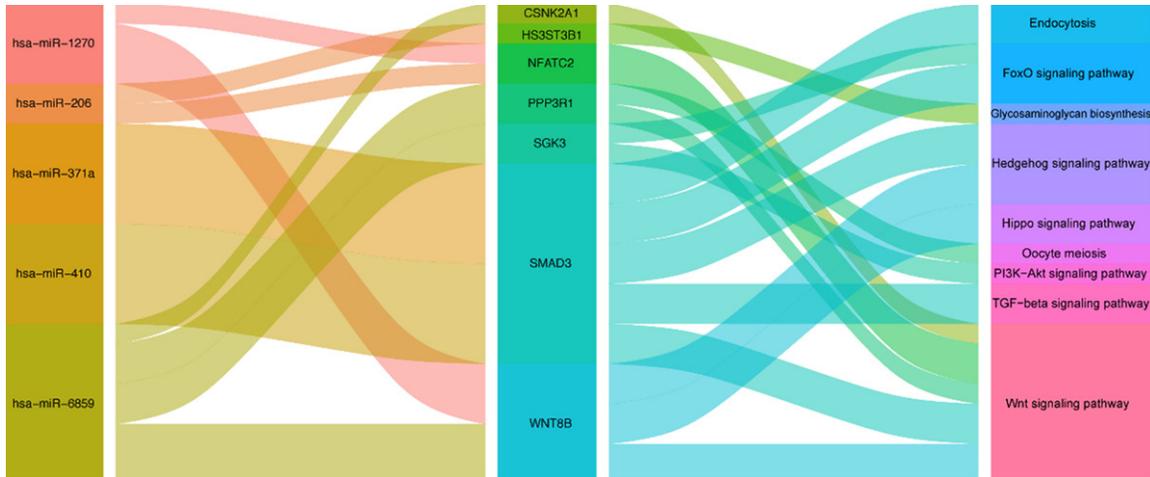
This is the first study to reveal the possible use of exosomal miRNAs as diagnostic markers of SA. According to the literature, several miRNAs are differentially regulated in SA, including miR-378a-3p [10], miR-133a [11], miR-103 [12], miR-27a [13], miR-449b [13], miR-98 [14], miR-365 [15], miR-19b [16], miR-494 [16] and miR-27a-3p [17], and so on. However, none of them were exosomal and serum originated. In our study, we found that exosomal miR-371a-5p, miR-147b, miR-6859-5p, miR-410-3p, and miR-1270 were up-regulated, while exosomal miR-

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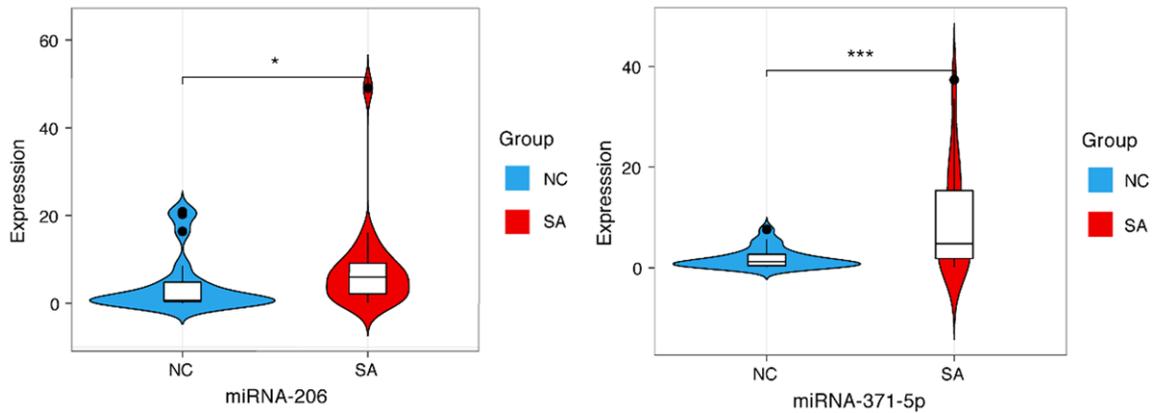
**Table 2.** Multiple genes regulated by five target microRNAs and KEGG pathway analysis of five target miRNAs

	TGF-beta signaling pathway	Hippo signaling pathway	Wnt signaling pathway	FoxO signaling pathway	Hedgehog signaling pathway	Glycosaminoglycan biosynthesis-heparan sulfate/heparin	HIF-1 signaling pathway	Endocytosis	PI3K-Akt signaling pathway	Oocyte meiosis	Notch signaling pathway	Estrogen signaling pathway
miR-371a-5p	SMAD3	SMAD3	DKK2 SMAD3	SMAD3	/	/	/	SMAD3	PPP2R5A TNR	PPP2R5A PGR	/	/
miR-206	E2F5	AJUBA AMOT DLG4 FZD7 WWC1 YWHAQ YWHAZ	FZD7 NFATC2 NFATC3 SFRP1	IGF1 KRAS	/	HS3ST3B1		ACAP2 ARF3 CAV2 CBL CLTC RAB11FIP1 RAB5A	/	/	/	ATF2 KRAS
miR-6859-5p	/	YAP1 LEF1 WNT8B	PPP3R1 CSNK1A1 LEF1 CSNK2A1 WNT8B	SGK3 HOMER1 GRM1	CSNK1A1 WNT8B	HS3ST3B1	EIF4E	RAB11FIP2 FGFR2 EPS15	FGFR2 CHRM2 EIF4E SGK3	PPP3R1 CPEB4	/	/
miR-410-3p	EP300 CREBBP	SMAD2 BMP5 SMAD3 SMAD4 BMP2 TGFB2 BMPR1A SMAD7 PPP2R1B BMPR2	SMAD3 SKP1 SMAD4 EP300 CREBBP	SMAD2 SMAD3 SMAD4 TGFB2 EP300 CREBBP	BMP2	/	EP300 CREBBP RPS6KB1	SMAD2 SMAD6 SMAD3 TGFB2 SMAD7	PPP2R1B RPS6KB1	SKP1 PPP2R1B	EP300 CREBBP	/
hsa-miR-1270	/	AFP WNT8B	CSNK2A1 NFATC2 PLCB1 PPARD PPP3R1 WNT8B	ARAF RAG1 SGK3 STK4	WNT8B	/	TFRC		LAMB4 SGK3	ppp3r1	/	/

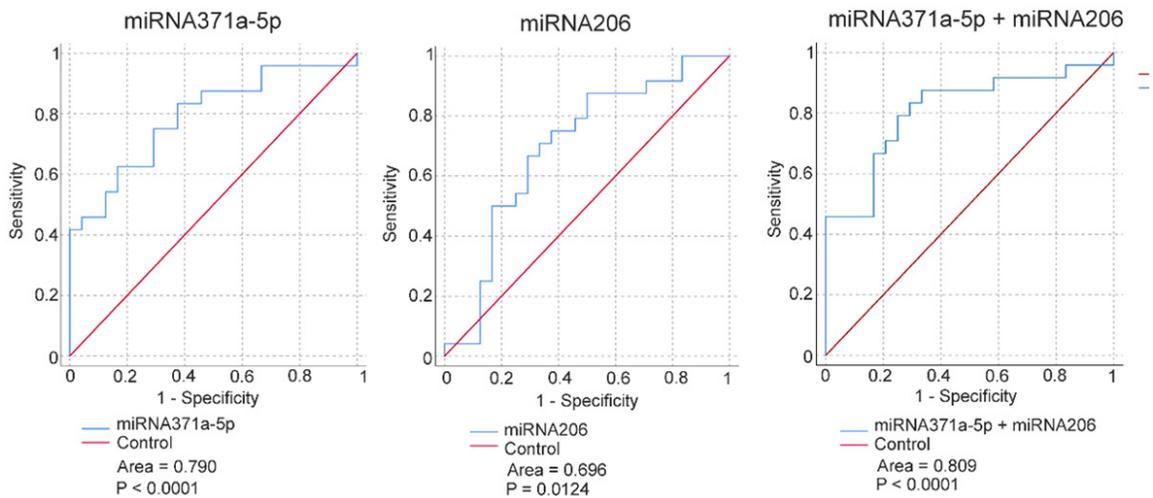
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**Figure 5.** Five exosomal miRNAs-target genes-KEGG pathway network showing the possible pathophysiological mechanism relating to SA.



**Figure 6.** Hsa-miR-371a-5p and hsa-miR-206 were significantly up-regulated in SA patients compared to normal pregnancy controls validating by qRT-PCR. (\* $P < 0.05$ , \*\*\* $P < 0.001$ ).



**Figure 7.** Receiver Operating Characteristic (ROC) curves and associated AUC showing the predictive value of exosomal miR-371a-5p and miR-206 for SA. ROC, receiver operating characteristic; AUC, area under the curve.

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206 and miR-524-5p were down-regulated in SA patients.

Validating by qRT-PCR, exosomal miR-371a-5p and miR-206 show a distinct difference between the SA and control groups. Previous studies indicated that in recurrent pregnancy loss, miR-371a-5p and XIAP was obviously decreased, and miR-371a-5p regulated apoptosis pathway by mediating the reduction of XIAP in RPL [18]. Besides, miR-371a-5p were also reported to have relations to inflammation in placenta [19], gestational trophoblastic disease [20], aging [21], cardiomyopathy [22], systemic lupus erythematosus [23], pancreatic carcinoma [24], hepatocellular carcinoma [25], nasopharyngeal carcinoma [26] and influenza A [27]. However, the function of exosomal miR-371a-5p was still unclear. Recently, miR-206 has been validated as a tumor suppressor in various cancers, such as breast cancer, lung cancer, and rhabdomyosarcoma [28-30]. It can regulate proliferation, apoptosis, and autophagy by influencing the Akt signaling pathway. However, the PCR result of miR-206 is opposite to that of sequencing. This inconsistent results may be due to some more complex mechanisms or small sample size, which needs to be further clarified.

In our study, we display a network of exosomal miRNA-target genes-signaling pathway in SA, which may help to elucidate the etiology and pathogenesis of abortion in an overall overview. Using bioinformatics prediction methods, we noted different miRNAs could relate to common genes: WNT8B, PPP3R1, CSNK2A1, SGK3, SMAD3, NFATC2, and HS3ST3B1. Until now, only SMAD3, targeted by miR-371a and miR-410 in our study, had been reported to be related to abortion caused by Chinese 1 strain of *Toxoplasma gondii* by inhibiting TGF $\beta$ RII/Smad2/Smad3/Smad4 signaling pathway [31]. However, the other genes need further validation.

Considering genes' interaction, these hub genes should involve some risk pathways contributing to SA's pathogenesis and epileptogenesis. Our study revealed nine high-risk pathways. Among these predicted pathways, we pointed out three major high-risk pathways: the Wnt signaling pathway, the Hippo signaling pathway, and the FoxO signaling pathway.

The Wnt signaling pathway, having five upstream genes regulated by three miRNAs, was highly conserved. This pathway extensively participates in various pathological events, including organogenesis, axis differentiation in multicellular organisms' cancer pathogenesis, and the epithelial-mesenchymal transition [32]. Activation of this pathway was reported to promote trophoblast invasion; otherwise, failures in this process were related to pregnancy complications, including abortions [33, 34].

The other two pathways were hippo and FOXO signaling pathway. The hippo signaling, involving two genes regulated by four miRNAs, is an evolutionarily conserved pathway that is notable in controlling organ size from flies to humans. The core components of Hippo pathway include a kinase cascade, MST1/2 and LATS1/2, and a transcription module, YAP and TAZ. This pathway combined a broad range of signals to control many critical cellular events. Many reviews have published its function in cancers, immunity, cardiovascular diseases, embryogenesis, and development [35-37]. In the last decade, mounting evidence showed that Hippo-YAP/TAZ played a unique role in normal pregnancy and pregnancy complications such as implantation failure, preeclampsia (PE), and RSA [38]. The FOXO signaling pathway involved two genes regulated by three miRNAs in our study. FOXO family of transcription factors are central regulators of cellular homeostasis. It regulates a various physiological events, such as apoptosis, cell-cycle control, glucose metabolism, oxidative stress resistance, and longevity. The best-known pathway negatively regulating FoxO transcription factors is the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB/Akt) signaling pathway. Although the FOXO signaling pathway was reported to be related to immune system diseases and cancers, and there are no relevant reports about FOXO signaling pathway and SA at present, the FOXO signaling pathway may have some extent relations to SA in immunology and metabolism aspects. However, a greater understanding of the regulation of FOXO target specificity and its relation to SA is still needed.

The limitations of this study are related to the databases we used. They may have limited data sources; thus, our analysis may have to be renewed as databases become more compre-

hensive and update. Secondly, the current sample size of 68 participants was inadequate to draw a conclusion. Additionally, without experimentation, we cannot verify the network of miRNA-mRNA-signaling pathway. Last but not the least, the thorough elucidation of the underlying molecular mechanisms about exosomes' biogenesis, actions, circulation, cargo, and so on is a prerequisite of the broad utilization of exosomes. With the development of more standardized purification and exosomes' analytical procedures, we will likely reveal their function more thoroughly.

In conclusion, our results suggest that the circulating miRNAs from exosomes might be potential biomarkers for patients with SA.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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