Serum miRNA-204-5p as a potential noninvasive biomarker for the diagnosis of endometrial cancer with sentinel lymph node mapping

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Research Article

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Abstract

Background
Lymph node status is one of the most important prognosis factors in determining adjuvant treatment in endometrial cancer (EC). However, lymphadenectomy bears significant surgical and postoperative risks. The use of the sentinel lymph node mapping (SLNM) has emerged as an alternative method to complete lymphadenectomy in EC. There remains controversy surrounding the SLNM in high-risk disease and its false negative rate (3%). We previously identified miR-204-5p is tumor suppressor miRNA associated with lymph node metastasis in endometrial cancer tissues. Here, we report serum miR-204-5p in EC patients have potential early diagnostic value combined with sentinel lymph node mapping.

Methods
The relative expression levels of miR-204-5p were detected by quantitative RT-PCR in the serum of 52 EC patients (total SLNM), 20 benign ovarian cyst patients and 20 myoma patients. The miR-204-5p expression was also detected in endometrial cancer tissues by in situ hybridization.

Results
Our results showed that serum miR-204-5p expression was down-regulated in EC patients than that in the benign ovarian cyst or myoma patients ($p < 0.01$). In accordance with final pathological evaluation, positive SLN EC patients have a significant lower level of miR-204-5p compared with negative SLN EC patients. The area under the ROC curve of miR-204-5p was 0.923, 95% CI(0.847, 1.000), and the diagnostic value with a sensitivity of 87.2% specificity of 80.0%.

Conclusions
Lower miR-204-5p expression is associated with lymph node metastasis in these SLN(+) EC tissues, indicate that down-regulation of serum miR-204-5p in EC patients have potential early diagnostic value combined with sentinel lymph node mapping.

Background
Endometrial cancer (EC) is the most common gynecological malignancy in the worldwide. In 2021, there were the predicted diagnosis of 66,570 new cases in the USA, resulting in 12,940 disease-related deaths[1]. The primary standard treatment of EC is surgery, followed adjuvant treatment with high risk EC including chemotherapy and brachytherapy. Lymph nodes is the first site of extra-uterine spread in EC patients, and the prevalence of lymph node metastases in clinical stage I or II EC patients is almost 10% [2], which is the most important prognostic factor [3]. However, lymphadenectomy is controversial as it
does not improve the long-term outcomes, such as the disease free survival and overall survival, but increasing the morbidity and inducing worse peri-operative outcomes [4, 5]. Newest evidence showed that lymphadenectomy even increases the 90-day risk of VTE [6].

Sentinel lymph node mapping (SLNM) is recommended in EC with uterine-confined malignancy [7]. SLNM with ultra-staging with low false-negative rates increases the detection of lymph node metastasis. A meta-analysis pooled the sensitivity of SLN mapping was 96% (95% CI 92-98%) for detection of lymphatic metastases [8]. However, the successful of SLN mapping rate is influenced by many factors, such as the dye tracer, the injection site and the BMI of patient, as the adipose tissues shield the colorimetric signal [9]. Besides, 56% metastatic SLN EC patients suffered para-aortic lymph node metastases [10], and 5% patients had skip metastases, and SLN ultra-staging is unavailable during operation, the additional systematic lymphadenectomy and adjuvant therapy are taken into account for some patients. Thus, a new method which increased the accuracy of SLNM detection helps guide the status of lymph node in EC patients needed to be developed.

Circulating microRNA (miRNA) were considered stable miRNA in the serum/plasma. These miRNAs represent potential biomarkers for evaluating cancer, and many circulating miRNAs indicative of breast cancer have been identified [11]. We previously uncovered a regulatory loop involving TrkB/miR-204-5p that is critical in the tumorigenesis of EC [12], and miR-204-5p expression correlates with lymph node metastasis in endometrial cancer patients. These observations led us to hypothesize that serum miR-204-5p in EC patients may have potential early diagnostic value combined with sentinel lymph node mapping.

Materials And Methods

Patients and clinical samples collection

This study was approved by the Institutional Review Board of Shanghai General Hospital affiliated with Shanghai Jiao Tong University. Fifty-two endometrial carcinoma patients who underwent hysterectomy with lymph node dissection (sentinel lymph node mapping first) at the Shanghai General Hospital affiliated with Shanghai Jiao Tong University from Oct 2018 to Nov 2020 were included in our study (Table 1). The stages and histological grades of these tumors were determined according to the criteria of the Federation International of Gynecology and Obstetrics (FIGO) surgical staging system (2009) [13]. Meanwhile, forty serum samples were obtained from patients who underwent treatment for benign diseases, such as ovarian cyst or myoma. None of the patients had received hormone therapy, radiotherapy, or chemotherapy before surgery. The resected specimen was stained with H&E for histological examination.

Blood samples were collected from patients with EC and benign diseases early in the morning. Up to 5 ml of fasting venous blood was collected in a serum separator tube from every participant. All blood samples were centrifuged at 2800 g for 10 min within half an hour after collection. Then the separated supernatant was stored in 1.5 ml tubes at -80°C until further use.
Total RNA isolation and quantitative real-time reverse transcription (RT)–PCR

As previously described [12], total RNA was extracted from serum samples using a miRVana PARIS Kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer’s protocols. RNA purity and concentration were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). For the miRNA analysis, TaqMan microRNA Reverse Transcription Kit was used to reverse transcribe mature miRNA from total RNA. According to the manufacturer’s instructions, real-time PCR was performed using TaqMan MicroRNA Assay primers with TaqMan Universal PCR Master Mix and analyzed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems; Foster City, CA, USA). U6 was used as an internal reference for the expression of microRNAs (Table 2). All reagents were purchased from Applied Biosystems (Foster City, CA, USA). For all the experiments, values on the y-axis were equal $2^{(-\Delta C_t)}$, where $\Delta C_t$ is the difference between gene Ct and normalizer gene Ct. The data were obtained in triplicate in three independent experiments.

In situ hybridization (ISH)

As previously described [14], the expression of miRNA in paraffin-embedded tissue specimens was determined by an in situ hybridization kit (MK1030, Boster, Wuhan, China). Briefly, 6-µm thick sections of paraffin-embedded specimens were deparaffinized with xylene and rehydrated in a series of ethanol. After Proteinase-K incubation for 15 min at 37 °C, the slides were prehybridized in a hybridization solution at 37°C for 2 h. Then, tissue sections were hybridized with 5'-digoxigenin-labeled (DIG-labelled) oligonucleotide probe at 37 °C overnight. After stringent washes with 5 × SSC, 1 × SSC, and 0.2 × SSC buffers, the sections were blocked with DIG blocking buffer at 37 °C for 30 min. An anti-DIG antibody was applied, and the sections were incubated at 37 °C for 1 h. After washing in a staining solution, the sections were developed by diaminobenzidine-hydrogen peroxide.

Statistical analyses

Each experiment was performed at least three times. Differences between two groups were assessed by the Mann-Whitney U test, and multiple comparisons between more than two groups were conducted with the Kruskal-Wallis test. Data are presented as the means ± SD. The area under the receiver operating characteristic curve (AUC) is calculated to assess the value of serum miR-204-5p in diagnosis lymph node metastases, and the sensitivity, specificity were calculated by discriminant analysis. All $p$-values are two-sided, and $P$-value < 0.05 was considered statistically significant. All statistical analyses were performed by SPSS 16.0 software.

Results

Surgical procedures and sentinel lymph node mapping

All EC patients underwent laparoscopic staging operation with the near-infra-red NOVADAQ Endoscopy system (Stryker, Portage, MI, USA) or robotic (da Vinci® Xi; Intuitive Surgery, Sunnyvale, CA, USA) staging
operation with Firefly®. All patients underwent SLNM with ICG fluorescence detection using NIR/ICG® and Firefly®, after which bilateral pelvic lymphadenectomy was performed. In accordance with the National Comprehensive Cancer Network guidelines, a total of 4ml of 1.25 mg/ml ICG solution (25mg of ICG mixed with 20ml of distilled water) was injected superficially (1-3 mm) and deep (1 cm) into the cervix at 3 o’clock and 9 o’clock (1 ml each). After the injection of ICG solution, Opening of the retroperitoneum in the left and right pelvic and paraaortic areas and development of the paravesical and pararectal spaces were performed 10-20 minutes after injection of the ICG solution. Fluorescent uptake was observed through an NIR laparoscopic camera, we defined the first twinkling lymph node as an SLN (Figure 1). Finally, a conventional laparoscopic- or robotic-assisted vaginal hysterectomy or robotic-assisted hysterectomy was performed after sending the frozen section of the SLN for pathological evaluation.

**Expression of miR-204-5p correlates with LN metastasis in EC tissues**

We previously identified miR-204-5p is tumor suppressor miRNA associated with lymph node metastasis in endometrial cancer tissues [12]. We observed a lower expression of miR-204-5p in endometrial cancer tissues compared with the normal endometrium (p<0.01) (Figure 2). Moreover, patients with positive lymph node metastasis having markedly lower levels of miR-204-5p compared with negative ones (p<0.01) (Figure 2). These results demonstrated that lower miR-204-5p expression is associated with lymph node metastasis in EC tissues.

**Down-regulation of serum miR-204-5p in EC patients and its diagnostic value**

To determine the feasibility of miR-204-5p detection in serum, qRT-PCR was used to detect the levels of serum miR-204-5p in 52 EC patients (total SLNM), 20 benign ovarian cyst patients and 20 myoma patients. The results showed that serum miR-204-5p expression was obvious lower in EC patients than that in the benign ovarian cyst or myoma patients (p<0.01), no statistical difference between the benign ovarian cyst patients and myoma patients (p>0.05) (Figure 3A).

To further examine the clinical implications of serum miR-204-5p in EC, we tested the correlation between serum miR-204-5p expression levels and clinicopathologic characteristics of EC. There were no statistical associations found with respect to age, FIGO stage, grade, myometrial invasion (p>0.05) (Table 1). However, a statistically significant correlation was observed between miR-204-5p expression and lymph node metastasis (p<0.01) (Table 1). Interestingly, in accordance with final pathological evaluation, positive SLN EC patients have a significant lower level of miR-204-5p compared with negative SLN EC patients (Figure 3A). We calculated the serum miR-204-5p is strongly associated with positive SLN, with a sensitivity of 87.2%, a specificity of 80.0% (AUC=0.923, 95%CI[0.847, 1.000], p=0.002) (Figure 3B). These results indicate that down-regulation of serum miR-204-5p in EC patients have potential early diagnostic value combined with sentinel lymph node mapping.

**Low miR-204-5p expression correlates with lymph node metastasis in SLN(+) EC patients**
Standard haematoxylin and eosin (H&E) staining of lymph nodes was also diagnosed as metastasis after the frozen section of SLN biopsy. In accordance with the serum miR-204-5p level, we demonstrated that lower miR-204-5p expression is associated with lymph node metastasis in these SLN(+) EC tissues (Figure 4).

Discussion

SLNM was first reported in lymphangiogram of penile carcinoma in 1977 [15]. Nowadays, SLNM has been applied in many malignancies, instead of lymphadenectomy, including the breast cancer and vulva cancer, and first applied in EC in 1996 [16]. Tracer injected in corpus uterus or cervix, which flowing along lymphatic channels, and accumulates in the first station, the so-called SLN, recognized as the first site of extra-uterus metastases [8]. Though the technology showed advantages throughout the treatment with similar survival but less surgical complications compared to systematic lymphonodectomy. Some drawbacks should not be ignored. Such as, the accuracy of intraoperative frozen section, researches figured that 46.58%-76.92% SLN positive cervical cancer patients were missed during the frozen section [17, 18]. Besides, on one hand, the rate of successful mapping range from 23% to 100%, and the difference may be influenced by the experience of surgeons [19]. On the other hand, there are 16% EC patients suffered para-aortic area metastases with pelvic nodes negative [20]. Additionally, for the tracer selection, the signal of blue dyes influenced by the adipose, while the technetium 99 and tricarbocyanine dye require complex imaging equipment, and the technologies are difficult to be popularized [21].

Early in 2014, researchers used CK19 mRNA at 250 copies from lymph node tissue during the operation to predict the metastases with sensitivity of 82.4%, specificity of 99.2%, however this method was also based on the dissection of lymph node during the surgery [22]. MicroRNAs as non-protein coding RNAs are associated with post-transcriptional regulation, and play roles in many cellular processes, and induced the cancer development. Previous studies also found that effective diagnostic value of microRNAs in cancers, Shimomura and colleagues figured that preoperative combination of serum miR-1246, miR-1307-3p, miR-4634, miR-6861-5p and miR-6875-5p detected the early breast cancer with a sensitivity of 97.3%, specificity of 82.9% [11]. The serum of miR-135a distinguished non-small cell lung cancer from healthy with specificity and sensitivity of 83.1% and 81.3% respectively [23]. Researchers also found that lower expression of serum miR-204 predicted worse survival in gastric cancer patients [24]. The current study has found serum miR-204-5p was a more convenient method pre-operation to predict the status of lymph node metastases combined with SLNM in treatment of EC patients, with a sensitivity of 87.2%, a specificity of 80.0% (AUC=0.923, 95%CI[0.847, 1.000], p=0.002).

MiR-204 regulated adipogenesis by inhibiting the activation of Wnt/β-catenin signaling pathway and reduced insulin production by downregulating insulin transcription factor-MAFA [25, 26]. Abnormal expression of miR-204 was found in kinds of cancer. Zanette and colleagues found that miR-204 over expression in acute lymphocytic leukemia [27]. While the level of miR-204 was significant lower than normal tissue, and was negative associated with lymph node metastases in gastric cancer and bladder cancer [28, 29], which consistence with our previous study, the expression of miR-204-5p was down-
regulation in EC tissue compared to normal tissue, and even lower in the positive lymph nodes [12]. Moreover, recent evidence figured out that the serum levels of miR-204-5p in gastric cancer patients were lower than benign patients, and pointed out that miR-204-5p targeted at CXCR4 and CXCL12 to suppress the lymph node metastases [30]. In accordance with the serum miR-204-5p level, we demonstrated that lower miR-204-5p expression is associated with lymph node metastasis in these SLN(+) EC tissues. Therefore, serum miR-204-5p may be an efficient biomarker in EC patients for detecting the status of lymph node metastases pre-operation, with little cost and more convenient.

There were some limitations in our study. Firstly, the samples included in this research was small, and the sentinel lymph node positive case was only five; Secondly, we didn’t explore the prognostic value of serum miR-204-5p in EC patients. In the further research, we will enlarge the size of study, and try to find the cut-off of serum miR-204-5p in lymph node metastatic EC patients.

In conclusion, our data demonstrated that the serum miR-204-5p was lower in positive SLN than negative SLN, and serum miR-204-5p may be an efficient biomarker in predicting the lymph node metastases pre-operation. Thus may be helpful for clinical decision making for lymphonodectomy or sentinel lymph node mapping in EC patients.

**Declarations**

**Ethics approval and consent to participate:**

The present study approved by the ethics committee of Shanghai General Hospital, and all the informed consent of included patients obtained, and if the participant is under 16 yrs, the informed consent is obtained from the parents, and the study was performed in accordance with the Declaration of Helsinki.

**Consent for publication:** Not applicable

**Availability of data and materials:**

The data and materials used in the present study are available from the corresponding authors.

**Competing interests:** Not applicable

**Funding:**

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**Authors’ contributions:**

B.W and W.S contributed to the design of the study. W.C. , Z.X. and L.J. performed the experiments, analysis the data and drafting the article. X.R., Y.Y. and Z.Y. collected the clinical data. D.L. and X.H.
collected the blood and surgery samples.

Acknowledgements: Not applicable

References


Tables

Table 1: Association between serum miR-204-5p expression and different clinicopathological features of endometrial cancer.
<table>
<thead>
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<th>Variable</th>
<th>n</th>
<th>miR-204-5p expression</th>
<th>p value</th>
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<tr>
<td>Total</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>≤50</td>
<td>11</td>
<td>2.53±2.38</td>
<td>1.86</td>
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<tr>
<td>&gt;50</td>
<td>41</td>
<td>2.67±2.24</td>
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<tr>
<td>FIGO stage</td>
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<td>Stage II</td>
<td>9</td>
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<td>Stage III</td>
<td>6</td>
<td>1.82±1.78</td>
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<tr>
<td>Grade (Endometrioid)</td>
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</tr>
<tr>
<td>G1</td>
<td>27</td>
<td>2.45±2.28</td>
<td>0.44</td>
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<td>G2</td>
<td>18</td>
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<tr>
<td>G3</td>
<td>7</td>
<td>1.88±1.82</td>
<td></td>
</tr>
<tr>
<td>Myometrial invasion</td>
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<tr>
<td>&lt;1/2</td>
<td>40</td>
<td>2.48±2.32</td>
<td>0.12</td>
</tr>
<tr>
<td>≥1/2</td>
<td>12</td>
<td>1.96±1.85</td>
<td></td>
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<tr>
<td>Lymph node metastasis</td>
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<td></td>
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<tr>
<td>negative</td>
<td>47</td>
<td>2.57±2.36</td>
<td>&lt;0.001</td>
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<td>positive</td>
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Table 2. Primers used for quantitative real-time PCR analysis.
<table>
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<th>miRNA</th>
<th>Primer sequence</th>
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<td>miR-204-5p</td>
<td>Forward 5′-CCCATCGTTAAGCAATGCATGAC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-GAGGGCCTCCTGATCATTTACC-3’</td>
</tr>
<tr>
<td>U6</td>
<td>Forward 5′-AGAGCCTGTGGTGTCGGC-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse 5′-CATCTTCAAAGCACTTCCCT-3’</td>
</tr>
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**Figures**

**Figure 1**

Mapping sites of sentinel lymph nodes.
Figure 2

MiR-204-5p expression correlates with lymph node metastasis in endometrial cancer patients. The expression of miR-204-5p was measured by TaqMan PCR in endometrial cancer tissues and the normal endometrium. Values were calculated relative to U6B expression. **$p < 0.01$. 

**p < 0.01  **p < 0.01
Figure 3

Serum miR-204-5p in EC patients correlates with SLN(+) lymph node metastasis. (A) Serum miR-204-5p was measured by TaqMan PCR in 52 EC patients (total SLNM operation), 20 benign ovarian cyst patients and 20 myoma patients. Values were calculated relative to U6B expression. **p < 0.01. (B) The area under the ROC curve of miR-204-5p was 0.923, 95% CI(0.847, 1.000), and the diagnostic value with a sensitivity of 87.2% specificity of 80.0%.

Figure 4
miR-204-5p expression correlates with lymph node metastasis in SLN(+) EC patients. Representative photographs of miR-204-5p immunoactivity in SLN(-) and SLN(+) endometrial cancer (scale bars: 100 μm).

Supplementary Files

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- Table.docx