Plasma-derived exosomal miR-15a-5p as a promising diagnostic biomarker for early detection of endometrial carcinoma

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Abstract

Endometrial cancer (EC) is a major cause of death among gynecologic malignancies. To improve early detection of EC in patients, we carried out a large plasma-derived exosomal microRNA (miRNA) studies for diagnostic biomarker discovery in EC. Small RNA sequencing was performed to identify candidate exosomal miRNAs as diagnostic biomarkers in 56 plasma samples from healthy subjects and EC patients. These miRNA candidates were further validated in 202 independent plasma samples by droplet digital PCR (ddPCR), 32 pairs of endometrial tumors and adjacent normal tissues by quantitative real-time PCR (qRT-PCR), and matched plasma samples of 12 patients before and after surgery by ddPCR. miR-15a-5p, miR-106b-5p, and miR107 were significantly upregulated in exomes isolated from plasma samples of EC patients compared with healthy subjects. Particularly, miR-15a-5p alone yielded an AUC value of 0.813 to distinguish EC patients with stage I from healthy subjects. The integration of miR-15a-5p and serum tumor markers (CEA and CA125) achieved a higher AUC value of 0.899. There was also a close connection between miR-15a-5p and clinical manifestations in EC patients. Its exosomal expression was not only associated with the depth of muscular infiltration and aggressiveness of EC, but also correlated with levels of reproductive hormones such as TTE and DHEAS. Collectively, plasma-derived exosomal miR-15a-5p is a promising and effective diagnostic biomarker for the early detection of endometrial cancer.

Keywords: Endometrial cancer, Liquid biopsy, Plasma-derived exosomal miRNA, ddPCR, Cancer diagnosis, Early detection

Main text

Endometrial cancer (EC) is the second highest incidence of gynecologic cancer [1]. Patients have to undergo uterine apopsis for accurate EC diagnosis, since there are no effective biomarkers [2]. Exosomes originate from the endosome, and then fuse with the plasma membrane under the traction of molecular motors, and are released to the extracellular environment [3, 4]. Exosomes are detected in body fluids such as plasma, urine, and amniotic fluid [5]. Exosomes encapsulate biomolecules such as proteins and miRNAs, maintain their integrity in the circulation, and transfer them to recipient cells [4]. MiRNA is the most abundant type in the RNA cargo of exosomes [6, 7], and exosomal miRNAs (exomiRs) are usually tumor-specific [8]. ExomiRs have received increasing attention in precision medicine, due to their non-invasiveness, and potential as diagnostic biomarkers.
high accessibility and stability [2, 9]. Recent studies have shown that exomiRs have the potential to be efficient biomarkers for the screening, diagnosis, and monitoring of cancers [10–13]. However, exomiRs as biomarkers have not yet been reported in EC.

To improve early detection of EC patients, we carried out a large plasma-derived exosomal miRNA study for biomarker discovery in EC (Supplementary Methods). Candidates were identified by miRNA sequencing in plasma samples from healthy controls (HC) vs. EC patients, and were further validated in independent plasma samples and endometrial tumor tissues (Table S1 and Figure S1). Plasma-derived exosomal miR-15a-5p was identified as a promising diagnostic biomarker for early detection of endometrial cancer.

Identification of exomiRs for EC diagnosis

Exosomes were isolated from plasma of EC patients and age-matched HC subjects. The previously reported method to identify the shape and size of exosomes [5]
was used with CD81, TSG101 and GM130 as positive or negative markers, respectively. Indeed, the fraction isolated from plasma was enriched in exosomes (Figure S2).

The miRNA sequencing was then performed in plasma-derived exosomes from 25 EC and 31 HC subjects. On average, approximately 50 million reads were generated in each library, and 384 exomiRs were detected in each sample (Table S2). Forty-nine miRNAs were differentially expressed between HC and EC groups (p < 0.01) (Fig. 1a and Table S3). Eighteen of them also differentially expressed between tumor and adjacent normal tissues in The Cancer Genome Atlas (TCGA) EC samples [14] (Table S4 and Figure S3). Next, a set of exomiRs from these 18 miRNAs were selected as a best
panel to distinguish EC from HC subjects using random forest algorithm (Fig. 1b). Clustering analysis showed that these samples were largely divided into two distinct groups (EC vs HC) by these six exomiRs (Fig. 1c). The AUC for each exomiR ranged from 0.693 to 0.819 with a mean of 0.757. The AUC of the combined six exomiRs achieved 0.983 (Fig. 1d). miR-106b-5p, miR-107, miR-15a-5p, and miR-3615 were significantly up-regulated, while miR-139-3p and miR-574-3p were significantly down-regulated in plasma-derived exosomes of EC compared with HC (Fig. 1e). Consistent trends in expression of these exomiRs were observed in tumor tissues from TCGA EC patients (Fig. 1f).

**Validation of diagnostic exomiRs by ddPCR in independent plasma samples**

Next, we applied droplet digital PCR (ddPCR) to verify these six exomiRs in an independent validation cohort including 115 EC and 87 HC plasma samples. Two stable high-abundance miRNAs (let-7b-5p and miR-26a-5p) were selected as endogenous references, due to their high consistency in expression across all samples (Figure S4). The miR-106b-5p, miR-107, and miR-15a-5p were consistently upregulated in plasma-derived exosomes from EC compared with HC (Fig. 2a). Their upregulation was further verified in 32 pairs of endometrial tumor tissues and adjacent normal tissues using qRT-PCR (Figure S5). While the other three exomiRs were not pursued because of no significant expression changes between EC and HC groups in the validation set or failure of primer design in ddPCR.

The average AUC of these three verified exomiRs was 0.705, ranging from 0.611 to 0.823, in the independent plasma samples. The combination of these exomiRs yielded a much higher AUC than tumor biomarkers (TB, including CEA and CA125) alone (0.873 vs. 0.736) (Figs. 2ba n dS 6). Integration of these exomiRs (TB, including CEA and CA125) alone (0.873 vs. 0.705, ranging from 0.611 to 0.823) in the independent set or failure of primer design in ddPCR.

**Pathway enrichment analysis of diagnostic exomiRs**

KEGG pathway enrichment analysis for target genes of the three diagnostic miRNAs were performed to investigate their potential functions involvement in EC (Figure S8). Among the 20 significant pathways (FDR < 0.05), most of them are cancer-related, such as TGF-beta, Hippo, MAPK, p53, FoxO, Wnt, mTOR, and ErbB signaling pathways. The other pathways are related to fatty acid metabolism and biosynthesis, whereas prolactin signaling, oocyte meiosis, and endocytosis pathways are known to be associated with exosome biogenesis and release [14]. These results suggested that these miRNAs can not only serve as diagnostic biomarkers, but also are potentially involved in various steps in EC carcinogenesis and progression.

**Exosomal miR-15a-5p associated with clinicopathologic characteristics**

We assessed the relationship between three diagnostic exomiRs and clinicopathologic characteristics in EC patients (Fig. 2g-i). The plasma-derived exosomal miR-15a-5p expression was significantly higher in patients with p53 positive than p53 negative staining (p = 0.010). The exosomal miR-15a-5p level increased with the increase of muscular infiltration depth in EC patients (p = 0.032). Patients with large tumor had higher exosomal miR-15a-5p expression compared with small tumor (p = 0.002). These results implies that exosomal miR-15a-5p expression is strongly predictive of the aggressiveness and p53 mutation status of EC tumors.

Exosomal miR-15a-5p was also associated with testosterone (TTE), dehydroepiandrosterone sulfate (DHEAS), and estradiol (E2) (p < 0.05). However, its expression was not associated with normal menstrual cycles (Figure S9). Given a
positive association of elevated circulating levels of TTE and DHEAS with EC risk [15], exosomal miR-15a-5p may also be a valuable clinical indicator for EC patients.

**Tissue specificity of miR-15a-5p expression**

Finally, we analyzed miR-15a-5p expression in other cancer types, including cervical, breast, ovarian and lung cancer. The miR-15a-5p expression is much more abundant (7–19 times) in EC tumor tissues than that in the other cancer types (Figure S10A). Compared with adjacent tissues, the miR-15a-5p expression was increased to other cancer types (Figure S10B). MiR-15a-5p was either downregulated in tumor tissues or showed small difference between tumor and adjacent tissues or normal controls of the other cancer types (Figure S10B).

**Conclusions**

Our study identified plasma-derived exosomal miR-15a-5p as a valuable diagnostic biomarker for the early detection of EC. Compared with uterine apoxesis, blood extraction is more convenient and carries less risk of vaginal/uterine cervix infection. It has the potential to be incorporated into routine blood examinations for screening endometrial cancer in the general population. Further validation of miR-15a-5p in large sample sizes is warranted before the clinical use as a diagnostic biomarker. Functional investigation of these diagnostic exo-miRs will help reveal the mechanisms that underlie the occurrence and development of EC.

**Abbreviations**

AUC: Area under receiver operating characteristics curve; CA125: Cancer antigen 125; CEA: Carcinoembryonic antigen; ddPCR: Droplet digital PCR; DHEAS: Dehydroepiandrosterone sulfate; E2: Estradiol; EC: Endometrial cancer; exomiRs: Exosomal miRNAs; FDR: False discover rate; FSH: Follicle stimulating hormone; HC: Healthy controls; LH: Luteinizing hormone; miRNA: microRNA; NTA: Nanoparticle tracking analysis; P: Progesterone; PRL: Prolactin; qRT-PCR: Quantitative real-time PCR; RPM: Reads Per Million mapped reads; TB: Tumor biomarker; TCGA: The Cancer Genome Atlas; TTE: Testosterone

**Supplementary Information**

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**Additional file 1.**

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**Authors’ contributions**

YL, WL and PL considered and designed the study. Lanyun Zhou, WW and FW performed experiments. Lanyun Zhou and JH did data analyses. SY, BL, ZP, YM, SL, PS and WL collected plasma samples, tissue specimens and clinical data. BL reviewed HE stained slides and confirmed the diagnosis. MZ and Luyuan Zhou provided assistance in the data analyses. Lanyun Zhou, PL and YL wrote the manuscript. All of the authors discussed and commented the study. The author(s) read and approved the final manuscript.

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**Availability of data and materials**

All data generated during this study are included in this published article and its supplementary files. The sequencing data were deposited in the Genome Sequence Archive (GSA) under accession HR000737 (http://bigd.big.ac.cn/gsa-human).

**Declarations**

**Ethics approval and consent to participate**

This study was reviewed and approved by the Ethics Committees of Women’s Hospital of Zhejiang University School of Medicine (Hangzhou, China; ID: 20170142). The study was conducted in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects. All samples have been collected and utilized following strict human subjects protection guidelines, written informed consent and IRB review of protocols.

**Consent for publication**

Not applicable.

**Competing interests**

No potential conflicts of interest were disclosed.

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