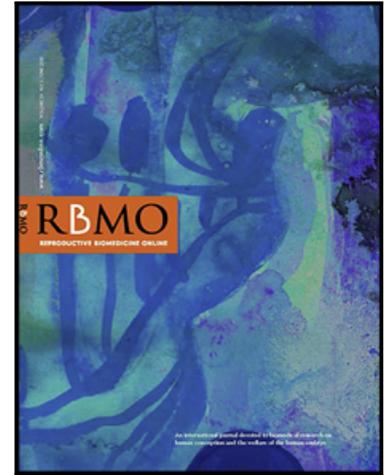# Journal Pre-proof

Extracellular vesicle-derived lncRNAs as circulating biomarkers for endometriosis

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Extracellular vesicle-derived lncRNAs as circulating biomarkers for endometriosis

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Abstract

Research question: Whether extracellular vesicle-derived lncRNAs could serve as promising circulating biomarkers for endometriosis?

Design: To obtain novel diagnostic markers, we enrolled 85 patients with endometriosis as the endometriosis group and 86 unaffected participants as the control group. RNA sequencing was performed to identify exosomal long non-coding RNAs (lncRNAs) that are differentially expressed between women with endometriosis (n=5) and unaffected participants (n=6). Messenger RNA and lncRNA sequences of the plasma exosomes were analyzed using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses. LncRNA expression levels were further validated using quantitative reverse transcriptase polymerase chain reaction. Moreover, receiver

operating characteristic curve analysis was performed to determine the diagnostic value of candidate lncRNAs. Clinical features were correlated to the expression levels of candidate lncRNAs.

Results: We found that 210 lncRNAs were significantly dysregulated; among these, expression of LINC01569, RP3-399L15.2, FAM138B, and CH507-513H4.6 was significantly decreased, whereas expression of RP11-326N17.2, KLHL7-AS1, and MIR548XHG was increased, in the plasma of patients with endometriosis. Combined expression level of RP3-399L15.2 and CH507-513H4.6 was used to distinguish patients with endometriosis from control participants; the results revealed a sensitivity of 80.00% and specificity of 85.45% at the cutoff point, and an area under the ROC curve of 0.9045. Our findings demonstrated the potential of these two lncRNAs as diagnostic biomarkers for endometriosis. Moreover, CH507-513H4.6 alone may be useful to detect early-stage endometriosis lesions.

Conclusions: The combination of RP3-399L15.2 and CH507-513H4.6 can be potential candidates for endometriosis biomarkers.

Keywords: lncRNA; exosome; endometriosis; diagnosis; biomarker; RNA sequencing

Introduction

Endometriosis, defined as the growth of endometrial-like tissue outside the uterus (Zondervan et al. 2018), is a benign gynecologic disease that mainly occurs in women of reproductive age. This estrogen-dependent disease, with a prevalence of 6%–10%, affects 50%–60% of women with chronic pelvic pain, and nearly 50% of infertile

women (Giudice 2010). Its major clinical manifestations, including dysmenorrhea and infertility, cause a heavy economic burden on those affected by the disease (Simoens et al. 2012). The diagnosis of endometriosis remains a challenge due to a lack of reliable biomarkers with both sensitivity and specificity (Falcone et al. 2018). Furthermore, diagnostic confirmation requires an invasive anatomopathological examination. Therefore, the diagnosis of endometriosis is generally delayed by approximately 7 years (Nnoaham et al. 2011).

Exosomes, an important component of extracellular vesicles (EVs), are membraned organelles of approximately 30–200 nm in diameter (Pegtel et al. 2019); they are secreted by different cells and enriched with certain proteins, nucleic acids, glycoconjugates, and lipids (Kalluri et al. 2020). Their membraned structure protects their contents from being degraded, making exosomes easier to isolate and detect. Exosomes play an important role in various pathophysiological processes such as intercellular communication, immune responses, inflammation, and tumor growth (Barile et al. 2017). Exosomes are also carriers of multiple types of RNA (Shurtleff et al. 2017), including microRNAs (miRNA), messenger RNAs (mRNA), and long non-coding RNAs (lncRNA).

Among them, lncRNAs—non protein-coding RNA transcripts of length exceeding 200 nucleotides (Ponting et al. 2009)—are involved in multiple biological processes (Rinn et al. 2012) such as cell proliferation, apoptosis, angiogenesis, and immune functions. It is reported that lncRNAs serve as epigenetic regulators of gene expression in multiple ways (Mercer et al. 2009), including the modulation of RNA

stability, coordination of chromatin dynamics, regulation of DNA methylation, etc. (Forrest et al. 2017). While the other important member of the non-coding RNA family, miRNAs, with a length of ~20 nucleotides, have posttranscriptional regulatory effects on gene expression by binding target RNAs (Beermann et al. 2016). Accumulating evidence have shown that lncRNAs and miRNAs interact with each other—lncRNAs as competing endogenous RNAs (ceRNAs) or sponges of miRNA (Tay et al. 2014)—to further modulate gene expression (Statello et al. 2021). Both lncRNAs and miRNAs are regarded as potential biomarkers of various diseases (Boon et al. 2016), including endometriosis (Ahn et al. 2017). They may play a vital role in enhancing angiogenesis, endometrial stromal cell invasion, and epithelial–mesenchymal transition (Wang et al. 2021) in endometriosis.

Studies have reported that exosomal lncRNAs can potentially act as diagnostic biomarkers for various diseases, including cancers (Bhan et al. 2017), cardiovascular diseases (Poller et al. 2018), and injuries (Viereck et al. 2017). A few studies have identified circulating lncRNAs or exosomal miRNAs as potential indicators of endometriosis, but knowledge on the potential importance of exosomal lncRNAs in this instance is insufficient and necessitates further study. Considering the dysregulation of exosomal miRNAs (Zhang et al. 2020) seen in patients, and their role in the pathogenesis of endometriosis (Bjorkman et al. 2019), we hypothesized that exosomal lncRNAs have significantly different expression levels in the plasma of patients, compared with healthy individuals' plasma. Therefore, in this study, we aimed to discover novel exosomal lncRNAs as potential biomarkers for endometriosis.

~~To discover novel exosomal lncRNAs as biomarkers for endometriosis, we performed RNA sequencing and identified differentially expressed exosomal lncRNAs in women with endometriosis compared with unaffected individuals. We further found that plasma exosomes from patients with endometriosis featured a unique lncRNA expression profile. Our findings indicate that exosomal lncRNAs can act as biomarkers for endometriosis. In particular, the combination of RP3-399L15.2 and CH507-513H4.6 may potentially serve as a novel non-invasive diagnostic biomarker for endometriosis.~~

Materials and methods

Ethics Approval and Informed Consent

This study was approved by the Scientific Research Projects Approval Ethics Committee of the International Peace Maternity and Child Health Hospital (IPMCH), Shanghai Jiao Tong University, School of Medicine, No. (GKLW) 2016-67. Informed consent was obtained from all recruited participants.

Sample Collection

With a power of 0.8, an alpha of 5%, and sensitivity and specificity value based on that of Carbohydrate antigen 125 (CA-125) (Muyldermans et al. 1995), a minimum of 59 subjects was calculated. Blood samples were collected from 85 patients with endometriosis—known in this study as the endometriosis group—and 86 unaffected participants—the control group—at IPMCH from September 2017 to December 2018.

Patients in the endometriosis group initially received a clinical diagnosis of ovarian endometriosis, which was later histologically confirmed through laparoscopy. 10 of the 80 patients which were enrolled for validation analysis had associated deep infiltrative endometriosis, none had associated superficial disease. Patients with malignancy, other benign ovarian cysts, and pelvic infection were excluded. The control group participants who were unaffected by endometriosis, were undergoing laparoscopy for fallopian tubal diseases without endometriosis lesions. In the control group, diagnoses like adenomyosis, leiomyoma, etc. were excluded according to pre-operative imaging examination. All participants were aged 20–45 years, of which five patients with endometriosis and six control participants, were recruited for exosomal RNA sequencing. An additional 80 pairs were selected for further validation analysis. The enrolled patients from both groups did not have a history of or present with signs of infection, tumors, or systemic or chronic diseases. They did not receive any hormonal medication within 3 months of undergoing laparoscopy.

The blood samples, approximately 5 mL, were collected into ethylenediaminetetraacetic acid tubes and stored at 15–20 °C for less than 1 h. The supernatant was transferred into centrifuge tubes after centrifugation at 820 g for 10 min at 4 °C. Prior to storage at -80 °C for further analysis, plasma was obtained by centrifuging the blood samples at 16000 g for 10 min at 4 °C.

Total Exosome Isolation and Identification

The total plasma exosome was isolated using the ExoRNeasy Serum/Plasma Midi Kit

(50) (Cat No. 77144, Qiagen, Hilden, Germany) and Buffer XE (100 μL; Cat No. 76214, Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The isolated exosomes were identified by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and western blotting. Exosomes were visualized by TEM in the laboratory of the Chinese Academy of Science, Shanghai, China. Exosomes were prepared in phosphate-buffered saline (Cat No. C0221A; Beyotime Biotechnology, Shanghai, China). A copper grid was used to sediment the exosome samples. We used 2.5% glutaraldehyde, 4% uranyl acetate, and 1% methylcellulose solution (Cat No. ST1510, Beyotime Biotechnology, Shanghai, China) to prepare exosome samples.

Exosomes were also identified by NTA using a ZetaView Nanoparticle Tracking Analyzer (Particle Metrix, Meerbusch, Germany). Data analysis was performed using ZetaView software (Particle Metrix, Meerbusch, Germany).

Exosomal marker CD63 (Cat No. MAB50482; R&D Systems, Shanghai, China) was selected to identify exosomes. Radioimmunoprecipitation assay buffer (Cat No. P0013B; Beyotime Biotechnology, Shanghai, China) and sodium dodecyl sulfate (SDS) loading buffer (Cat No. P0015F; Beyotime Biotechnology, Shanghai, China) were used to prepare exosomes. Protein samples were further run on a 12.5% SDS-polyacrylamide gel (Cat No. PG113; EpiZyme, Shanghai, China), and then transferred onto polyvinylidene fluoride membranes. After blocking with Quickblock buffer (Cat No. P0252; Beyotime Biotechnology, Shanghai, China), the membranes were incubated with primary antibodies against CD63 (1:1000, Cat No. MAB50482;

R&D Systems, Shanghai, China) at 4 °C overnight. The membranes were then incubated with secondary anti-mouse or anti-rabbit antibodies at 15–20 °C for 1 h, to identify exosomes.

RNA Isolation, Library Construction, and Sequencing

RNA quality was examined using the Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA, USA), based on which the RNA integrity number values were determined. Starting from using 1 μL of total exosomal RNA, a library was constructed using TruSeq Stranded Total RNA with Ribo-Zero Globin (Illumina, San Diego, CA, USA). The Agilent 2100 Bioanalyzer System was used to check the quality of complementary DNA (cDNA). Samples were prepared according to the method described in the HiSeq 4000 User Guide (Illumina, San Diego, CA, USA). The second-generation sequencer HiSeq 4000 was used for sequencing with the 150 bp paired-end sequencing method. Differentially expressed lncRNAs were identified based on the criteria of $P < 0.05$ and $|\log_2 \text{fold change}| \geq 1$.

GO and KEGG Enrichment Analyses

The main functions and pathways of significantly dysregulated mRNAs and lncRNAs were predicted by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) using DAVID v6.8(Huang da et al. 2009). The GO terms included biological processes (BP), cellular components (CC), and molecular functions (MF). The P value was corrected using the Bonferroni method.

Evaluation of Candidate lncRNAs with qRT-PCR

Blood samples from both endometriosis and control groups were collected as described in the sample collection section. An ExoRNeasy Serum/Plasma Midi Kit (50) (Cat No. 77144; Qiagen, Hilden, Germany) was used to extract plasma exosomal RNA; 14 µL of RNA was obtained. The NanoDrop 2000C UV-Vis spectrophotometer (ThermoFisher, MA, USA) was used to determine the RNA OD260/OD280 value for evaluating RNA concentration and purity. The RNA concentration ranged from 10 to 30 ng/µL, and the OD260/OD280 values were between 1.8 and 2.0.

RNA was reverse transcribed using the Takara PrimeScript™RT Master Mix (Perfect Real Time) kit (Cat No. RR036A; Takara, Beijing, China). The obtained cDNA was stored at -20 °C for further use.

Ten lncRNAs were selected as candidates for further analysis. To detect the lncRNA expression levels, we used the TB Green® Premix Ex TaqTM (Tli RNaseH Plus) kit (Cat No. RR420A; Takara, Beijing, China) and the Applied Biosystems QuantStudio instrument (ThermoFisher, MA, USA). The reaction was conducted according to the manufacturer's protocols. Human 18S ribosomal RNA was used as the internal control, based on a previous report (Li et al. 2020). The results were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The expression levels of lncRNAs were given as mean ± standard error of the mean

(SEM). They were analyzed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA) and SPSS 26.0 (SPSS Inc., Chicago, IL, USA). The mean expression levels of plasma lncRNAs between the endometriosis and control groups were compared using the Mann–Whitney U test. Bonferroni correction was performed for multiple comparisons. Relative expression levels were standardized using the following formula: lncRNA / SD (lncRNA). Receiver operating characteristic (ROC) curve analysis was performed, and the area under the ROC curve (AUC) was calculated to evaluate the diagnostic utility of candidate lncRNA biomarkers. Results with a P value of <0.05 were considered statistically significant.

Results

Patient Demographics

The clinical characteristics of patients with endometriosis (endometriosis group) and unaffected participants (control group) are shown in Table 1. As summarized, no significant differences were found in age, body mass index, and gravidity or parity between the groups. The patients with endometriosis presented with relatively advanced stages of disease according to the revised American Society of Reproductive Medicine (rASRM) score. No patients from either group were administered hormonal medication during the 3 months prior to enrolling in the study.

Exosome Identification

Plasma exosomes were isolated and identified by using TEM, NTA, and western

blotting. TEM demonstrated that the extracted EVs were round or oval (Figure 1a). The size distribution of exosomes, typically ranging from 50 to 150 nm with a peak at 81.1 nm diameter, was analyzed using NTA (Figure 1b). The exosomes were further quantified for protein marker CD63—enriched in exosomes—by western blotting (Figure 1c), confirming the successful isolation of plasma exosomes.

Differentially Expressed mRNAs and lncRNAs

mRNA and lncRNA expression levels were assessed by RNA sequencing of plasma exosomes from five patients with endometriosis and six control participants. We sorted 159 mRNAs and 210 lncRNAs that were significantly dysregulated according to the criteria of $P < 0.05$ and $|\log_2 \text{fold change}| \geq 1$.

A total of 159 mRNAs were differentially expressed between the endometriosis and control group plasma samples, among which 63 mRNAs were upregulated and 96 downregulated. RP11-371E8.4 was the most downregulated mRNA with $\log_2 FC$ of -3.875526485, while NPB ($\log_2 FC$ of 3.135949705) was the most upregulated. All dysregulated mRNAs are demonstrated on a volcano plot (Figure 2a). A hierarchical cluster heat map shows the variation in expression levels of the significantly dysregulated mRNAs (Figure 2b).

Similarly, 210 differentially expressed lncRNAs were found between the endometriosis and control group plasma samples, 122 of which were upregulated and 88 downregulated. CTD-2161F6.1 ($\log_2 FC$ -3.802773144) was the most downregulated lncRNA, while CTD-2151A2.1 ($\log_2 FC$ 4.388261022) was the most

upregulated lncRNA. A volcano plot is used to illustrate all dysregulated lncRNAs (Figure 2c). The variation in the expression level of significantly dysregulated lncRNAs is shown on a hierarchical cluster heat map (Figure 2d).

Correlation Between the Dysregulated lncRNAs and mRNAs

The correlation between dysregulated mRNAs and lncRNAs was evaluated by calculating the Pearson correlation coefficient (PCC). The coding–non-coding co-expression (CNC) network of the dysregulated mRNAs and lncRNAs was then established based on the criteria of $|PCC| \geq 0.8$ and $P < 0.05$ (Figure 3). Due to the lack of studies on lncRNAs, the selected mRNAs were used to perform further enrichment and pathway analyses of lncRNAs to predict their functions.

Enrichment and Pathway Analyses of the dysregulated mRNAs and lncRNAs

The functions of significantly dysregulated mRNAs between the endometriosis and control groups were predicted using the GO and KEGG pathway analyses. In the GO analysis (Figure 4a), the enriched terms included BP, MF, CC, and KEGG pathway, of which the G protein-coupled receptor (GPCR) activity was the most enriched. These enriched terms are crucial to the pathogenesis of endometriosis. The G protein-coupled estrogen receptor (GPER) is intensively expressed in ovarian surface epithelium and upregulated in ovarian endometriosis (Heublein et al. 2012), promoting angiogenesis and cell proliferation induced by hypoxia-inducible factor 1α (Zhang et al. 2017; Mori et al. 2015). Meanwhile, CXCR7—a C-X-C

motif-containing GPCR, essential for angiogenesis and cell proliferation—was reportedly overexpressed in patients with endometriosis as well as in a murine model of the disease (Pluchino et al. 2018). Moreover, leucine-rich repeat-containing G protein-coupled receptor 5-positive (LGR5+) cells were enriched in deep-infiltrating endometriosis lesions (Vallvé-Juanico et al. 2017), indicating the role of GPCRs in the pathogenesis of endometriosis.

As described above, the functions of the dysregulated lncRNAs were predicted according to the correlated mRNAs. Similarly, GO and KEGG analyses were conducted (Figure 4b), yielding results that were nearly consistent with those of the mRNAs. The results demonstrated that the lncRNAs were enriched in various important biological processes and molecular functions involved in the pathogenesis of endometriosis. Additionally, such enrichment in proteasome binding, protein destabilization, and extracellular region suggested that lncRNAs likely mediate the degradation and regulation of the extracellular matrix in endometriosis. It has been reported that the use of a proteasome inhibitor results in a significantly reduced volume of endometriotic implants in murine models (Celik et al. 2008). Meanwhile, aberrant regulation of matrix metalloproteinases may be indispensable for lesion formation in endometriosis (Bałkowiec et al. 2018).

Validation of Differentially Expressed mRNAs and lncRNAs

Based on the results of RNA sequencing, 10 dysregulated lncRNAs were selected for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

validation in plasma exosomes for 25 pairs of patients, each consisting of a patient with endometriosis and a control participant. In terms of seven of the selected lncRNAs, namely LINC01569, RP3-399L15.2, FAM138B, CH507-513H4.6, RP11-326N17.2, KLHL7-AS1, and MIR548XHG, the endometriosis group samples could be clearly and confidently distinguished from the controls (Figure 5). The former four lncRNAs were significantly downregulated, while the latter three were upregulated, in plasma exosomes from the endometriosis group.

Diagnostic Value of lncRNAs for Patients with Endometriosis

Next, RP3-399L15.2, CH507-513H4.6, and RP11-326N17.2 were chosen for further validation in another 55 pairs, consisting of patients with endometriosis and control participants, as these lncRNAs tended to demonstrate more significance and less deviation between groups.

To evaluate the diagnostic value of dysregulated lncRNAs, boxplots were drawn, and ROC curve analysis was conducted for the three lncRNAs mentioned above (Figure 6a). The associated AUC was also applied to confirm the diagnostic potency.

Individually, RP3-399L15.2 manifested the highest diagnostic value among the three lncRNAs validated, with an AUC of 0.8602 (95% confidence interval (CI): 0.7920–0.9283, P < 0.0001), as well as a sensitivity of 67.27% and specificity of 98.18% at the cutoff point. CH507-513H4.6 presented an AUC of 0.8096 (95% CI: 0.7307–0.8885, P < 0.0001), with a sensitivity of 67.27% and specificity of 81.82% at the cutoff point. To increase the diagnostic value, the two lncRNAs were combined to

differentiate between patients with and without endometriosis (Figure 6b). The AUC of the combined lncRNAs reached 0.9045 (95% CI: 0.8513–0.9576, P < 0.0001), with a sensitivity of 80.00% and specificity of 85.45% at the cutoff point.

Meanwhile, the dysregulated lncRNAs, CH507-513H4.6 and RP3-399L15.2, were also examined to determine whether they could be associated with certain clinical features in patients with endometriosis. The endometriosis group was divided into mild (I/II) and severe (III/IV) disease groups according to the rASRM stage to determine the diagnostic value of lncRNAs for disease severity (Figure 7a). As shown, the expression of RP3-399L15.2 consistently decreased with endometriosis progression; however, no significant difference was observed in RP3-399L15.2 expression between the groups with mild and severe disease. CH507-513H4.6 was more promising in the detection of early-stage lesions of endometriosis than RP3-399L15.2. We also correlated the expression level of lncRNAs with dysmenorrhea. Neither the two lncRNAs individually, nor their combination could distinguish patients with dysmenorrhea from those without dysmenorrhea (Figure 7b).

Previously, certain studies regarding circulating microRNAs as potential biomarkers for endometriosis have found different profile expression during menstrual cycle phase (Zhou et al. 2020). Since studies regarding circulating lncRNAs as potential biomarkers for endometriosis were relatively few, to clarify the influence of physiological hormonal status on lncRNAs expression, the expression level of lncRNAs RP3-399L15.2 and CH507-513H4.6 was compared between the sample of proliferative phase and secretory phase. No significant difference was

demonstrated (Supplementary Figure 1). And studies of circulating microRNAs might further support our results (Rekker et al. 2013; Vanhie et al. 2019; Papari et al. 2020).

Thus, our results indicated that CH507-513H4.6 alone may be useful to detect early-stage endometriosis and the combination of lncRNAs CH507-513H4.6 and RP3-399L15.2, may be a potential non-invasive diagnostic method for endometriosis.

Discussions

Difficulties in the non-invasive diagnosis of endometriosis delays the confirmation and management of the disease, thereby aggravating patients' symptoms and causing a substantial medical and economic burden on them. It should thus be a priority to identify sensitive and specific diagnostic biomarkers for this condition. To discover novel exosomal lncRNAs as biomarkers for endometriosis, we performed RNA sequencing and identified differentially expressed exosomal lncRNAs in women with endometriosis compared with unaffected individuals. We further found that plasma exosomes from patients with endometriosis featured a unique lncRNA expression profile. Our findings indicate that exosomal lncRNAs can act as biomarkers for endometriosis. In particular, the combination of RP3-399L15.2 and CH507-513H4.6 may potentially serve as a novel non-invasive diagnostic biomarker for endometriosis.

Previously, Sun et al. first reported differential lncRNA expression between the eutopic and ectopic endometrium, determined by microarray in patients with ovarian endometriosis (Sun et al. 2014). A total of 948 dysregulated lncRNAs and 4088

dysregulated mRNAs were found. Wang et al. identified 1277 dysregulated lncRNAs (Wang et al. 2015), of which 488 were upregulated and 789 downregulated, in eutopic endometrium of patients in endometriosis and control groups; their biological functions were predicted, which included cell cycle and immune-related pathway regulation. The lncRNA expression profile in serum samples between the endometriosis and control groups was also evaluated (Wang et al. 2016), indicating their potential as noninvasive biomarkers for endometriosis. However, isolated circulating lncRNAs are relatively unstable and only present in small quantities, which may influence their diagnostic value as disease biomarkers. Therefore, exosomal lncRNAs may be more suitable in clinical application (Kalluri et al. 2020).

To our knowledge, we are the first to report plasma exosomal lncRNA expression profiles in patients with endometriosis. We identified 210 significantly dysregulated lncRNAs, among which 122 were upregulated and 88 downregulated, in the plasma of patients with endometriosis, compared with that of healthy controls. We then conducted enrichment analysis of BP, MF, and CC terms and KEGG pathways. qRT-PCR validation of dysregulated lncRNAs was performed, and the diagnostic value of lncRNAs for patients with endometriosis evaluated through ROC curve analysis. Expression of lncRNAs LINC01569, RP3-399L15.2, FAM138B, and CH507-513H4.6 was significantly lower in the plasma samples of patients with endometriosis compared with the control samples, whereas RP11-326N17.2, KLHL7-AS1, and MIR548XHG expression was higher in patients with endometriosis. RP3-399L15.2 and CH507-513H4.6 were then combined to distinguish patients with

endometriosis from the controls, as the combination demonstrated an 80.00% sensitivity and 85.45% specificity at the cutoff point, along with an AUC of 0.9045, demonstrating potency for the diagnosis of endometriosis. The underlying mechanisms of these candidate lncRNAs in the pathogenesis of endometriosis remain unknown and require further research.

We analyzed the correlated mRNAs of seven lncRNAs through GO and KEGG analyses. Terms such as GPCR activity, GPCR signaling pathway, and methyl-CpG binding were enriched. The mRNAs related to these enriched items were sorted, for which a CNC network was constructed (Figure 8). Among these mRNAs, CXCL10, ERH, and GPR15 are involved in endometriosis-related pathophysiological processes. Existing studies have already revealed the role of cytokine CXCL10 in modulating the biological function of T cells in cancers and autoimmune diseases (Karin et al. 2018). In patients with endometriosis, serum and peritoneal fluid CXCL10 concentrations were significantly lower than in patients without endometriosis (Galleri et al. 2009). However, the underlying mechanism of CXCL10 in endometriosis remains unknown. It has been reported that ERH promotes human bladder cancer cell metastasis and regulates *MYC* expression (Pang et al. 2019). ERH is also necessary for the survival of cancer cells with KRAS mutation (Weng et al. 2012), indicating its role in enhancing cancer cell growth and metastasis. This might explain the infiltration and implantation behavior of endometriotic lesions. Furthermore, G protein-coupled receptor 15 (GPR15) is an extensively studied orphan GPCR involved in the immune processes of human colonic inflammation (Adamczyk

et al. 2017) and certain cancers (Wang et al. 2019). While immune dysfunction plays a crucial role in pathogenesis of endometriosis. Therefore, the value of candidate lncRNAs as biomarkers of endometriosis was enhanced by the involvement of their correlated mRNAs in the pathophysiology of endometriosis or immunological diseases. The validation of mRNA expression levels and the study of related pathophysiological mechanisms of endometriosis should be conducted in future.

Studies of the involvement of lncRNAs in various pathogenic processes of endometriosis have already been carried out. For example, lncRNA MALAT1 that is upregulated in ectopic lesions, has been reported to promote cell proliferation and invasion (Yu et al. 2019). LINC00261 could partially inhibit cell proliferation and migration and enhance cell apoptosis in endometriosis (Sha et al. 2017). LncRNAs CCDC144NL-AS1(Zhang et al. 2019), AC002454.1(Liu et al. 2019), and TC0101441(Qiu et al. 2020) can also facilitate cell migration and invasion in the disease process. Furthermore, lncRNA antisense hypoxia-inducible factor is reported to play a crucial role in angiogenesis (Qiu et al. 2019). LncRNA AFAP1-AS is involved in epithelial–mesenchymal transition (Lin et al. 2019). However, more studies, including functional studies of the dysregulated lncRNAs that we have identified, are required to obtain an overall understanding of the role of lncRNAs in the pathogenesis of endometriosis.

Despite the potential lncRNA biomarkers that we have identified, our research has some limitations. Firstly, the number of patients included was relatively small. In our study, all the patients enrolled had ovarian endometriosis, while patients with only

superficial lesion or deep infiltrative endometriosis were not taken into consideration. Large-scale cohort studies and patients with extra-ovarian lesions only are required to validate our results. Secondly, although the relationships between the expression level of candidate lncRNAs and some clinical features were analyzed, clinical features such as the severity of dysmenorrhea and years of infertility, could not be properly related to the expression level of candidate lncRNAs, due to insufficiently detailed clinical information. Finally, the action mechanism of the candidate lncRNAs and correlated mRNAs in the pathogenesis of endometriosis remains unknown. Further investigations including cellular and molecular experiments are warranted.

In summary, through RNA sequencing and qRT-PCR analysis, we found that plasma exosomes from patients with endometriosis have a unique lncRNA expression profile. Our findings indicate that exosomal lncRNAs can serve as promising biomarkers for endometriosis. Furthermore, we suggest that a combination of RP3-399L15.2 and CH507-513H4.6 can be potentially used as a novel non-invasive diagnostic biomarker for endometriosis. Future studies in larger cohorts are needed to confirm the clinical value of exosomal lncRNAs as biomarkers for endometriosis, as well as more basic researches are necessary to elucidate the underlying mechanism.

List of abbreviations

extracellular vesicles (EVs); microRNA (miRNA); messenger RNA (mRNA); long non-coding RNA (lncRNA); International Peace Maternity and Child Health Hospital (IPMCH); carbohydrate antigen 125 (CA-125); transmission electron microscopy

(TEM); nanoparticle tracking analysis (NTA); sodium dodecyl sulfate (SDS); complementary DNA (cDNA); Gene Ontology (GO); Kyoto Encyclopedia of Genes and Genomes (KEGG); biological processes (BP); cellular components (CC); molecular functions (MF); standard error of the mean (SEM); receiver operating characteristic curve (ROC); area under the ROC curve (AUC); revised American Society of Reproductive Medicine (rASRM); Pearson correlation coefficient (PCC); coding–non-coding co-expression (CNC); G protein-coupled receptor (GPCR); G protein-coupled estrogen receptor (GPER); quantitative reverse transcriptase polymerase chain reaction (qRT-PCR); 95% confidence interval (95% CI)

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

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

Code availability

Not applicable.

Authors' contributions

Conceptualization, S.S., Yeping Yang, Y.L. and H.X.; Data curation, S.S. and J.J.; Funding acquisition, H.X.; Investigation, S.S., Yeping Yang and B.Y.; Methodology, Y.L.; Project administration, Y.L. and H.X.; Resources, J.J, Yisai Yang. and F.S.; Supervision, F.S., J.Z., Y.L. and H.X.; Visualization, S.S., Yeping Yang. and B.Y.; Writing – original draft, S.S.; Writing – review & editing, J.Z. and Y.L. All authors read and approved the final manuscript.

Ethics approval

This study was approved by the Scientific Research Projects Approval Ethics Committee of the International Peace Maternity and Child Health Hospital (IPMCH), Shanghai Jiao Tong University, School of Medicine, No. (GKLW) 2016-67. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Consent to participate

Informed consent was obtained from all recruited participants.

Consent for publication

Not applicable.

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Figure legends
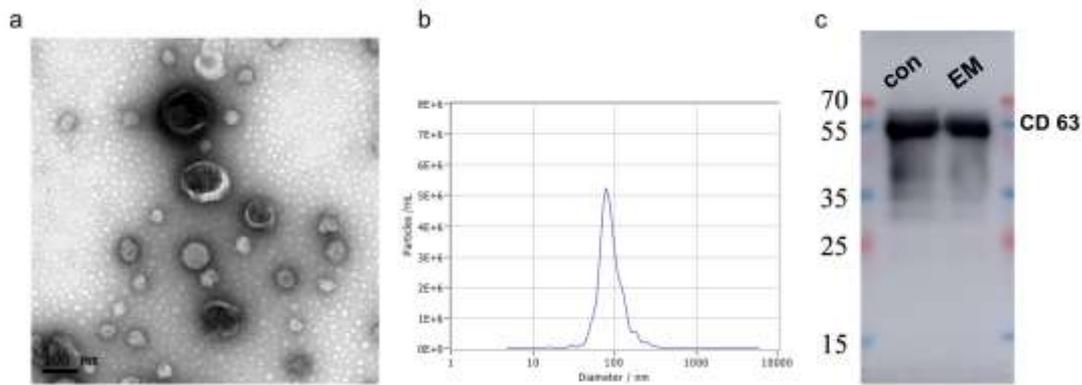
Figure 1. Isolation and identification of plasma exosomes. (**a**) Transmission electron microscopy (TEM) of the isolated exosomes. (**b**) Nanoparticle tracking analysis (NTA) of plasma exosomes. (**c**) Western blotting of exosomal marker, CD63. con: control group sample, EM: endometriosis group sample.
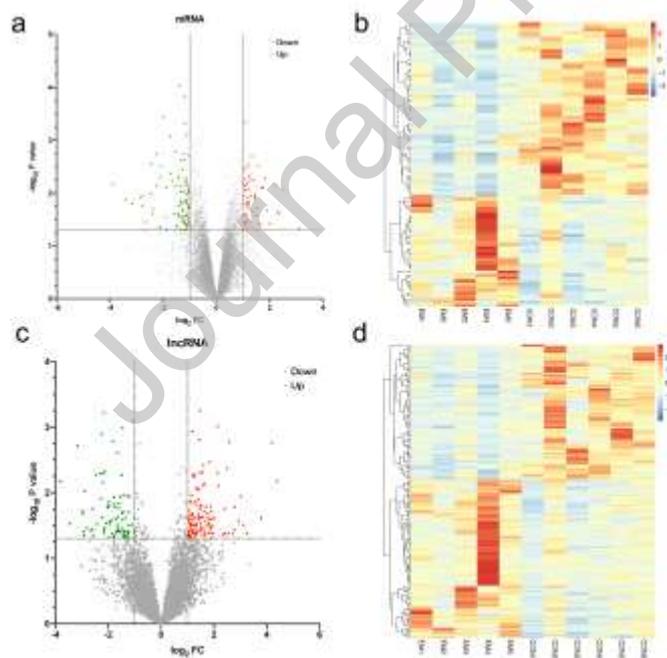

Figure 2. Differentially expressed exosomal mRNAs and lncRNAs in patients with endometriosis and the control. (**a**) Volcano plot of all dysregulated mRNAs. Data are expressed as -$\log_{10}$ (P Value) and $\log_2$ (FC). Green: relatively low

expression; grey: no significant difference in expression; red: relatively high

expression. FC: fold change. (**b**) Hierarchical cluster heat map of mRNAs with P

value < 0.05 and |log$_2$fold change|≥1. (**c**) Volcano plot of all dysregulated

lncRNAs. (**d**) Heat map of lncRNAs with P value < 0.05 and |log$_2$fold change|≥1.
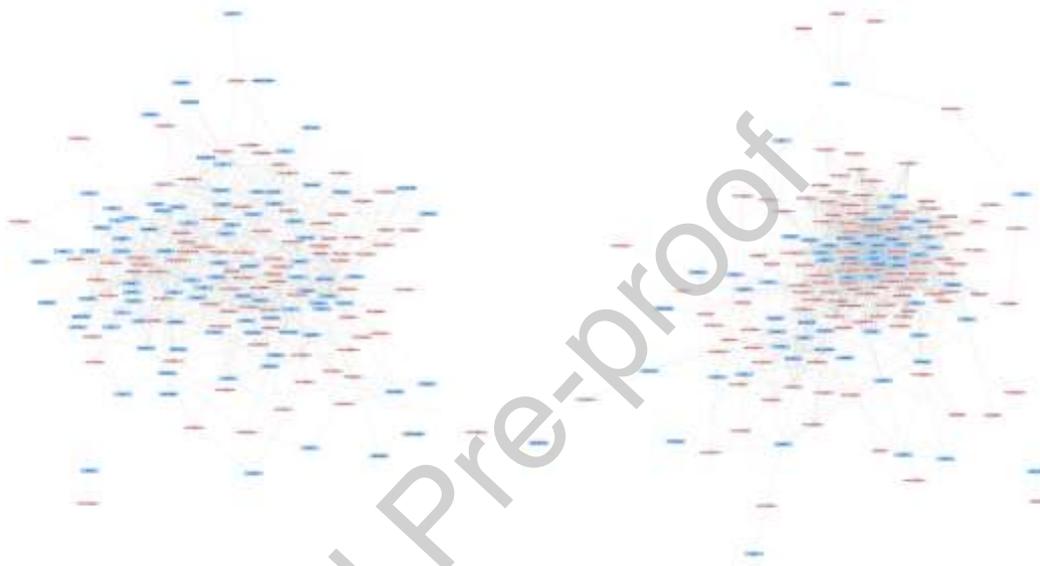
Figure 3. Coding–non-coding co-expression network (CNC) network of

lncRNA–mRNA co-expression (P < 0.05 and |PCC|≥0.8). Pink circular node:

lncRNA; blue rectangular node: mRNA. PCC: Pearson correlation coefficient.
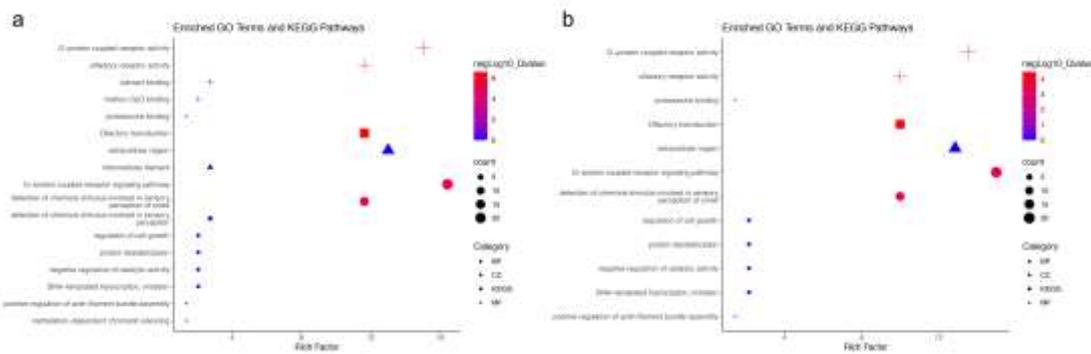
Figure 4. GO and KEGG pathway analyses of significantly dysregulated mRNAs and lncRNAs. (**a**) The enriched GO terms and pathways of dysregulated mRNAs. (**b**) The enriched GO terms and pathways of dysregulated lncRNAs. The horizontal axis shows the rich factor, and the vertical axis demonstrates the GO terms or KEGG pathways. The size of the symbol represents the number of genes, and the color indicates the q value.
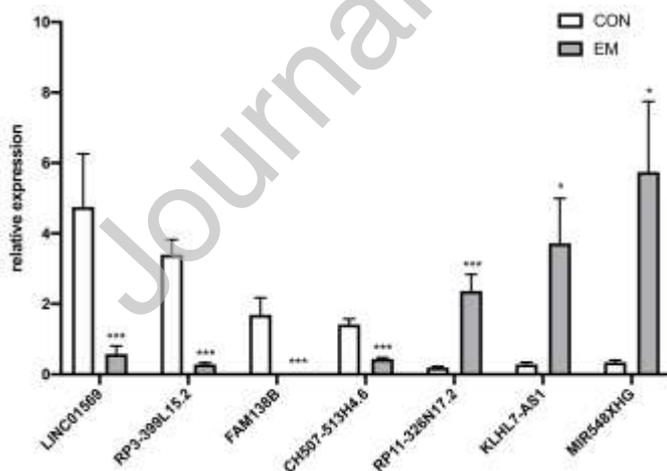

Figure 5. Expression levels of seven lncRNAs. The expression of LINC01569, RP3-399L15.2, FAM138B, CH507-513H4.6, RP11-326N17.2, KLHL7-AS1, and MIR548XHG in plasma exosomes, normalized to 18S as an internal control, using Mann–Whitney U test followed by the Bonferroni correction for multiple

comparisons. Error bars show the standard error of the mean (SEM). $*P < 0.05$,

$**P < 0.01$, $***P < 0.001$.
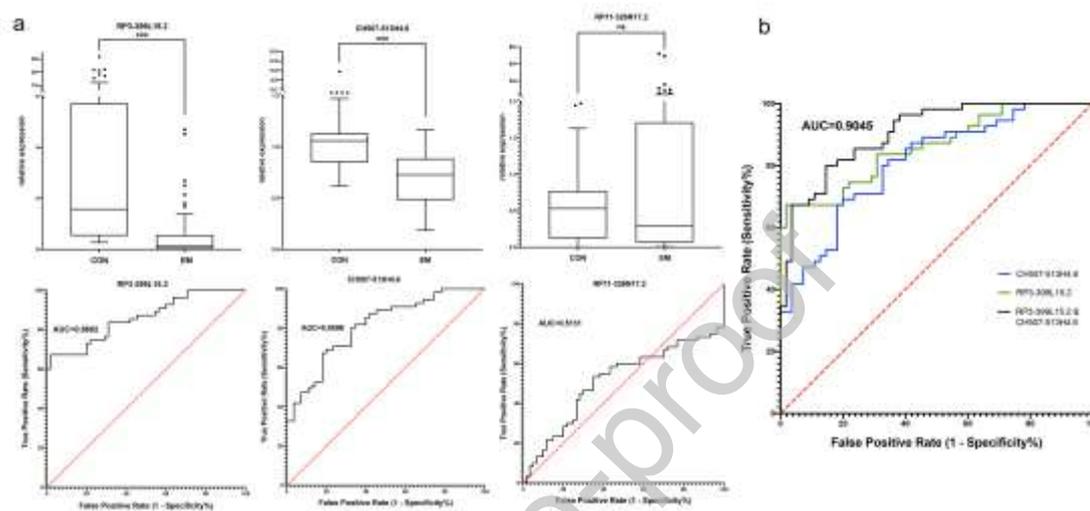
Figure 6. (**a**) Expression and ROC analysis of three lncRNAs (i.e., RP3-399L15.2, CH507-513H4.6, and RP11-326N17.2) in plasma exosomes. Expression levels are plotted showing the median and interquartile range (IQR), using Mann–Whitney U test followed by the Bonferroni correction for multiple comparisons. Whiskers and outliers are displayed according to the Tukey method, with points falling outside this range plotted individually. The ROC analysis was performed with the calculated AUC. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (**b**) Diagnostic value of combined lncRNAs RP3-399L15.2 and CH507-513H4.6 for endometriosis**.**
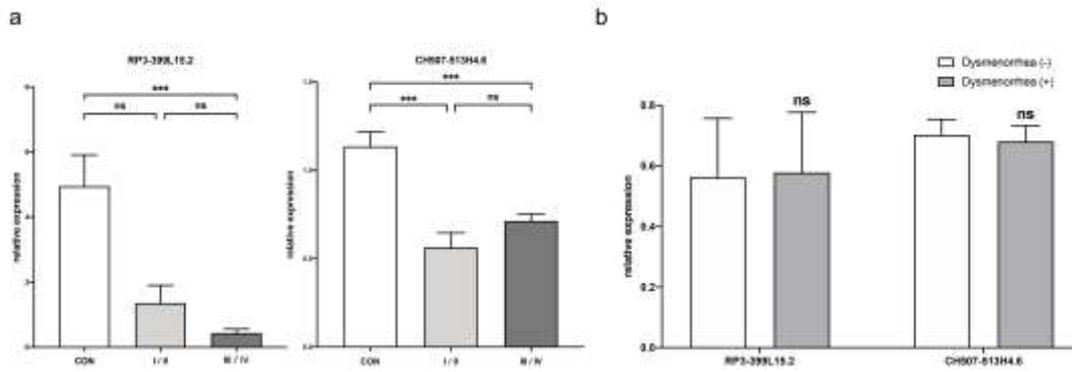
Figure 7. (a) Expression levels of RP3-399L15.2 and CH507-513H4.6 in plasma exosomes of controls and patients with endometriosis. The endometriosis group was divided into a mild disease group (rASRM stage I/II) and a severe disease group (III/IV). Error bars show the standard error of the mean (SEM). Kruskal–Wallis test was used, followed by the Dunn's multiple comparisons test. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001. (b) Expression levels of lncRNAs RP3-399L15.2 and CH507-513H4.6 in plasma exosomes of endometriosis patients with or without dysmenorrhea, per Mann–Whitney U test.
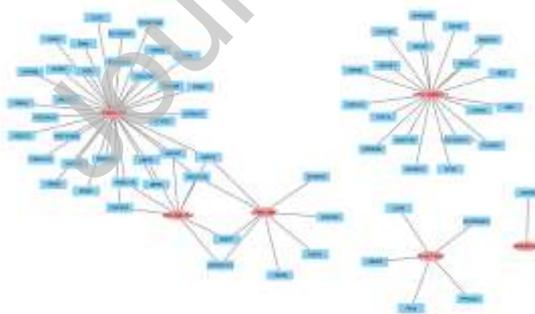
Figure 8. CNC network of selected lncRNA–mRNA co-expression pairs (P < 0.05 and |PCC|≥0.8). The pink oval node represents lncRNA, and the blue rectangular node represents mRNA. PCC: Pearson correlation coefficient.

Table 1. Demographic data and clinical features of endometriosis patients and healthy

controls

	Endometriosis (n=85)	Control (n=86)
Age (Mean ± SD)	33.52±6.794	33.04±6.521
BMI (Mean ± SD)	21.52±2.952	21.82±2.939
Gravidity (Mean ± SD)	1.01±1.006	1.04±0.781
Parity (Mean ± SD)	0.59±0.603	0.63±0.571
Menstrual cycle phase (n)		
Proliferative phase	33	40
Secretory phase	43	43
Unable to determine	9	3
Control diagnoses (n)		
No abnormality	63	78
Adenomyosis	12	0
leiomyoma	5	8
endometrial polyps	5	0
rASRM stage (n)		
I	7	NA
II	6	NA
III	35	NA
IV	37	NA
Dysmenorrhea (n)		
With dysmenorrhea	49	NA
Without dysmenorrhea	36	NA
Hormonal treatment (n)	-	-

Table 2. Candidate lncRNAs

Gene ID	Symbol	Log$_2$FoldChange	P value
ENSG00000262468	LINC01569	-1.066723169	0.000813014
ENSG00000226079	RP3-399L15.2	-2.986813947	0.018794151
ENSG00000226516	FAM138B	-1.996444058	0.01386978
ENSG00000280800	CH507-513H4.6	-2.660718385	0.03794923
ENSG00000274281	RP11-326N17.2	1.460693756	0.001550628
ENSG00000224141	MIR548XHG	1.352839007	0.008452462
ENSG00000230658	KLHL7-AS1	1.511496463	0.01544057
ENSG00000261480	RP11-578F21.6	4.198180819	0.001730671
ENSG00000249941	CTD-2046I8.1	-2.173700287	0.000596213
ENSG00000267073	AC005256.1	-1.821115703	0.00164872

Biography

Hong Xu, who had received her MD from Universität zu Lübeck, Germany, is a chief physician and doctoral supervisor of The International Peace Maternity & Child Health Hospital, School of medicine, Shanghai Jiao Tong University. She mainly focuses on the basic and clinical research of endometriosis and adenomyosis.

Key message

210 dysregulated lncRNAs were identified in the plasma exosomes of endometriosis patients compared with unaffected participants. A combination of RP3-399L15.2 and CH507-513H4.6 was used to distinguish patients; results revealed a sensitivity of 80% and specificity of 85.45%, and an AUC of 0.9045, demonstrating their promise as diagnostic biomarkers for endometriosis.