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Exosomal miR-202 derived from leukorrhea as a potential biomarker for endometriosis

Li Zheng, Dai Fei Sun and Yanyan Tong

Abstract

Objective: Endometriosis (EMS) is a chronic gynecological disorder with an urgent need of a reliable non-invasive diagnostic strategy. Recently, there has been increasing interest in using the contents of exosomes, especially exosomal microRNAs (miRNAs), as potential biomarkers for various types of diseases. In this study, we assessed the differentially expressed miRNAs in exosomes derived from primary normal and ectopic endometrial cells.

Methods: We used miRNA microarray analysis to identify differentially expressed exosomal miRNAs. Among the selected exosomal miRNAs, we focused on hsa-miR-202-3p and hsa-miR-202-5p and validated their expression levels using quantitative reverse transcription polymerase chain reaction analysis. We then further examined their expression in exosomes derived from vaginal discharge (leukorrhea) from patients with EMS and the negative control group.

Results: The data show that hsa-miR-202-3p and hsa-miR-202-5p were expressed significantly higher in leukorrhea-derived exosomes from EMS patients compared with the negative controls.

Conclusion: Taken together, our results suggest that leukorrhea-derived exosomal hsa-miR-202 could serve as a potential useful biomarker for diagnosing EMS.

Keywords

Endometriosis, miR-202, vaginal discharge, biomarker, quantitative reverse transcription polymerase chain reaction, microarray, exosome

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Introduction

Endometriosis (EMS) remains one of the most common gynecological conditions, and is characterized by the implantation and growth of endometrial-like tissue outside of the uterus. EMS is classified into extra-abdominal and abdominal lesions,

Department of Obstetrics and Gynaecology, Ningbo Women and Children's Hospital, Zhejiang, China

Corresponding author:
Yanyan Tong, Department of Obstetrics and Gynaecology, Ningbo Women and Children's Hospital, 339 Liuting Street, Zhejiang 315000, China.
Email: 415201324@qq.com

ovarian cysts, and deep EMS lesions based on the location of the ectopic endometrium colonization.[1] EMS affects about 10% to 15% of reproductive-age women, with many suffering from severe pelvic pain, heavy periods, and infertility.[2,3] The standard diagnostic approach for EMS is laparoscopic surgery, in which the endometriotic lesion can be obtained for pathological examination. Because of the invasive nature of laparoscopic surgery, many patients are reluctant to participate. As a result, a non-invasive diagnostic method that can diagnose EMS and reduce pain in the patient is desired.[4] Despite the great efforts that have been made to identify sensitive biomarkers over the past decades, there is still an urgent need to discover a definite diagnostic biomarker for EMS.

Exosomes are small extracellular vesicles (EVs) with a diameter ranging in size from 60 to 150 nm. They mediate cell–cell communication by carrying biological information, such as nucleic acids, proteins, lipids, and enzymes, between cells. Exosomes are secreted by almost all types of cells and are widely distributed in various body fluids, including blood, urine, ascites, saliva, and sputum.[5,6] The involvement of exosomes in different types of diseases has been extensively studied, with a particular interest on the use of exosomes in diagnostic and therapeutic applications. In recent years, the potential role of exosomal components as valuable non-invasive diagnostic and/or prognostic biomarkers has garnered more attention. Many studies have shown that serum exosomal microRNAs (miRNAs) are frequently upregulated in inflammatory disorders and cancers.[7–9] A recent study reported that serum-derived exosomal miRNAs, such as miR-22-3p and miR-320a, are significantly upregulated in the sera of patients with EMS. These molecules may therefore serve as potential diagnostic biomarkers for EMS.[10] However, the role of exosomes, particularly exosomal miRNAs derived from vaginal discharge (leukorrhea), in patients with EMS has not been well studied.

The aim of our study was to investigate the potential utility of leukorrhea exosomal miRNAs as diagnostic biomarkers for EMS. In this study, we used miRNA microarrays to explore the differentially expressed exosomal miRNAs between exosomes derived from the endometrial tissue of patients with EMS and those from individuals with other gynecological conditions and/or healthy women. We then assessed the aberrantly expressed exosomal miRNAs in leukorrhea, which provided a rationale for using these molecules as biomarkers for EMS diagnosis.

Materials and methods

Patients and samples

All samples were collected at Ningbo Women and Children's Hospital from January 2019 to January 2020. For the EMS group, ectopic endometrial tissue and leukorrhea samples were collected from patients diagnosed with EMS by laparoscopy and histopathological examination. For the negative control group, normal endometrial tissue and leukorrhea were obtained from normally cycling, reproductive-age women who underwent hysteroscopic submucosal myomectomy and women who had regular physical examinations (obtaining vaginal secretions only, exclusion of malignancy and EMS by vaginal ultrasound and abdominal ultrasound, ruling out dysmenorrhea, dyspareunia, pelvic pain, or infertility). Inclusion criteria: 1. No history of treatment with hormones or antibiotics within three months before laparoscopic surgery; 2. No hepatitis, tuberculosis, tumors, or other diseases. Exclusion criteria: 1. Treated with hormones or antibiotics

recently; 2. Has a serious disease; 3. Has any other gynecological disease, such as inflammation of the reproductive system or tumors. All subjects included were women who had regular menstrual cycles and no hormonal treatment for at least 3 months before sample collection. All vaginal secretions were obtained by scraping with a cotton swab, then stored at −80°C. The study was approved by the ethical committee of Ningbo Women and Children's Hospital (approval no. EC2019-037). The patients who gave tissue samples provided written informed consent and the participants who donated leukorrhea samples provided verbal informed consent before specimen collection.

Cell culture

Primary ectopic and normal endometrial cells were isolated from endometrial tissues obtained from patients in the EMS and negative control groups. Briefly, tissues were minced and digested in DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA) containing type IV collagenase and penicillin-streptomycin (Solarbio, Beijing, China) for 1 hour at 37°C in a shaking incubator. The cell suspension was centrifuged for 10 minutes at 400 × g and 4°C, then resuspended in fresh DMEM/F12 supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific). Cells were separated by filtering through 200-μm mesh, then resuspended and cultured in DMEM/F12 containing 10% FBS and 500 mg/mL penicillin-streptomycin in a humidified incubator at 37°C with 5% CO_2.

Exosome isolation and identification

Endometrial cell-derived exosomes were isolated form the cell supernatants of normal endometrial cells (NECs), ectopic endometrial cells (EECs), and leukorrhea by differential ultra-centrifugation, as previously described.[11] The isolated exosome pellets were sent to Hibio Technology Co., Ltd. (Hangzhou, China) for transmission electron microscope (TEM) observation, validation, and size distribution analysis.

Western blotting

Western blot analysis was performed to identify the exosomal markers CD63 and heat-shock protein 70 (HSP70). Briefly, exosomes isolated from endometrial tissue and leukorrhea were washed with phosphate-buffered saline (PBS), then lysed with RIPA buffer supplemented with proteinase inhibitor (Beyotime Biotechnology, Shanghai, China) and centrifuged at 14,000 × g for 10 minutes at 4°C. The exosomal protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific). Equal amounts of protein from each sample were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto polyvinyliene difluoride (PVDF) membranes. The blots were incubated with primary antibodies at 4°C overnight as follows: anti-CD63 (1:1000; Cell Signaling Technology (CST), Danvers, MA, USA), anti-HSP70 (1:1000; CST), and anti-GAPDH (1:3000; Bios, Shanghai, China). GAPDH was used as a loading control. After incubation with horseradish peroxidase-conjugated secondary antibodies for 2 hours at room temperature, the protein bands were visualized with chemiluminescence reagents (CST), followed by imaging on an electrophoresis gel imaging analysis system (D-Digital, Los Angeles, CA, USA).

Exosomal RNA extraction and miRNA microarray analysis

Extraction of exosomal miRNAs was performed using TRIzoI™ reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's suggested protocol.

The concentration and quality of each RNA sample were determined by a Nanodrop Spectrophotometer (Thermo Fisher Scientific). The extracted exosomal miRNAs were sent for miRNA microarray profiling (Askomics Co., Shanghai, China).

Quantitative reverse transcription polymerase chain reaction

The isolated exosomal miRNAs were reverse transcribed using a miRNA cDNA synthesis kit (CWBio, Beijing, China) according to the manufacturer's instructions. Forward primers for hsa-miR-202-3p (5′-AGAGGTATAGGGCATGGGAA-3′) and hsa-miR-202-5p (5′-TTCCTATGCATATACTTCTTTG-3′) were purchased from Sangon Biotech Co. (Shanghai, China), and quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis was performed using the SYBR Green PCR Kit (CWBio) on an Applied Biosystems 7500 Real-time PCR system and related software (Applied Biosystems, Waltham, MA, USA). U6 snRNA was used as an internal control to normalize miRNA expression levels. Forward U6 primer: 5′-CGCTTCGGCAGCACATATAC-3′; Reverse U6 primer: 5′-TTCACGAATTTGCGTGTCAT-3′. All samples were run in triplicate, with the average value used for fold change values.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA) and SPSS software (version 20.0; IBM Corp., Armonk, NY, USA). Two-tailed Student's t-tests were used to identify statistically significant differences among the EMS and control groups. All experiments were performed in triplicate and the results are expressed as mean ± standard deviation (SD). A P-value <0.05 was considered statistically significant.

Results

Characterization of endometrial cells and leukorrhea-derived exosomes

Overall, 11 patients were included in the EMS group. Of the 11 women in the negative control group, 6 underwent hysteroscopic submucosal myomectomy and 5 had regular physical examinations. Exosomes were isolated from patients' leukorrhea and EEC cell supernatants, which were previously cultured in media supplemented with exosome-free FBS for 48 hours, by ultracentrifugation as previously described. We used TEM, NanoSight analysis, and western blot analysis to validate exosome purification. Representative TEM images show that the majority of isolated exosomes were round-shaped and membrane-bound (Figure 1a). Particle size analysis demonstrated that the diameter distribution of the exosomes ranged from 60 to 150 nm, with an average of 95.5 nm (Figure 1b). Through western blotting, we examined protein expression of CD63 and HSP70 to confirm the presence of these specific exosomal markers (Figure 1c).

Exosomal miRNA microarray profiling in endometrial cells from patients with EMS and normal controls

To identify differentially expressed miRNAs in the exosomes derived from individuals with and without EMS, we extracted miRNAs from the exosomes of both groups and performed a miRNA microarray assay. Overall, 217 differentially expressed miRNAs were identified from the microarray profiling (Figure 2a–b). We validated the results of the microarray analysis by examining the expression levels of 11 identified miRNAs in exosomes from EMS patients ($n = 6$) and negative controls ($n = 5$) through RT-qPCR assays. The data show that hsa-miR-202-3p/-5p expression

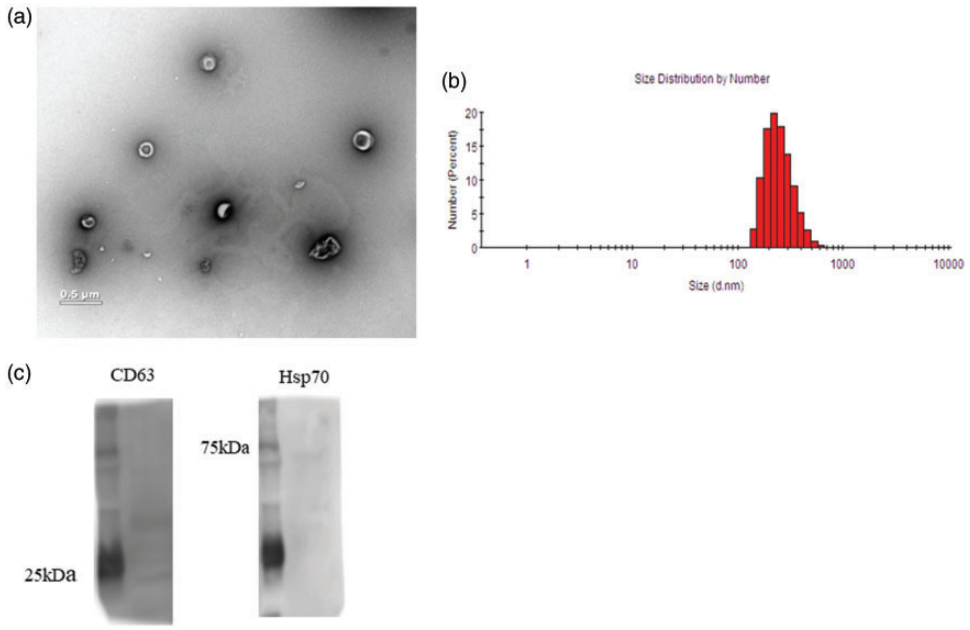

Figure 1. Characterization of endometrial cells and leukorrhea-derived exosomes. (a) Transmission electron microscope (TEM) images of exosomes isolated form endometrial cells. Scale bar = 20 nm by DLS and (b) Size distribution analysis of isolated exosomes and (c) Western blot analysis of exosome-specific markers CD63 and HSP70. Exosome depleted supernatant (EDS) was included as a control.

levels were significantly higher in exosomes from EMS patients compared with those from the controls ($P = 0.0194$ (miR-202-3p), $P = 0.004$ (miR-202-5p), Figure 2c). However, there was no significant differences in the expression levels of the other exosomal miRNAs between the EMS and negative control groups.

Hsa-miR-202-3p/5p expression in exosomes from EMS patient leukorrhea

We aimed to determine if the exosomal miRNAs detected in endometrial cells could also be identified in patients' leukorrhea. This would suggest that they could be used as non-invasive biomarkers for diagnosing EMS. Thus, we extracted exosomal miRNAs from leukorrhea in patients with EMS (n = 11) and negative controls (n = 11), then used RT-qPCR assays to examine the expression levels of the 11 validated miRNAs. The data suggest that exosomal hsa-miR-202 expression levels were significantly higher in leukorrhea from EMS patients than in that from the negative control group ($P = 0.0126$ (miR-202-3p), $P = 0.0238$ (miR-202-5p), Figure 3).

Discussion

EMS remains one of the most common gynecological disorders, yet a non-invasive and specific diagnostic biomarker is still urgently needed to improve early detection and treatment. Exosomes can be detected in almost all types of body fluids, including blood, urine, and leukorrhea, which has provided new opportunities to develop less invasive diagnostic approaches for various diseases. Recent studies have shown that exosomal contents, including miRNAs, can be detected in serum, demonstrating

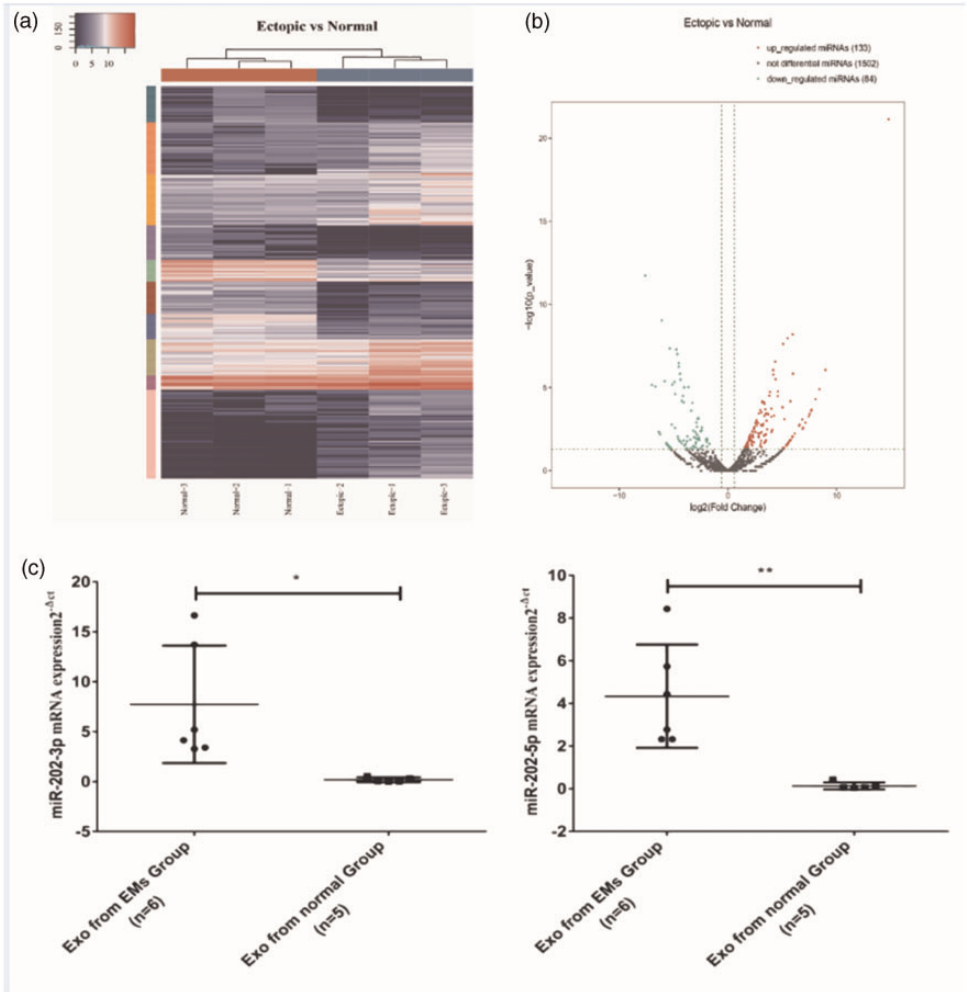

Figure 2. Exosomal microRNA (miRNA) microarray profiling and validation of differentially expressed exosomal miRNAs. (a) Heatmap of differential miRNAs in tissue-derived exosomes. (b) Volcano map of differential miRNA genes in tissue-derived exosomes with 133 upregulated and 54 downregulated differential miRNAs and (c) Hsa-miR-202-3p/5p expression levels were significantly increased in tissue exosomes with ectopic endometrial tissue.

their potential for use as less invasive biomarkers for disease diagnosis.[12]

MiRNAs, small non-coding RNAs that are about 22 to 24 nucleotides long, are highly conserved across species and can regulate protein expression levels by targeting specific mRNAs.[13] MiRNAs are involved in various biological processes, such as cell proliferation, differentiation, and apoptosis.[14] These molecules can be highly enriched in exosomes and associated with different biological effects.[15] A previous study reported the role of exosomal miRNAs in relation to EMS pathogenesis.[16] Numerous dysregulated and mutated miRNAs have been identified in EMS, which can potentially control the aggressiveness and angiogenesis associated with the disease.[17] Moreover, a recent

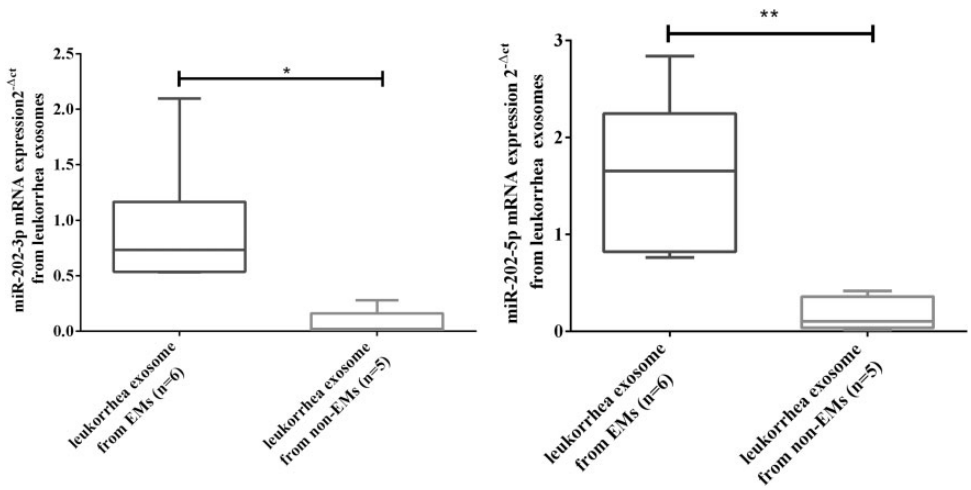

Figure 3. Exosomal hsa-miR-202-3p/5p expression is significantly higher in leukorrhea from patients with endometriosis.

study reported different expression profiles of exosomal miRNAs in serum from patients with EMS, suggesting their potential role as biomarkers.[10] However, exosomes and exosomal miRNAs in leukorrhea from patients with EMS have not been well characterized. Our data suggest that miRNAs derived from leukorrhea are more representative of the microenvironmental conditions inside the uterus and adnexa than those derived from circulating exosomes.

In the current study, we isolated and identified exosomes from both endometrial cells and vaginal discharge (leukorrhea) from individuals with and without EMS. We first analyzed the endometrial cell-derived exosomal miRNA expression profiles of the EMS and negative control groups using miRNA microarray. The results revealed that 217 exosomal miRNAs were differentially expressed. We further validated the expression levels of 11 exosomal miRNAs by performing RT-qPCR analysis, finding that hsa-miR-202-3p and hsa-miR-202-5p were significantly upregulated in exosomes derived from patients with EMS compared with negative controls. Next, we observed that both hsa-miR-202-3p and hsa-miR-202-5p were also significantly upregulated in leukorrhea exosomes from patients with EMS. Taken together, these observations suggest that leukorrhea exosomal hsa-miR-202-3p and hsa-miR-202-5p may serve as potential non-invasive diagnostic biomarkers for EMS.

Aberrant expression patterns of hsa-miR-202-3p and hsa-miR-202-5p can reportedly contribute to the progression of different types of diseases, including cancers, metabolic disorders, and cardiovascular disease. For example, hsa-miR202-3p was reported to be involved in suppression of tumor cell proliferation, migration, and invasion in gastric cancer.[18,19] Additionally, another study showed that high expression of hsa-miR-202-5p inhibited the tumorigenic potential of colorectal carcinoma cells by downregulating oncogenic SMARCC1.[20] However, another study stated that higher blood miR-202-3p expression levels were associated with an increased risk of essential hypertension.[21] Furthermore, in EMS, a study on differentially expressed miRNAs in eutopic and ectopic endometria showed that both hsa-miR-202-3p and

hsa-miR-202-5p expression levels were upregulated in ectopic endometrium.[22]

Here, we assessed and compared differentially expressed exosomal miRNA levels in normal and ectopic endometrial cells and further identified their expression in leukorrhea-derived exosomes. Overall, our study demonstrates the possibility of using leukorrhea-derived exosomal hsa-miR-202 as a biomarker for the non-invasive diagnosis of EMS. However, because the number of patients and healthy subjects included in this study was low, the expression profiles of exosomal miRNAs in endometrial cells and leukorrhea need further verification by analyzing a larger cohort. Furthermore, the implication of exosomal miR-202 in EMS, specifically the mechanism underlying the pathogenesis of EMS mediated by exosomal miR-202, requires further exploration. In addition, the diagnostic value of miR-202, along with any correlations between exosomal miR-202 and clinicopathological features of EMS patients, should be further investigated in a subsequent study.

Conclusion

The identification and characterization of leukorrhea-derived exosomes and exosomal miRNAs could provide a basis for the future development of less/non-invasive biomarkers for diagnosing EMS. However, future studies are needed to validate the roles of such miRNAs in the development of EMS.

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Author contributions

YT, DS, and ZL designed the study and collected and analyzed the data. YT and DS drafted the initial manuscript. YT and ZL helped draft the manuscript. All authors have read and approved the final submitted manuscript.

Declaration of conflicting interests

The authors declare that there is no conflict of interest.

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ORCID iD

Yanyan Tong https://orcid.org/0000-0002-4537-2786

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