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

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Abstract. The lymph node status is one of the most critical prognostic factors used in determining adjuvant treatment in endometrial cancer (EC). Lymphadenectomy is associated significant surgical and postoperative risks. The use of sentinel lymph node mapping (SLNM) has emerged as an alternative method to complete lymphadenectomy in EC. However, there remains controversy surrounding the use of SLNM in high-risk disease and its false-negative rate (3%). The authors previously identified miR-204-5p as a tumor-suppressor miRNA associated with lymph node metastasis in EC tissues. The present study demonstrated that serum miR-204-5p in patients with EC has the potential for use as an early diagnostic biomarker combined with SLNM to assess the lymph node status prior to surgery. The present study also aimed to identify the optimal cut-off value of serum miR-204-5p. The relative expression levels of miR-204-5p were detected using reverse transcription-quantitative PCR in the serum of 52 patients with EC (total SLNM). A total of 20 patients diagnosed with ovarian cysts, 20 patients diagnosed with myoma, and 20 participants diagnosed with endometrial polyps or endometrial hyperplasia were included as the control group. miR-204-5p expression was also detected in lymph node tissues using *in situ* hybridization. The results revealed that serum miR-204-5p expression was downregulated in patients with EC compared with its expression in patients with benign ovarian cysts, myoma and endometrial hyperplasia/polyps (P<0.01). In accordance with the final pathological evaluation, patients with EC with a positive SLN status had a significantly lower level of miR-204-5p compared with those with a negative SLN status (P<0.01). The area under the ROC curve of miR-204-5p was 0.923, 95% CI (0.847-1.000), and the diagnostic value had a sensitivity of 87.2% and specificity of 80.0%, with an optimal cut-off value of 0.253. On the whole, it was demonstrated that a lower miR-204-5p expression is associated with lymph node metastasis in these SLN(+) EC tissues, indicating that the downregulation of serum miR-204-5p in patients with EC has potential for use as an early diagnostic biomarker combined with SLNM. In addition, with a cut-off value of 0.253, it appeared optimal for the prediction of lymph node metastasis in EC.

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Introduction

Endometrial cancer (EC) is the most common gynecological malignancy worldwide. In 2021, 66,570 new cases were diagnosed in the USA, resulting in 12,940 disease-related deaths (1). The primary standard treatment for localized EC is surgery, followed by adjuvant therapy for high-risk or advanced EC, including chemotherapy and brachytherapy. The lymph nodes are the first site of extra-uterine spread in patients with EC; thus, the lymph node status is regarded as a critical prognostic factor (2). As previously demonstrated, the rate of pelvic lymph node metastasis (LNM) is 11.5% and the rate of isolated para-aortic LNM is 1.28% (3); LNM is associated with the tumor grade, as this has been shown to be 20% in high-grade disease, and 14% in low-grade disease (4).

The use of lymphadenectomy is controversial as it does not improve the long-term outcomes, such as recurrence-free survival and overall survival, whereas it increases morbidity and leads to more severe peri-operative outcomes (5). There is evidence to indicate that a lymphadenectomy even increases the risk of developing 30-day complications (6) and the 90-day risk of venous thromboembolism (7). Of note, lymphadenectomy is not recommended in patients with low-grade or low-risk EC (8).

Sentinel lymph node mapping (SLNM) is recommended in EC with uterine-confined malignancy (9). SLNM under

ultra-staging increases the detection of LNM with low false-negative rates. A previous meta-analysis demonstrated that the sensitivity of SLNM was 96% (95% CI, 92-98%) for the detection of lymphatic metastases (10). However, the success rate of SLNM is affected by several factors, such as the dye tracer, the injection site and the body mass index of the patient, as adipose tissues shield the colorimetric signal (11). Moreover, 5% of patients with EC suffer from para-aortic LNM with a negative pelvic lymph node status (12), and SLN ultra-staging is unavailable during surgery; thus, additional systematic lymphadenectomy and adjuvant therapy are taken into account for such patients. It is evident that a novel method with an increased the accuracy for SLNM detection to determine the lymph node status in patients with EC is required.

Circulating microRNAs (miRNAs/miRs) are considered stable miRNAs in the serum/plasma. These miRNAs represent potential biomarkers for evaluating cancer, and a number of circulating miRNAs indicative of breast cancer have been identified (13). However, there are only a limited number of studies reporting the utility of potential serum miRNAs in gynecologic cancer. The authors previously uncovered a regulatory loop involving TrkB/miR-204-5p that is critical in the tumorigenesis of EC (14), and miR-204-5p expression is associated with LNM in patients with EC. These observations led us to hypothesize that serum miR-204-5p in patients with EC may have potential for use as an early diagnostic biomarker combined with SLNM.

Patients and methods

Patients and clinical sample collection. A total of 52 patients with EC who underwent hysterectomy with lymph node dissection (sentinel lymph node mapping first) at the Shanghai General Hospital Affiliated with Shanghai Jiao Tong University from October, 2018 to November, 2020 were included in the study (Table I). 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) (15).

All patients with EC underwent laparoscopic staging surgery with the near-infra-red NOVADAQ Endoscopy system (Stryker) or robotic (da Vinci® Xi; Intuitive Surgery) staging surgery 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 4 ml of 1.25 mg/ml ICG solution (25 mg ICG mixed with 20 ml distilled water) was injected superficially (1-3 mm) and deep (1 cm) into the cervix at the 3 o'clock and 9 o'clock positions (1 ml each). At 10-20 min after the injection of ICG solution, the opening of the retroperitoneum in the left and right pelvic and paraaortic areas and the development of the paravesical and pararectal spaces were performed. Fluorescent uptake was observed through an NIR laparoscopic camera; the first twinkling lymph node was defined as an SLN (Fig. 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.

In addition, 20 serum samples each were obtained from patients diagnosed with ovarian cysts, 20 patients with myoma, and 20 serum samples were obtained from patients diagnosed with endometrial polyps or endometrial hyperplasia. None of the patients had received hormone therapy, radiotherapy, or chemotherapy prior to surgery. The resected specimens from patients with EC (4-μm thick) were stained with hematoxylin and eosin (H&E) at room temperature (RT) (cat. no. ab245880; Abcam) and *in situ* hybridization (ISH) was performed for a histological examination.

Blood samples were collected from patients with EC and benign diseases early in the morning. Up to 5 ml fasting venous blood was collected in a serum separator tube from each participant. All blood samples were centrifuged at 2,800 x g for 10 min RT within half an hour after collection. The separated supernatant was then stored in 1.5 ml tubes at -80˚C until further use. The present study was approved by the Ethics Committee of Shanghai General Hospital (Shanghai, China), and informed consent was written and obtained from all included patients (no. 2018SQ307-1).

Total RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). As previously described (14), total RNA was extracted from the serum samples using a miRVana PARIS kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocols. RNA purity and concentration were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). For the miRNA analysis, a TaqMan microRNA Reverse Transcription kit was used to reverse transcribe mature miRNA from total RNA. According to the manufacturer's instructions, qPCR 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; Thermo Fisher Scientific, Inc.). U6 was used as an internal reference for the expression of miRNAs (Table II). All reagents were purchased from Applied Biosystems; Thermo Fisher Scientific, Inc. For all the experiments, values on the y-axis were equal $2^{(-\Delta Ct)}$, where ΔCt is the difference between gene Ct and normalizer gene Ct. The data were obtained in triplicate in three independent experiments.

ISH and scoring. As previously described (16), the expression of miRNAs in paraffin-embedded tissue specimens was determined using an *in situ* hybridization kit (MK1030, Wuhan Boster Biological Technology, Ltd.). Briefly, 6-μm-thick sections of paraffin-embedded specimens were deparaffinized with xylene and rehydrated in a series of ethanol. Following proteinase-K incubation for 15 min at 37˚C, the slides were prehybridized in a hybridization solution at 37˚C for 2 h. The tissue sections were then hybridized with 5'-digoxigenin-labeled (DIG-labeled) oligonucleotide probe at 37˚C overnight. Following stringent washes with 5X SSC, 1X SSC and 0.2X SSC buffers (cat. no. 11666681001, Roche), the sections were blocked with DIG blocking buffer at 37˚C for 30 min. An anti-DIG antibody (1:2,000; cat. no. 76907, Abcam) 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. Scoring

Table I. Association between serum miR-204-5p expression and the different clinicopathological features of the endometrial cancer samples.

	Clinical serum sample		
Variable	n	miR-204-5p expression	P-value
Total	52		
Age (years)			
≤50	11	2.53±2.38	1.86
>50	41	2.67±2.24	
FIGO stage			
Stage I	37	2.34±2.24	0.42
Stage II	9	2.05±1.96	
Stage III	6	1.82±1.78	
Grade (endometrioid)			
G1	27	2.45±2.28	0.44
G2	18	2.12±2.05	
G3	7	1.88±1.82	
Myometrial invasion			
<1/2	40	2.48±2.32	0.12
≥1/2	12	1.96±1.85	
Lymph node metastasis			
Negative	47	2.57±2.36	<0.001
Positive	5	0.17±0.03	

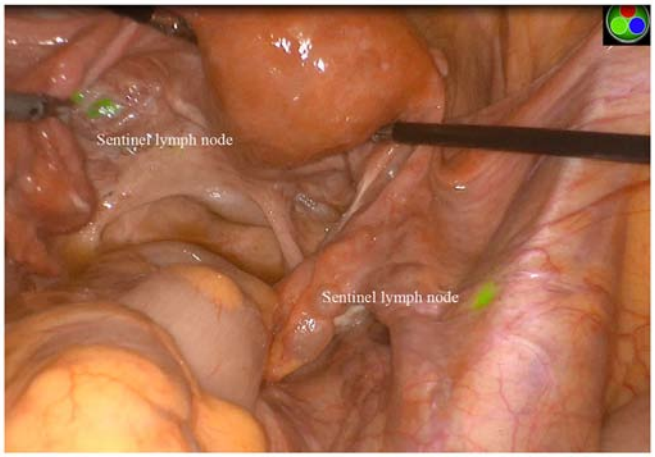

Figure 1. Mapping sites of sentinel lymph nodes.

was measured by the cell cytoplasm staining. The sections were evaluated based on the percentage of positively stained cells (0-3) and the intensity of staining (0-3). The score of miRNA expression was then calculated as a percentage x intensity of the staining. Therefore, score 0 presents negative (-) staining, 1-2 weak positive (+), 3-4 moderately positive (++), and 6-9 strong positive (+++) staining. Scoring with '-' and '+' was regarded as a lower miR-204-5p expression, whereas '++'and '+++' represented a higher expression of miR-204-5p.

Table II. Primers used for reverse transcription-quantitative PCR analysis.

miRNA	Primer sequence
miR-204-5p	Forward: 5'-CCCATCGTTAAGCAATGCATGAC-3'
	Reverse: 5'-GAGGGCCTCCTGATCATTTACC-3'
U6	Forward: 5'-AGAGCCTGTGGTGTCCG-3'
	Reverse: 5'-CATCTTCAAAGCACTTCCCT-3'

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

Results

SLNM. After the ICG injection, fluorescent uptake was observed through an NIR laparoscopic camera; the first twinkling lymph node was defined as an SLN (Fig. 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.

Downregulation of serum miR-204-5p in patients with EC and its diagnostic value. To determine the feasibility of miR-204-5p detection in serum, RT-qPCR was used to detect the levels of serum miR-204-5p in 52 patients with EC (total SLNM), 20 patients diagnosed with benign ovarian cysts, 20 patients with myoma, and 20 participants diagnosed with endometrial polyps or endometrial hyperplasia. The results revealed that serum miR-204-5p expression was evidently lower in patients with EC than that in the control group patients ($P<0.01$; Fig. 2A).

To further examine the clinical implications of serum miR-204-5p in EC, the association between the serum miR-204-5p expression levels and the clinicopathological characteristics of patients with EC was assessed. No statistically significant associations were found as regards age, FIGO stage, grade and myometrial invasion ($P>0.05$; Table I). However, a statistically significant association was observed between serum miR-204-5p expression and LNM ($P<0.01$; Table I). It was found that the serum miR-204-5p expression was strongly associated with a positive SLN status, with a sensitivity of 87.2% and a specificity of 80.0% (AUC, 0.923; 95% CI, 0.847-1.000; $P=0.002$) (Fig. 2B), with an optimal cut-off value of 0.253. These results indicate that the

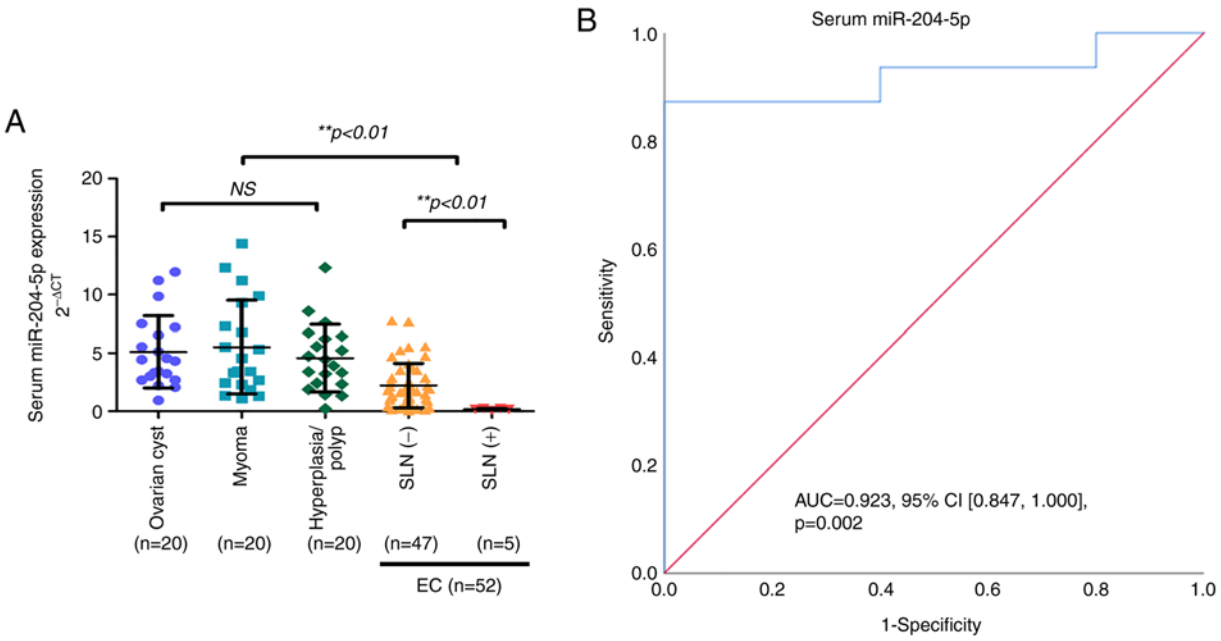

Figure 2. Serum miR-204-5p in patients with EC is associated with SLN(+) lymph node metastasis. (A) Serum miR-204-5p levels were measured using reverse transcription-quantitative PCR in 52 patients with EC (total SLNM operation), 20 patients with benign ovarian cysts, 20 patients with myoma and 20 participants diagnosed with endometrial polyps or endometrial hyperplasia. Values were calculated relative to U6B expression. **P<0.01. (B) The ROC curves for the diagnostic value of miR-204-5p for EC. 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% and a specificity of 80.0%. EC, endometrial cancer; SLN, sentinel lymph node; SLNM, sentinel lymph node mapping; ROC, receiver operating characteristic.

downregulation of serum miR-204-5p in patients with EC has potential for use as an early diagnostic biomarker combined with SLNM.

A low miR-204-5p expression is associated with LNM in patients with SLN(+) EC. Standard H&E staining of lymph nodes was also used to reveal metastasis in the frozen sections. The expression of miR-204-5p in SLN tissue resected from patients with EC was examined using ISH. In accordance with the serum miR-204-5p level, it was demonstrated that a lower miR-204-5p expression was associated with LNM in these SLN(+) EC tissues (Fig. 3A), and the scores of the expression of miR-204-5p in SLN(+) by ISH were markedly lower than in the SLN(-) group (P<0.01; Fig. 3B).

Discussion

Sentinel lymph node mapping (SLNM) was first reported in a lymphangiogram of penile carcinoma in 1977 (17). Currently, SLNM has been applied as an alternative to lymphadenectomy in numerous malignancies, including breast cancer and vulvar cancer, and was first applied in EC in 1996 (18). A tracer is injected into the corpus uterus or cervix, which flows along the lymphatic channels, and accumulates in the first station, termed the SLN, recognized as the first site of extra-uterus metastases. This technology has certain advantages for treatment, with similar survival rates and fewer surgical complications compared to systematic lymphonodectomy. However, certain drawbacks should not be ignored. For example, as regards the accuracy of intraoperative frozen sections, researchers have found that 46.58-76.92% SLN-positive cervical cancer patients were not identified (19,20). On the one hand, the rate of successful mapping ranges from 23 to 100%, and the difference may be influenced by the experience of surgeons (10). On the other hand, as previously demonstrated, there are 1.28% patients with EC suffering from para-aortic area metastases with negative pelvic nodes (3). Additionally, for the tracer selection, the signal of blue dyes is influenced by adipose tissue, while technetium 99 and tricarbocyanine dye require complex imaging equipment, and the technologies are difficult to be fully implemented (21).

Early in 2014, researchers used CK19 mRNA at 250 copies from lymph node tissue during the surgery to predict the metastases with a sensitivity of 82.4% and a specificity of 99.2%; however, this method was also based on the dissection of lymph nodes during the surgery (22). miRNAs as non-protein coding RNAs are associated with post-transcriptional regulation, and play roles in a number of cellular processes, and can induce cancer development. The expression of miRNAs is tissue-specific; miR-204 has been found to be highly expressed in renal, eye and pulmonary tissue (23,24), and promotes pulmonary artery smooth muscle cell proliferation and resistance to apoptosis by activating the Src/STAT3/NFAT pathway, leading to the development of pulmonary arterial hypertension (25). Another study found that miR-204-5p exhibited a higher expression in mouse mammary glands than other tissues, stimulating milk lipid secretion by targeting sirtuin 1 (26). Previous studies have also demonstrated that miR-204 regulates adipogenesis by inhibiting the activation of the Wnt/β-catenin signaling pathway and reducing insulin production by downregulating the insulin transcription factor, MafA (27,28). Additionally, miR-204 has been found to be abnormally expressed in various types of cancer. Zanette *et al* (29) found that miR-204

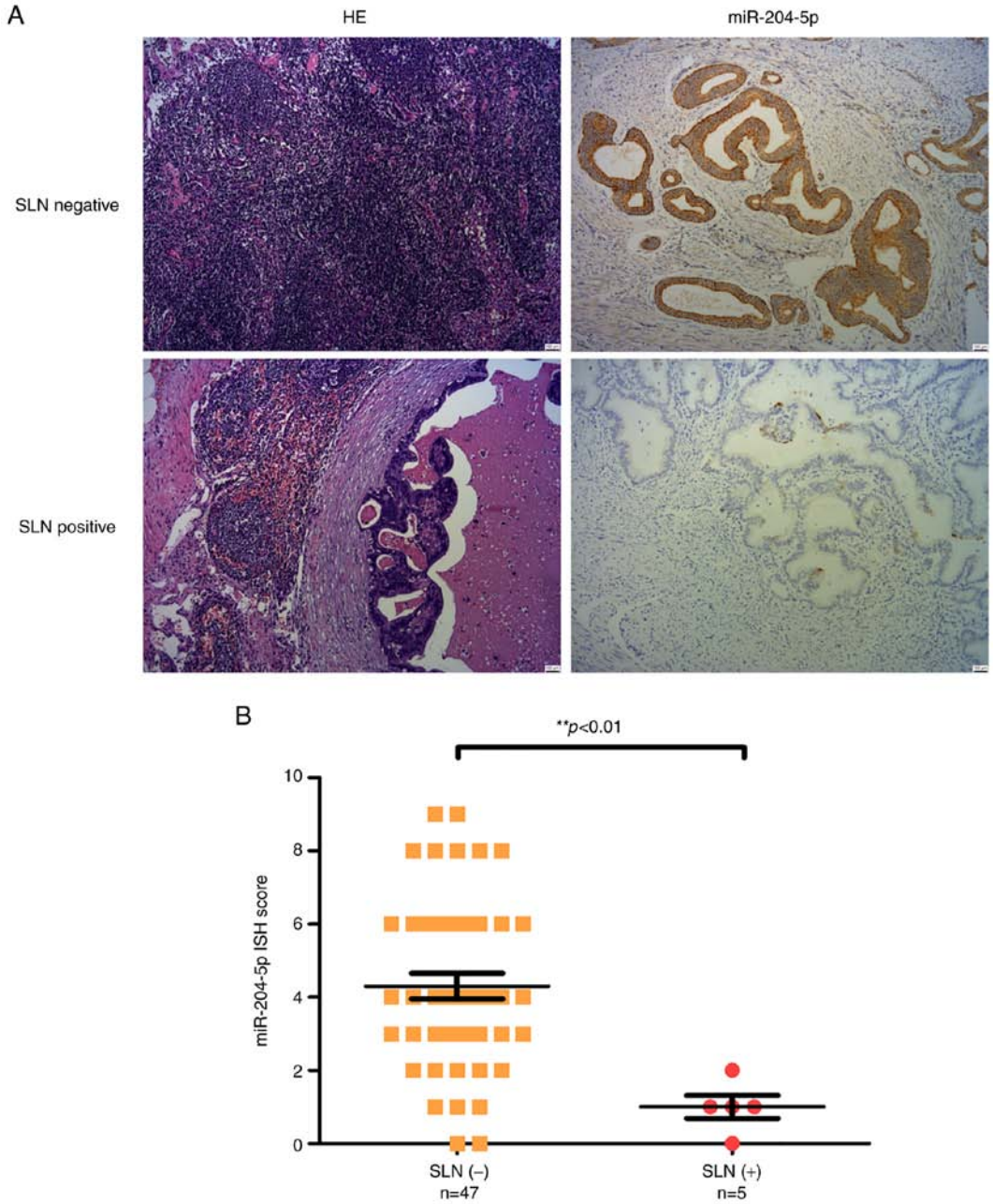

Figure 3. miR-204-5p expression is associated with lymph node metastasis in patients with SLN(+) EC. (A) Representative images of miR-204-5p immunoactivity in SLN(-) and SLN(+) EC (scale bars, 100 μm). (B) The scores of serum miR-204-5p expression in the SLN(+) group obtained using *in situ* hybridization were lower than those in the SLN(-) group. **P<0.01. EC, endometrial cancer; SLN, sentinel lymph node.

was overexpressed in acute lymphocytic leukemia. However, the level of miR-204 has been shown to be significantly lower in cancer than in normal tissue, and to be negatively associated with LNM in gastric and bladder cancer (30,31). Moreover, there is recent evidence to indicate that the serum levels of miR-204-5p in patients with gastric cancer are lower than those in patients with benign lesions, and indicate that miR-204-5p targets CXCR4 and CXCL12 to suppress the LNM (32). In accordance with these findings, the present study demonstrated that a lower miR-204-5p expression was associated with LNM in these SLN(+) EC tissues. Previous studies have also revealed the effective diagnostic value of miRNAs in cancers. Shimomura *et al* (33) demonstrated that the pre-operative combination of serum miR-1246, miR-1307-3p, miR-4634, miR-6861-5p and miR-6875-5p detected early-stage breast cancer with a sensitivity of 97.3% and a specificity of 82.9%. The serum levels of miR-135a have also been shown to distinguish non-small cell lung cancer tissue from healthy tissue with a specificity and sensitivity of 83.1 and 81.3%, respectively (34). Another study demonstrated that miR-204-5p was a potential biomarker for the prediction of the prevalence of frontotemporal dementia, and the area under the ROC curve of miR-204-5p was 0.89 (90% CI, 0.79-0.98) (35). The present study found the serum miR-204-5p level was a more convenient biomarker pre-operation to predict the status of LNM combined with SLNM in the treatment of patients with EC, with a sensitivity of 87.2% and a specificity of 80.0% (AUC, 0.923; 95% CI, 0.847-1.000); P=0.002), and the optimal

cut-off value was 0.253. Therefore, serum miR-204-5p levels may be an efficient biomarker for detecting the status of LNM pre-operation in patients with EC, with minimal costs.

There were some limitations to the present study. Firstly, the sample size of the present study was small, and the number of SLN-positive cases was only five. Secondly, the present study did not examine the prognostic value of serum miR-204-5p in patients with EC. Thus, further research is required with larger sample sizes, and to explore the prognostic value of miR-204-5p in patients with EC.

In conclusion, the present study demonstrated that serum miR-204-5p levels were lower in SLN-positive than in SLN-negative cases, and serum miR-204-5p may be an efficient biomarker for predicting LNM pre-operation with an AUC of 0.923, and an optimal cut-off value of 0.253. Thus, serum miR-204-5p levels may prove to be useful for clinical decision-making for lymphadenectomy or SLNM in patients with EC.

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Availability of data and materials

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

Authors' contributions

CW, YZ and WB conceived and designed the experiments. CW, XZ, JL, RX, LD and HX performed the experiments, and LD and HX analyzed the data. CW, XZ and JL wrote the manuscript. YZ and WB confirm the authenticity of all the raw data. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Shanghai General Hospital (Shanghai, China) on Oct, 2018, and informed consent was obtained from all included patients (no. 2018SQ307-1); if the participant was <16 years of age, the informed consent was obtained from the parents. The study was performed in accordance with the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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