Identification of Endometrioid Endometrial Carcinoma-associated microRNAs in Tissue and Plasma

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Highlights

1. A set of endometrioid endometrial carcinoma (EEC)-associated miRNAs in tissue and plasma was identified by next-generation sequencing approach.

2. EEC-associated miRNAs in tissues and plasma samples could distinguish EEC sample from NE sample with high accuracy.

3. EEC-associated miRNA levels in EEC tissues and plasma samples were associated with pathological characteristics.

Abstract (247 words)

Objective: This study aimed to identify a set of endometrioid endometrial carcinoma EEC-associated microRNAs (miRNAs) in tissue and plasma, and evaluate their clinical significance.

Methods: A set of EEC-associated miRNAs in tissue and plasma were identified by next-generation sequencing (NGS), which could enable in-depth characterization of the global repertoire of miRNAs.

Results: NGS identified 11 candidate EEC-associated miRNAs. Quantitative reverse-transcriptase PCR identified 8 EEC-associated miRNAs in tissue (upregulated: miR-499, miR-135b, miR-205, downregulated: miR-10b, miR-195, miR-30a-5p, miR-30a-3p and miR-21). Expression of hsa-miR-499 in International Federation of Gynecology and Obstetrics (FIGO) Stage IA and Grade 1 tissues was significantly lower than in others (FIGO Stage IB or more advanced, and Grade 2 or 3). By receiver operating characteristic (ROC) curves analysis, compared with single EEC-associated miRNA, two miRNA signatures (miR135b/miR195 and miR135b/miR30a-3p) could distinguish between EEC and normal endometrial tissue samples yielding a high area under the curve (AUC) of 0.9835 [95% confidence interval (CI): 0.9677–1.0], and 0.9898 (95% CI: 0.9677–1.0), respectively. As possible non-invasive markers for EEC, four EEC-associated miRNAs (increased level:

miR-135b and miR-205, decreased-level: miR-30a-3p and miR-21) in plasma were identified. Circulating levels of three EEC-associated miRNAs (miR-135b, miR-205 and miR-30a-3p) in plasma were significantly decreased after hysterectomy. ROC curves analysis revealed that miR-135b and miR-205 levels in plasma yielded AUCs of 0.9722 (95% CI: 0.913–1.0) and 1.0 (95% CI: 1.0–1.0), respectively.

Conclusion: Measurement of tissue and plasma EEC-associated miRNAs may be useful for early detection, diagnostic, and follow-up tests for EEC.

Introduction

Endometrial cancer is a common malignancy of the female reproductive tract. The most dominant subtype, endometrioid endometrial carcinoma (EEC) accounts for ~80% of cases (1). Accumulation of several genetic and epigenetic alternations in oncogenes and tumor suppressor genes is involved in the development of endometrial carcinoma (2). However, such alterations are not uniformly found in all EEC cases, and information regarding the molecular mechanisms of EEC etiology is still limited. The search for novel molecular markers for early detection and predicting outcomes has been ongoing in most cancers with a view to identifying molecular targets for therapeutic agents.

MicroRNAs (miRNAs) are non-protein-coding small RNAs (21–25 nucleotides) that function as regulators of gene expression by antisense complimentarily to specific mRNAs (3,4). As miRNAs are expressed in tissue-specific patterns (3), miRNAs predominantly expressed in EEC tissues are probably involved in cell proliferation, differentiation, apoptosis, and carcinogenesis of the endometrium (5, 6). Recently, by searching a panel of microarray assays, miRNA signatures in tissue and plasma could be used to distinguish EEC from normal endometrium (NE) (7, 8). This suggests that EEC-associated miRNAs have the potential to be developed as novel diagnostic and therapeutic molecules. However, the data regarding EEC-associated miRNAs in tissue and plasma are limited; therefore, investigation of EEC-associated miRNAs is likely to shed light on the molecular mechanisms of EEC etiology.

Microarray technology is high throughput, but can only detect a limited number of miRNAs because of the nature of probe hybridization (9). Next-generation sequencing (NGS) technology using Illumina technology generates short reads (35 bp) but more than 1 million bp of sequence data per run, and can be used to measure the abundance of small-RNA sequences in a sample. miRNAs are only 21–25 bp in length; therefore, this technology can

enable in-depth characterization of the global repertoire of miRNAs (10).

In this study, to get a clue regarding novel diagnostic and therapeutic molecules of EEC, we tried to identify EEC-associated miRNAs in tissue and plasma. First, by comparative analysis of NGS-generated miRNA expression profiles of EEC tissue, NE tissue and blood cells from the same patient, we selected candidate EEC-associated miRNAs, whose expression level was negative in the blood of patients, and in EEC tissues was >2 times up- or downregulated compared with that in NE tissue. Second, by comparative analysis of EEC and NE tissues using real-time quantitative RT-PCR (qRT-PCR), we identified EEC-associated miRNAs in tissue. Subsequently, to identify and characterize EEC-associated miRNAs in plasma, the circulating levels of EEC-associated miRNA in plasma in women with EEC or NE. Finally, the relationship between EEC-associated miRNA expression and clinicopathological characteristics, and the diagnostic value of EEC-associated miRNA expression in tissue and plasma were analyzed tentatively.

Materials and Methods

Sample collection

Study subjects were recruited at the Department of Obstetrics and Gynecology, Nagasaki University Hospital, Japan. All samples were obtained after receiving written informed consent, and the study protocol was approved by the Institutional Review Board for Ethical, Legal and Social Issues of Nagasaki University.

For NGS analysis, EEC tissue, NE tissue, and blood cells were obtained from an identical patient with International Federation of Obstetricians and Gynecologists (FIGO) Stage IA (Grade 1) EEC. EEC and NE tissue samples were obtained immediately after total

hysterectomy with bilateral salpingo-oophorectomy. EEC was diagnosed by endometrial biopsy prior to the operation. Diagnosis of EEC and NE tissue was confirmed by pathological analysis. EEC and NE tissue samples were placed in RNAlater (Ambion, Austin, TX, USA). The blood samples (7 mL) were collected before the operation and placed in tubes containing EDTA. Using a mirVana miRNA Isolation Kit (Ambion), total RNA containing small RNA molecules was extracted from each sample immediately after sampling. Quality assessment and concentration measurements of total RNA, including small RNAs, were performed using a Bioanalyzer (Agilent Technologies, South Queensferry, UK) and a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively.

For subsequent expression analysis by qRT-PCR, EEC tissues were obtained from 28 cases of EEC (EEC group) and NE tissues from 14 cases of non-EEC (NE group). In addition to total hysterectomy with bilateral salpingo-oophorectomy, lymphadenectomy was performed in 25 cases. Tumor stage was determined according to 2009 revised FIGO classification (11). In cases of NE, total hysterectomy was performed because of uterine myoma. Final diagnosis of EEC or NE was confirmed by pathological analysis. None of the EEC patients had a history of other malignant disease or had received neoadjuvant therapy. After the operation, patients were submitted to radiotherapy and/or chemotherapy according to FIGO guidelines. Between the EEC and NE groups, there were no significant differences in body mass index (BMI), history of parity, smoking, diabetes, or family history of endometrial cancer (data not

shown). The mean (SD) patient age was 60.6 (10.8) years in the EEC group and 42.8 (5.1) years in the NE group (Student's *t* test, $P<0.001$). Clinicopathological characteristics in the EEC group are listed in Supplementary Table 1.

To obtain cell-free plasma miRNAs, blood samples (7 mL) were collected from 12 cases of EEC and 12 of NE. Blood sampling was performed 1 day before the operation and 7 days after. Between the EEC and NE groups, there were no significant differences in BMI, history of parity, smoking, diabetes, or family history of endometrial cancer (data not shown). The mean (SD) patient age was 50.8 (8.3) years in the EEC group and 36.5 (8.3) years in the NE group (Student's *t* test, $P=0.001$). Pathological characteristics in the EEC group are listed in Supplementary Table 2. Cell-free plasma samples were prepared from blood by a double centrifugation method as described previously (12). Total RNA containing small RNA molecules was extracted from 1.2 mL cell-free plasma samples as described previously (12). Extracted total RNAs were stored at −80°C. Although there were differences in age between the EEC and NE groups in both tissue and plasma, there was no significant correlation between expression of studied miRNAs and age of patients (data not shown).

In miRNA expression analysis in endometrial tissue, there is no consensus on universal endogenous normalization controls because small RNAs, including RNU48 and RNU6B, have been suggested as reference RNAs, but exhibit high variability (13). In addition, it is recommended that the quantitative mRNA measurements in plasma are expressed as an

absolute concentration (14). Therefore, we considered that the quantitative miRNA measurements may be the same as quantitative mRNA measurements in plasma. In this study, absolute real-time qRT-PCR analysis was performed.

Small RNA library construction and NGS analysis

To screen for EEC-associated miRNAs, NGS was applied to a set of EEC tissues, NE tissues and blood from the same EEC patient. Isolation of total RNA including small RNAs, their quality assessment, concentration measurements, small RNA library construction, NGS and miRNA mapping were performed as described previously (15–18).

To compare miRNA levels across data sets, the sequencing read count for each miRNA was normalized to the total read count of 1 000 000 in each sample, and expressed as reads per million (RPM) (15-17). For mapped data, when the normalized miRNA read count was negative in patient's blood, and was >2 times up- or downregulated in EEC tissues than in NE tissue, these miRNAs were selected as candidate EEC-associated miRNAs. These miRNAs were then analyzed by RT-PCR in tissue and plasma from the EEC and NE group.

Real-time qRT-PCR analysis of miRNAs

All specific primers and TaqMan probes were purchased from TaqMan MicroRNA Assays (Applied Biosystems). For real-time qRT-PCR of miRNAs in tissues and plasma samples was performed as described previously (12, 15). For each miRNA assay, we prepared a calibration curve by 10-fold serial dilution of single-stranded cDNA oligonucleotides corresponding to each miRNA sequence from 1.0×10^2 to 1.0×10^8 copies/mL. Each sample and each calibration dilution was analyzed in triplicate. Each assay could detect down to 100 RNA copies/mL. Every batch of amplifications included three water blanks as negative controls for each of the

reverse transcription and PCR steps. All data were collected and analyzed using an ABI Prism 7900 Sequence Detector (Applied Biosystems).

Statistical analysis

Patient backgrounds were compared by Student's *t* test and Pearson's χ^2 test for continuous and discrete variables, respectively, of EEC and NE cases. Absolute quantification data were analyzed with SDS 2.3 software (Applied Biosystems). The expression levels of EEC-associated miRNAs in tissues and the cell-free plasma concentrations of EEC-associated miRNAs in cases of EEC and NE were converted into multiples of the median (MoM) of concentration in the cases of NE. Differences between the two groups were evaluated with Mann–Whitney's *U* test or Kruskal–Wallis test. Changes in the cell-free plasma concentration of EEC-associated miRNAs before and after the operation were compared by the Wilcoxon signed-rank test. Statistical analyses were performed with SPSS version 19 (IBM Japan, Tokyo, Japan). To determine the ability of miRNAs to classify EEC and NE samples, receiver operating characteristic (ROC) curves were plotted with an R package, pROC (19). To develop miRNA signatures featuring the best accuracy in distinguishing between EEC and NE samples a multivariate logistic regression model was utilized. Evaluation of obtained regression models was performed with the Wald test. Statistical analyses were performed using R (R Core Team, Vienna, Austria). Significant differences were defined as $P<0.05$.

Results

Screening of candidate EEC-associated miRNAs by NGS

NGS analysis yielded 20 674 015 reads from EEC tissue, 19 107 722 reads from blood cells,

and 20 375 081 reads from NE tissue. All the above sequence data were deposited in DDBJ Sequence Read Archive (DRA) (Accession ID: DRA001166). High-throughput sequencing assays can be susceptible to noise and variability; therefore, measurement of miRNA expression was normalized using the library size (1 000 000 reads). Eleven candidate EEC-associated miRNAs were identified (Table 1). Candidate EEC-associated miRNAs identified were located on various chromosomal regions. Out of 11 candidate EEC-associated miRNAs, 5 (miR-10b, miR-499, miR-184, miR-195 and miR-135b) were upregulated in EEC tissue, while 6 (miR-203, miR-10a, miR-30a-5p, miR-205, miR-30a-3p and miR-21) were downregulated in EEC tissue than NE (Table 1).

Confirmation of EEC-associated miRNAs in tissue by qRT-PCR

Expression levels of the 11 candidate EEC-associated miRNAs in 28 EEC and 14 NE tissues were measured by qRT-PCR. Eight miRNAs showed significantly different expression between EEC and NE tissues, and were identified as EEC-associated miRNAs in tissue. The expression levels of 3 EEC-associated miRNAs (miR-499, miR-135b and miR-205) were significantly higher in EEC than NE tissues (Mann–Whitney *U* test, $P=0.003$, $P<0.001$ and $P=0.002$, respectively), while those of 5 EEC-associated miRNAs (miR-10b, miR-195, miR-30a-5p, miR-30a-3p and miR-21) were significantly downregulated in EEC tissue (Mann–Whitney *U* test, $P=0.006$, $P<0.001$, $P=0.019$, $P=0.001$, and $P=0.011$, respectively;

Table 2). Meanwhile, there was no significant difference in the levels of 3 candidate EEC-associated miRNAs (miR-184, miR-203 and miR-10a) between EEC and NE tissues (Table 2). Using a database search of predicted miRNA targets in mammals (www.targetscan.org), we searched the candidate target mRNAs of EEC-associated miRNAs in tissue. Three mRNAs (MutS homolog 2: MSH2, Leukotriene B4 12-hydroxydehydrogenase: LTB4DH and IκB kinase α: IKKα) were selected as common target mRNAs of 3 upregulated EEC-associated miRNAs in EEC tissue, while there was no common target mRNAs of 5 downregulated EEC-associated miRNAs.

Identification of EEC-associated miRNAs in plasma

Regarding the 8 EEC-associated miRNAs in tissue, circulating levels of each miRNA in plasma from 12 women with EEC and 12 with NE tissue were measured by qRT-PCR. Four miRNAs showed significantly different circulating levels between the EEC and NE groups, and were identified as EEC-associated miRNAs in plasma. The expression levels of 2 EEC-associated miRNAs (miR-135b and miR-205) were significantly higher in plasma samples from the EEC group than the NE group (Mann–Whitney *U* test, $P<0.001$), while those of 2 EEC-associated miRNAs (miR-30a-3p and miR-21) were significantly lower in plasma samples from the EEC group than the NE group (Mann–Whitney *U* test, $P=0.009$ and $P=0.033$, respectively). Meanwhile, there was no significant difference in the levels of 4

EEC-associated miRNAs (miR-10b, miR-30a-5p, miR-195 and miR-499) between plasma samples from the EEC and NE groups (Table 3).

Identification of EEC-associated miRNAs that showed significantly decreased concentrations in plasma after hysterectomy

The 4 EEC-associated miRNAs that showed significantly increased or decreased levels in plasma in the EEC group compared with the NE group (Table 3, increased level: miR-135b and miR-205, decreased level: miR-30a-3p and miR-21) were selected for analysis before and after hysterectomy. The plasma concentrations of 3 miRNAs (Table 4, miR-135b, miR-205 and miR-30a-3p) were significantly decreased after hysterectomy (Wilcoxon signed-rank tests, P=0.003, Table 3), and were considered as possible molecular markers in plasma. Meanwhile, there was no significant difference in the plasma level of hsa-miR-21 before and after hysterectomy (Table 3).

Relationship between EEC-associated miRNA expression and clinicopathological characteristics

To investigate the clinical significance of EEC-associated miRNAs in tissue and plasma, we compared EEC-associated miRNA expression in groups distinguished based on FIGO stage, histopathological grade, or relapse. Significant relationships were found between expression of miR-499 and FIGO stage, and between expression of miR-205 and histological grade. The

expression level of miR-499 in 14 cases of FIGO Stage II or more advanced was significantly higher than that in 4 cases of FIGO Stage IA and IB (Mann–Whitney *U* test, *P*=0.019, Supplementary Table 1). The expression level of miR-205 in EEC cases with Grade 3 tumor (*n*=2) was significantly higher than that in cases with Grade 1 (*n*=15) and 2 (*n*=11) tumors (Kruskal–Wallis test, *P*=0.024, Supplementary Table 1). The expression level of miR-499 in 7 cases of FIGO Stage IA and Grade 1 tumor was significantly lower than in 21 cases of other tumors (FIGO Stage IB or more advanced, and Grade 2 or 3) (Mann–Whitney *U* test, *P* =0.047, Table 6). Meanwhile, there was no significant difference in the tissue levels of all EEC-associated miRNAs between groups distinguished according to the presence of lymph node metastasis or occurrence of relapse (Table 4).

Circulating miRNA levels of EEC-associated miRNAs in plasma were compared in groups distinguished according to FIGO stage and histopathological grade. We compared FIGO Stage IA Grade 1 tumors with others (more advanced FIGO stage and/or histopathological grade). The plasma concentration of miR-21 in 4 cases of FIGO Stage IA and Grade 1 tumors was significantly higher than that in 8 cases of more advanced tumors (Mann–Whitney *U* test, *P*=0.017, Table 7). Meanwhile, there was no significant difference in the plasma concentrations of other EEC-associated miRNAs (miR-135b, miR-205 and miR-30a-3p) and carbohydrate antigen (CA)125 before and after hysterectomy (Table 5).

Diagnostic value of EEC-associated miRNA expression in tissue and plasma

ROC curves for discriminating EEC samples from NE were constructed based on EEC-associated miRNA expression in tissues (EEC, *n*=28; NE, *n*=14). Analysis of the ROCs revealed high area under curve (AUC) values for each EEC-associated miRNA in tissues (Figure 1): miR-499, miR-30a-5p, miR-21, miR-10b, miR-205, miR-30a-3p, miR-195 and miR-135b yielded AUC of 0.7143 [95% confidence interval (CI): 0.5537–0.8749), 0.7245 (95% CI: 0.5445–0.9045), 0.7423 (95% CI: 0.5744–0.9103), 0.7602 (95% CI: 0.6132–0.9072), 0.8112 (95% CI: 0.666–0.9565), 0.8265 (95% CI: 0.6953–0.9578), 0.8736 (95% CI: 0.7145–1.0) and 0.9184 (95% CI: 0.8285–1.0), respectively (Figure 1A). The miRNA signatures consisting of 2 miRNAs yielded elevated AUCs in comparison to single miRNAs. miR135b/miR195 and miR135b/miR30a-3p yielded AUCs of 0.9835 (95% CI: 0.9677–1.0, $P<0.048$, Wald test, Figure 1A), and 0.9898 (95% CI: 0.9677–1.0, $P<0.038$, Wald test, Figure 1A), respectively.

ROC curves for discriminating women with EEC from those with NE were constructed based on EEC-associated miRNAs levels in plasma samples (EEC, *n*=12; NE, *n*=12). Analysis of the ROCs revealed high AUC values for each EEC-associated miRNA in plasma (Figure 1B); miR-21, miR-30a-3p, miR-135b and miR-205 yielded AUC of 0.7569 (95% CI: 0.5611–0.9528), 0.8125 (95% CI: 0.6381–0.9869), 0.9722 (95% CI: 0.913–1.0) and 1.0 (95% CI: 1.0–1.0), respectively.

Discussion

In this study, we identified EEC-associated miRNAs in tissue and plasma, and evaluated their clinical significance.

NGS can be used to investigate all known and unknown miRNAs, while oligonucleotide microarray methods can only be used to examine a limited number of known miRNAs present on each array. Therefore, using NGS allows whole genome analysis to be performed to identify candidate miRNAs that are differentially expressed. In addition, the miRNA expression in each case of EEC depends on the heterogeneity of cancer. Our NGS analyses identified 11 candidate EEC-associated miRNAs (upregulated: miR-10b, miR-499, miR-184, miR-19 and miR-135b, downregulated: miR-203, miR-10a, miR-30a-5p, miR-205, miR-30a-3p and miR-21) at various chromosomal regions. Although all miRNAs were previously known, nine of the 11 were newly identified as candidate EEC-associated miRNAs (except for miR-203 and miR-205) that had not been identified in previous microarray studies (5–7, 20). Therefore, this indicates that NGS enables a more in-depth characterization of the global repertoire of miRNAs compared with oligonucleotide microarray analysis and/or the heterogeneity of cancer because the discovery set used for NGS analysis was obtained from a single cancer patient, a limitation of this study. Therefore, it is critical to explore additional studies of miRNAs based on the heterogeneity of EEC. Consistent with a previous study, several dysregulated miRNAs in EEC tissues were identified in our analyses (5–7, 20).

However, in previous studies of EEC tissues, miR-200 family, miR-9, miR-203, miR-205 and miR-210 were upregulated, while miR-410, miR-17-5p, miR-214, miR-99a,b, miR-199b, miR-100, miR-20a, miR-221, miR-222 and miR-424 were downregulated (5, 7, 20–26). The discrepancy between our study and the previous studies reflects the difference in the way to select the candidate EEC-associated miRNAs at the beginning of each study. Previous studies have selected EEC-associated miRNAs with predominantly dysregulated expression in the EEC tissues at the beginning of their study (7). In contrast, we selected the miRNAs that had predominantly dysregulated expression in EEC tissues compared with NE tissues, but negative expression (<100 read counts) in blood cells as candidate EEC-associated miRNAs. This was because one of our goals was to identify the EEC-associated miRNAs in plasma as non-invasive diagnostic markers for EEC. Another reason for the discrepancy between the present and previous studies may be related to the method of obtaining samples for high-throughput analysis. Previous studies obtained EEC and NE samples from different individuals. However, each case had a heterogeneous background and each miRNA expression pattern in EEC and NE was affected by various factors, for example, the phase during the menstrual cycle, and the background affecting the molecular pathways of EEC and NE differed among individuals. Therefore, in the present study, to make uniform the influence of backgrounds affecting miRNA expression in EEC and NE, we compared EEC and NE tissue from the same EEC patient (FIGO Stage IA, Grade 1) at the same time.

Subsequent confirmation analysis using qRT-PCR identified 8 EEC-associated miRNAs in tissue (upregulated: miR-499, miR-135b and miR-205, downregulated: miR-10b, miR-195, miR-30a-5p, miR-30a-3p and miR-21). miR-205 was upregulated in the qRT-PCR study, although it was downregulated in the NGS experiment. Additionally, miR-10b and miR-195 were downregulated in the qRT-PCR study, although they were upregulated in the NGS experiment. This discrepancy was also found in a previous study (7), and might have been because single cases were analyzed by NGS but multiple cases by qRT-PCR.

We identified novel and already known EEC-associated miRNAs (5, 7, 20–26). miR-205 is frequently dysregulated in many human cancers, suggesting its important roles in initiation and progression of cancer. Previous studies identified significantly overexpressed hsa-miR-205 in endometrial cancer compared with NE tissue, and JPH4, ESRRG and PTEN were the candidate tumor suppressor genes in EEC (5, 27, 28). In contrast, miRNA-205 was significantly suppressed in renal cancer cell lines and tumors when compared with normal tissues and a non-malignant cell line (29). The expression of miRNA-205 is significantly high in some malignancies but significantly low in other malignancies, depending on the organs from which the malignancy comes. These observations suggest that a miRNA has more than one target mRNA.

By using the database search, 3 mRNAs (MSH2, LTB4DH and IKKα) were selected as common targets of 3 up-regulated EEC-associated miRNAs in EEC tissue. An oncogenic

345 miRNA acts as an oncogene and has increased expression in tumor cells, while a tumor
346 suppressor miRNA acts as a tumor suppressor gene and has decreased expression in tumor
347 cells. All 3 candidate target mRNAs are tumor suppressor genes (30-34), thus, it is compatible
348 that they are candidate target mRNAs of upregulated EEC-associated miRNAs (oncogenic
349 miRNAs) in EEC tissue.

350 The relationship between EEC-associated miRNA expression in EEC tissue and
351 clinicopathological characteristics was investigated. The expression level of has-miR-499 in
352 tissues of FIGO Stage II or more advanced tumors was significantly higher than in tissues of
353 Stages IA and IB tumors. The expression level of miR-205 in EEC of FIGO Grade 3 tumor
354 was significantly higher than that in Grade 1 and 2 tumors. The expression level of
355 has-miR-499 in tissues of FIGO Stage IA and Grade 1 tumors was significantly lower than in
356 tissues of other tumors (FIGO Stage IB or more advanced, and Grade 2 or 3). ROC curve
357 analysis revealed that single regulated EEC-associated miRNAs in tissues could distinguish
358 between EEC and NE tissue samples yielding high AUCs. In addition, 2 miRNA signatures,
359 miR135b/miR195 and miR135b/miR30a-3p, classified EEC tumor tissues with higher
360 accuracy than single miRNAs. These observations suggest that EEC-associated miRNA
361 signatures in tissue could be a diagnostic marker, a supportive marker to estimate the
362 pathological stage and grade of EEC, and potential markers to decide treatment strategies for
363 each EEC case (7).

Finally, as non-invasive markers for EEC, four EEC-associated miRNAs (increased level: miR-135b, miR-205, decreased-level: miR-30a-3p and miR-21) in plasma were identified. Increased levels of EEC-associated miRNAs in plasma also showed higher expression level in EEC tissue, and decreased levels of EEC-associated miRNAs in EEC plasma showed lower expression level in EEC tissue. This suggests that circulating levels of EEC-associated miRNA in plasma reflect the expression status of EEC-associated miRNA in tissue. Torres *et al*. evaluated miRNA profiles in matched tissue and plasma samples from EEC patients, and showed diagnostic and prognostic significance of plasma miRNA signatures in EEC (7). Although invasive procedures including biopsies or surgery were performed in the current clinical diagnosis, plasma-based biomarkers may lead to development of a non-invasive test of EEC. To date, miRNA expression pattern is known to be aberrant in cancer, and tumor-cell-derived miRNAs in circulation may be stored in microvesicles that are secreted by various cell types. Additionally, cell-free miRNAs are remarkably stable molecules in plasma (35). Although the source of plasma EEC-associated miRNAs has not been determined so far, they might derive from exosomes shed from apoptotic or broken cells in EEC and NE (35–37). In this study, circulating levels of 3 EEC-associated miRNAs (miR-135b, miR-205 and miR-30a-3p) in plasma were significantly decreased after surgery, suggesting that these miRNAs in plasma were mainly from EEC and NE, and may serve as a non-invasive biomarker for diagnosis of EEC, for example, early

383 detection of early-stage EEC or relapse.

384 As for the clinical significance of plasma EEC-associated miRNAs, circulating levels
385 of EEC-associated miRNAs in plasma were compared in groups distinguished according to
386 FIGO stage and histopathological grade. Comparison of FIGO Stage IA (Grade 1) tumors
387 with others (more advanced FIGO stage and/or histopathological grade), the plasma
388 concentration of miR-21 in cases of FIGO Stage IA (Grade 1) tumors was significantly higher
389 than in more advanced tumors, suggesting that this miRNA may have the potential to detect
390 early-stage EEC. ROC curve analysis revealed that 4 single regulated EEC-associated
391 miRNAs in plasma could distinguish between EEC and NE cases yielding high AUCs (Figure
392 1B). Two single miRNAs, miR-135b and miR-205, yielded 0.9722 (95% CI: 0.913–1.0) and
393 1.0 (95% CI: 1.0–1.0), respectively. CA125 is a current tumor marker for EEC, and can be
394 measured simply and non-invasively, and provide a useful indicator of tumor status. However,
395 the sensitivity and positive predictive value of CA125 is relatively low in detecting EEC (38).
396 In contrast, EEC-associated miRNAs have different expression profiles in NE and EEC,
397 suggesting that EEC-associated miRNAs in plasma may be used as additional biomarkers for
398 EEC diagnosis.

399 In conclusion, a set of EEC-associated miRNAs in tissue and plasma of EEC patients
400 were identified by NGS, which could enable in-depth characterization of the global repertoire
401 of miRNAs. EEC-associated miRNA levels in tissue and plasma were associated with

pathological characteristics, and could distinguish EEC from NE samples with high accuracy. Although our data are still preliminary because of the small sample size, the measurement of EEC-associated miRNAs in the tissue and plasma may be used as a diagnostic, prognostic, and follow-up test for EEC. Future studies regarding the biological pathway of EEC-associated miRNAs in tissue and plasma may contribute to the elucidation of molecular pathogenesis of EEC, endometrium development, and discovery of novel therapeutic targets of EEC.

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Table Legends

Table 1. Candidate EEC-associated miRNAs detected by Next-generation sequencing analysis.

Normalized read counts are described as reads per million.

Table 2. Expression of candidate EEC-associated miRNAs in carcinoma tissues from patients with EEC group and NE tissues from patients without carcinoma.

Expression levels are described as MoM values [median (minimum – maximum)]. Significant differences between groups were analyzed by Mann–Whitney *U* test. $P<0.05$ was considered significant. NS, not significant.

Table 3. Circulating levels of EEC-associated miRNAs in plasma samples from patients without carcinoma (NE plasma) and patients with EEC (EEC plasma) before and after surgery.

Expression levels are described as MoM values [median (minimum – maximum)]. Significant differences between control and EEC plasma before surgery were analyzed by Mann–Whitney *U* test, and significant differences between EEC plasma before and after operation were analyzed by Wilcoxon singed-rank test. $P<0.05$ was considered significant.

–, not analyzed; ND, not detected; NS, not significant.

Table 4. Association between clinicopathological characteristics and EEC-associated miRNA levels in EEC tissues.

Expression levels of miRNAs are described as MoM values [median (minimum – maximum)]. Significant differences between groups were analyzed by Mann–Whitney *U* test. $P<0.05$ was considered significant.

[a]Includes tumors with more advanced FIGO stage and/or histopathological grade than Stage IA, Grade 1. NS, not significant.

Table 5. Association between pathological characteristics, EEC-associated miRNA levels and CA 125 levels in plasma from patients with EEC.

Expression levels of miRNAs are described as MoM values [median (minimum – maximum)], and CA 125 levels as U/mL. Significant differences between groups were analyzed by Mann–Whitney *U* test. $P<0.05$ was considered significant.

[a]Includes tumors with more advanced FIGO stage and/or histopathological grade than Stage IA, Grade 1. NS, not significant.

553 **Figure Legends**

554 **Figure 1.** ROC curve analysis using tissue and plasma miRNAs profiles for discriminating
555 EEC samples from NE samples. (A) Tissue miRNA profiles (EEC, *n*=28; control, *n*=14);
556 miR-10b, miR-499, miR-195, miR-135b, miR-30a-5p, miR-205, miR-30a-3p and miR-21
557 yielded AUC of 0.7602 (95% CI: 0.6132–0.9072), 0.7143 (95% CI: 0.5537–0.8749), 0.8736
558 (95% CI: 0.7145–1.000), 0.9184 (95% CI: 0.8285–1.0), 0.7245 (95% CI: 0.5445–0.9045),
559 0.8112 (95% CI: 0.666–0.9565), 0.8265 (95% CI: 0.6953–0.9578) and 0.7423 (95% CI:
560 0.5744–0.9103), respectively. The miR signatures consisting of 2 miRNAs yielded elevated
561 AUC values in comparison to single miRNAs. The miR135b/miR30a-3p yielded an AUC of
562 0.9898 (95% CI: 0.9677–1.0, $P<0.038$, Wald test), miR135b/miR195 yielded AUC of 0.9835
563 (95% CI: 0.9677–1.0, $P<0.048$, Wald test). (B) Plasma miRNA profiles (EEC, *n*=12; control,
564 *n*=12); miR-135b, miR-205, miR-30a-3p and miR-21 yielded AUC values of 0.9722 (95% CI:
565 0.913–1.0), 1.0 (95% CI: 1.0–1.0), 0.8125 (95% CI: 0.6381–0.9869), and 0.7569 (95% CI:
566 0.5611–0.9528), respectively.

Table 1. Candidate EEC-associated miRNAs detected by Next-generation sequencing analysis

miRNA	Chromosome localization	Blood cell (reads per million)	EEC tissue (reads per million)	NE tissue (reads per million)	EEC/NE
hsa-miR-10b	2q31.1	3	2220	770	2.88
hsa-miR-499	20q11.22	0	2010	705	2.85
hsa-miR-184	15q25.1	11	2006	851	2.36
hsa-miR-195	17p13.1	0	12901	6320	2.04
hsa-miR-135b	1q32.1	0	133	66	2.02
hsa-miR-203	14q32.33	0	1712	3514	0.48
hsa-miR-10b	17q21.32	2	263	602	0.44
hsa-miR-30a-5p	6q13	50	15732	45694	0.34
hsa-miR-205	1q32.2	0	285	1356	0.21
hsa-miR-30a-3p	6q13	29	1610	9476	0.17
hsa-miR-21	17q23.1	30	219	1369	0.16

Normalized read counts are described as reads per million.

Table 2. Expression of candidate EEC-associated miRNAs in carcinoma tissues from patients with EEC group and NE tissues from patients without carcinoma

miRNA	NE group (*n*=14)	EEC group (*n*=28)	*P* value
miR-10b	1.0 (0.68–1.62)	0.79 (0.17–2.81)	0.006
miR-499	1.0 (0.28–1.99)	2.49 (0.22–40.08)	0.003
miR-184	1.0 (0.1–18.2)	0.82 (0.06–169.3)	NS
miR-195	1.0 (0.024–1.96)	0.32 (0.10–0.87)	<0.001
miR-135b	1.0 (0.57–3.40)	5.13 (0.49–15.13)	<0.001
miR-203	1.0 (0.38–1.49)	1.13 (0.18–3.92)	NS
miR-10b	1.0 (0.29–1.55)	0.79 (0.17–2.81)	NS
miR-30a-5p	1.0 (0.23–2.0)	0.61 (0.27–1.62)	0.019
miR-205	1.0 (0.06–4.07)	2.47 (0.0–6.19)	0.002
miR-30a-3p	1.0 (0.54–2.04)	0.53 (0.1–2.45)	0.001
miR-21	1.0 (0.39–2.76)	0.64 (0.27–1.22)	0.011

Expression levels are described as MoM values [median (minimum – maximum)]. Significant differences between groups were analyzed by Mann–Whitney *U* test.

$P<0.05$ was considered significant.

NS, not significant.

Table 3. Circulating levels of EEC-associated miRNAs in plasma samples from patients without carcinoma (NE plasma) and patients with EEC (EEC plasma) before and after surgery

miRNA	A	B	C	*P* value	
	NE plasma (*n*=12)	EEC plasma before operation (*n*=12)	EEC plasma after operation (*n*=11)	A vs B	B vs C
miR-135b	1.0 (0.57–3.40)	5.13 (0.49–15.13)	0.0062 (0–0.96)	<0.001	0.003
miR-205	1.0 (0.06–4.07)	2.34 (0.0–6.19)	0.56 (0.34–0.94)	<0.001	0.003
miR-30a-3p	1.0 (0.54–2.04)	0.53 (0.096–2.25)	0.062 (0–0.12)	0.009	0.003
miR-21	1.0 (0.39–2.76)	0.64 (0.27–1.22)	0.59 (0.023–3.11)	0.033	NS
miR-10b	1.0	0.745	–	NS	–

	(0.34–2.49)	(0.05–1.55)			
miR-30a-5p	1.0 (0.21–2.74)	0.48 (0.05–1.3)	–	NS	–
miR-195	1.0 (0.24–2.1)	0.615 (0.05–1.73)	–	NS	–
miR-499	ND	ND	–	–	–

Expression levels are described as MoM values [median (minimum – maximum)]. Significant differences between control and EEC plasma before surgery were analyzed by Mann–Whitney *U* test, and significant differences between EEC plasma before and after operation were analyzed by Wilcoxon singed-rank test. $P<0.05$ was considered significant.

–, not analyzed; ND, not detected; NS, not significant.

Table 4. Association between clinicopathological characteristics and EEC-associated miRNA levels in EEC tissues

	FIGO Stage and Grade			Lymph node metastasis			Relapse		
	IA G1 (*n*=7)	Others[a] (*n*=21)	*P* value	– (*n*=21)	+ (*n*=4)	*P* value	– (*n*=25)	+ (*n*=3)	*P* value
miR-135b	5.64 (3.51–12.13)	4.39 (0.49–15.13)	NS	5.55 (0.49–12.13)	6.03 (1.49–15.13)	NS	5.67 (0.86–15.13)	4.09 (0.49–4.68)	NS
miR-205	2.92 (1.14–6.09)	2.24 (0–6.19)	NS	2.14 (0–6.19)	3.03 (1.43–6.12)	NS	2.74 (0–6.19)	1.43 (1.4–2.34)	NS
miR-21	0.89 (0.43–1.22)	0.58 (0.27–0.96)	NS	0.7 (0.32–1.22)	0.55 (0.46–0.96)	NS	0.7 (0.27–1.22)	0.55 (0.32–0.81)	NS
miR-30a-3p	0.595 (0.22–2.45)	0.43 (0.1–2.15)	NS	0.28 (0.24–0.45)	0.57 (0.13–2.45)	NS	0.55 (0.1–2.45)	0.45 (0.2–0.72)	NS
miR-499	1.09	2.92	0.047	2.09	5.24	NS	2.48	3	NS

	(0.41–2.54)	(0.22–40.08)		(0.22–40.48)	(2.48–26.26)		(0.21–40.48)	(2.73–7.21)	
miR-10b	0.8 (0.49–2.59)	0.66 (0.17–2.81)	NS	0.79 (0.21–2.81)	0.88 (0.3–1.15)	NS	0.79 (0.17–2.81)	0.85 (0.56–1.15)	NS
miR-30a-5p	0.69 (0.39–1.26)	0.61 (0.27–1.62)	NS	0.62 (0.27–1.62)	0.58 (0.46–0.68)	NS	0.61 (0.28–1.62)	0.61 (0.27–1.39)	NS
miR-195	0.28 (0.13–0.87)	0.35 (0.1–0.74)	NS	0.26 (0.13–0.87)	0.35 (0.1–0.54)	NS	0.33 (0.13–0.87)	0.18 (0.1–0.74)	NS

Expression levels of miRNAs are described as MoM values [median (minimum – maximum)]. Significant differences between groups were analyzed by Mann–Whitney *U* test. $P<0.05$ was considered significant.

[a]Includes tumors with more advanced FIGO stage and/or histopathological grade than Stage IA, Grade 1.

NS, not significant.

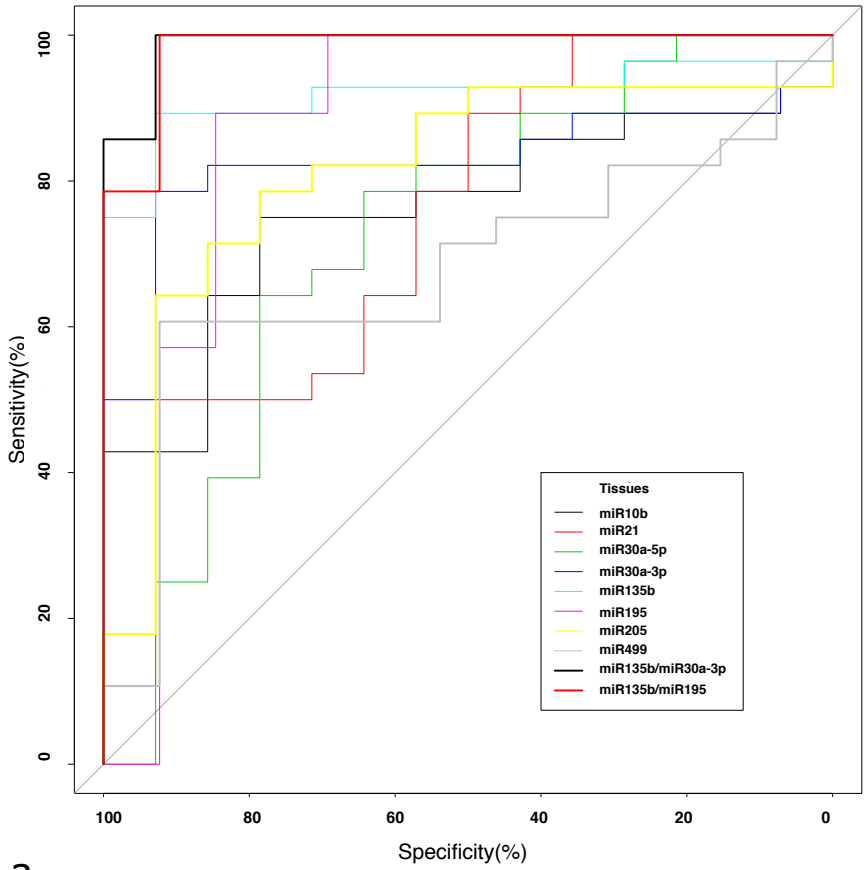
Table 5. Association between pathological characteristics, EEC-associated miRNA levels and CA 125 levels in plasma from patients with EEC

	FIGO Stage and Grade		
	IA G1 (*n*=4)	Others[a] (*n*=8)	*P* value
miR-135b	3.71 (3.61–7.67)	6.52 (1.47–9.22)	NS
miR-205	4.95 (3.95–5.79)	5.38 (2.96–7.36)	NS
miR-21	0.24 (0.013–0.45)	0.82 (0.31–1.73)	0.017
miR-30a-3p	0.52 (0.34–0.65)	0.69 (0.52–1.08)	NS
CA125	22.5 (17.6–51.2)	17.5 (7.7–127.4)	NS

Expression levels of miRNAs are described as MoM values [median (minimum – maximum)], and CA 125 levels as U/mL. Significant differences between groups were analyzed by Mann–Whitney *U* test. $P<0.05$ was considered significant.

[a]Includes tumors with more advanced FIGO stage and/or histopathological grade than Stage IA, Grade 1.

NS, not significant.


Sensitivity(%)
100
80
60
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20
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Specificity(%)
100
80
60
40
20
0
Tissues
miR10b
miR21
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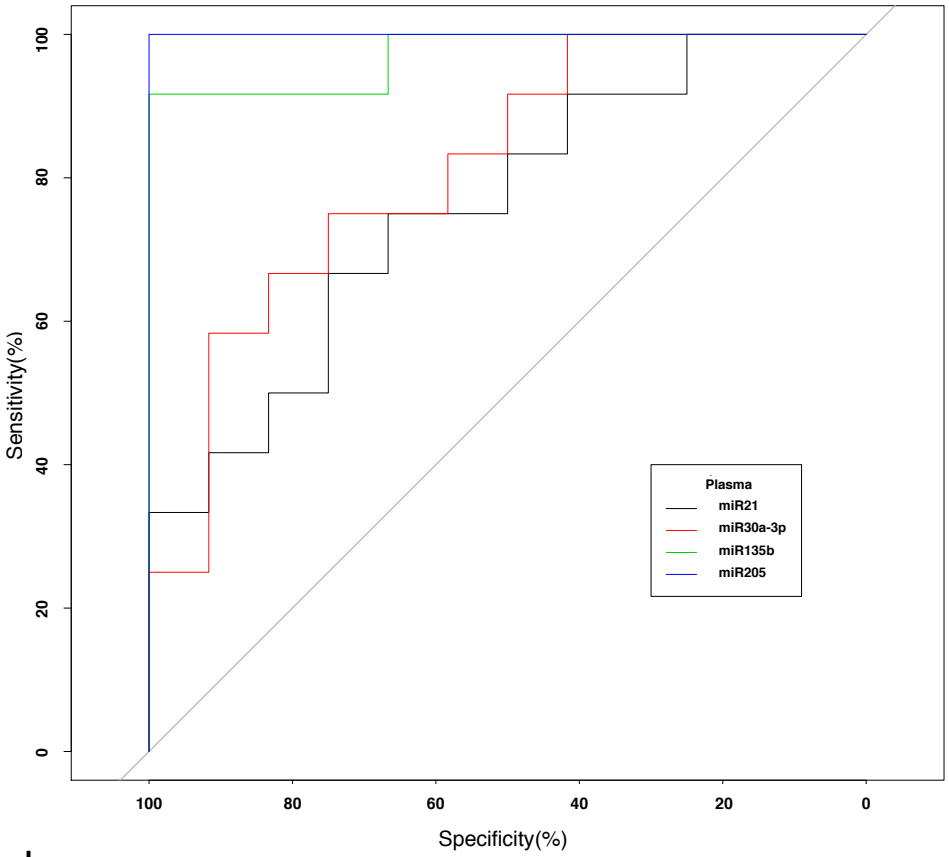
miR30a-3p
miR135b
miR195
miR205
miR499
miR135b/miR30a-3p
miR135b/miR195

a


Sensitivity(%)
100
80
60
40
20
0
Specificity(%)
100
80
60
40
20
0
Plasma
miR21
miR30a-3p
miR135b
miR205

b

Figure 1