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Utility of microRNA analysis using liquid-based cytology samples for uterine endometrial cancer screening

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ABSTRACT

Objectives: This study was performed to investigate microRNA (miRNA) expression in cytological specimens processed under clinical conditions to determine the potential of miRNA detection as a diagnostic biomarker of uterine endometrial cancer. **Methods:** We collected 40 lysates from cytology (LBC) specimens obtained during screening for endometrial cancer and normal endometrium, 18 surgically resected tissue specimens containing endometrial cancer and normal endometrium, and 14 LBC specimens of equivocal diagnosis at Hiroshima University Hospital. We compared the expression of four miRNAs (*miR-96*, *miR-182*, *miR-183*, and *miR-143*) and selected two (*miR-96* and *miR-143*) that we predicted would be useful. **Results:** We confirmed that *miR-96* expression was significantly elevated and *miR-143* expression was significantly depressed in cancer samples relative to normal endometrial samples. Among the 14 LBC specimens diagnosed with equivocal results, seven were pathologically diagnosed with cancer and showed the same profile as described above. **Conclusions:** Our data suggest that miRNA analysis of LBC samples may be useful for endometrial cancer screening.

Key words: *Uterine endometrial cancer*, *Screening*, *microRNA*, *LBC*

INTRODUCTION

Endometrial cytology is the standard examination method in screening for uterine endometrial cancer. Its respective sensitivity, specificity, and percentage of false-positive results have been reported to be 88.8% (approximately 90%), 98.5% (84% to 100%), and 25.2%[6,14,16,23,24)]. indicating that current diagnostic criteria and specimens are insufficiently standardized. Uterine endometrial cytology may not produce an adequate specimen or high quality smear. Consequently, some patients undergo more invasive testing.

MicroRNAs (miRNAs) are 18- to 23-nucleotide long noncoding RNAs that modulate the translation of specific messenger RNAs (mRNAs) and play important roles in tumorigenesis[4)]. Various studies have shown that miRNAs are candidate diagnostics for early cancer detection. Recently, miRNAs have attracted attention as disease-specific biomarkers that can be rapidly and stably amplified using the polymerase chain reaction (PCR). miRNAs are novel biomarkers for various neoplasms and are dysregulated in various tumor types[9)].

miR-96 is upregulated in various cancers and represses forkhead box protein O1 (FOXO1). FOXO1 is involved in promoting apoptosis, and FOXO1 suppression promotes tumor growth[7, 8, 13, 15, 19)]. *miR-182* and *miR-183* are members of the *miR-183* cluster belonging to the polycistronic miRNA cluster located in a 5-kb region at human chromosome 7q32.3. *miR-182* and *miR-183* have been reported to be upregulated in human malignancies[8,18)]. *miR-143* is downregulated in cancer and is involved in the overexpression of DNA methyltransferase 3B (DNMT3B). Reduced DNMT3B expression is a poor prognostic factor[12)]. Based on previous findings described in The Cancer Genome Atlas (TCGA) database, we focused on *miR-96*, *miR-182*, *miR-183*, and *miR-143* in this study.

To date, no studies of uterine endometrial cancer focused on the validity or utility of miRNA profiles extracted from liquid-based cytology (LBC) samples. We thus studied miRNA expression in cytological samples to investigate their usefulness in uterine endometrial cancer screening.

MATERIALS AND METHODS

All study participants provided written informed consent and the study design was approved by the Epidemiology Research Ethics Committee of Hiroshima University (#E2023-0166).

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Table 1 Clinical Characteristics of 80 Patients

	N (n = 40)	T (n = 40)	Total (n = 80)
Median age (range)	49.7 y (29–88)	50.8 y (30–84)	50.1 y (29–88)
Number of deliveries			
0	13 (32.5%)	6 (15.0%)	19 (23.75%)
1	11 (27.5%)	6 (15.0%)	17 (21.25%)
> 2	16 (40.0%)	28 (70.0%)	44 (55.0%)
Complications			
None	30 (75.0%)	19 (47.5%)	49 (61.25%)
Obesity (BMI ≥ 25)	6 (15.0%)	19 (47.5%)	25 (31.25%)
DM	1 (2.5%)	4 (10.0%)	5 (6.25%)
HT, other	6	16	22

N: Samples with negative cytological diagnoses that were later histologically diagnosed as normal uterine endometrium.
T: Samples with positive cytological diagnoses also histologically diagnosed as uterine endometrial cancer
n, number; y, years; BMI, body mass index; DM, diabetes mellitus; HT, hypertension

Table 2 Clinical Characteristics of 14 Patients

	N (n = 7)	T (n = 7)	Total (n = 14)
Median age (range)	47.7 y (31–70)	54.3 y (32–83)	51.1 y (31–83)
Number of deliveries			
0	4 (57.1%)	1 (14.3%)	5 (35.7%)
1	0	1 (14.3%)	1 (7.1%)
> 2	3 (42.9%)	5 (71.4%)	8 (57.1%)
Complications			
None	6 (85.7%)	3 (42.9%)	9 (64.3%)
Obesity (BMI ≥ 25)	0	3 (42.9%)	3 (21.4%)
DM	0	2 (28.6%)	2 (14.3%)
HT, other	1	3	4

N: Samples with equivocal results in the cytology test that were later histologically diagnosed as normal uterine endometrium.
T: Samples with equivocal cytology test results that were histologically diagnosed as uterine endometrial cancer.
n, number; y, years; BMI, body mass index; DM, diabetes mellitus; HT, hypertension

Samples

Cytology samples

At our hospital, smears are used for diagnosis in endometrial cytology, and the brush used to prepare a smear is subsequently used to prepare and store a liquid specimen. Collected cells were directly transferred into Cellprep Solution (Roche Diagnostics) and stored at −80°C until use.

We collected 40 cytological samples with positive cytological diagnoses that were later histologically diagnosed as uterine endometrial cancer, and 40 cytological samples with negative cytological diagnoses that were later histologically diagnosed as normal uterine endometrium. Only G1 or G2 endometrioid carcinomas were selected for histological analyses. Patient characteristics are shown in Table 1.

We also collected cytological samples with equivocal cytology test results. Among these, seven cases were histologically diagnosed as uterine endometrial cancer, while another seven were histologically diagnosed as normal uterine endometrium. These patients' characteristics are shown in Table 2.

Tissue samples

Eighteen surgically resected uterine endometrial cancer samples and 18 normal uterine tissue samples were collected. Cancer cases were histologically confirmed as G1 or G2 endometrioid carcinomas. Normal uterine tissue samples were obtained from patients with benign disease. Malignant and normal tissue samples were acquired during routine surgeries. All study participants provided written informed consent and all samples were stored as formalin-fixed paraffin-embedded tissues until further use.

RNA extraction and reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from tissues using a *mir*VANA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Total RNA was extracted from LBC samples using a Magcore Compact Automated Nucleic Acid Extractor (RBC Bioscience) and Magcore Total RNA Cultured Cells Kit (RBC Bioscience) according to the manufacturer's protocol. The RNA

concentration was measured using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific). Extracted RNA samples were stored at −80°C until use.

Total RNA was diluted to 2 ng/μL with nuclease-free water, and 10 ng of total RNA was reverse-transcribed into complementary DNA using a TaqMan miRNA reverse transcription kit and TaqMan miRNA assays (Thermo Fisher Scientific). According to the manufacturer's protocol, reverse transcriptase reactions were performed with a total volume of 15 μL per reaction, under the following conditions: 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min, then held at 4°C. The following primers were used: *RNU6B* (U6 small nuclear RNA; assay 001093), *miR-96* (assay ID: 000434), *miR-182* (assay ID: 000483), *miR-183* (assay ID: 002269), and *miR-143* (assay ID: 002146).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed using an Illumina Eco Real-Time PCR System. Real-time PCR was performed in a total volume of 20 μL per reaction. After the reverse transcriptase reaction, 1.33 μL of product was mixed with 10 μL of TaqMan Universal Master Mix II (Thermo Fisher Scientific), 1 μL of specific probes for TaqMan miR assays (Thermo Fisher Scientific), and nuclease-free water, then subjected to real-time PCR. *RNU6B* was used as an endogenous control to normalize expression levels. The relative expression levels of target miRNAs were calculated using the ΔCt method (Ct_{sample} – $Ct_{RNU6B_{sample}}$) and samples obtained from uterine endometrial cancer were compared with samples from normal tissues. All experiments were performed in triplicate.

Statistical analyses

Data were analyzed using JMP Pro version 16.1.0 (SAS Institute). The Mann–Whitney U test was used to analyze differences in miRNA expression levels between samples. Receiver operating characteristic (ROC) curves and areas under the curve (AUC) were used to determine the sensitivity and specificity of each miRNA. $P < 0.05$ was considered statistically significant.

RESULTS

We analyzed the expression of four miRNAs (*miR-96*, *miR-182*, *miR-183*, and *miR-143*) in 40 pairs of uterine endometrial cancer and non-cancerous cytological samples. As shown in Fig. 1, relative *miR-96* expression was significantly higher in cancer cells than in noncancerous cells. In contrast, relative *miR-143* expression was significantly lower in cancer cells than in noncancerous cells. The expression levels of *miR-182* and *miR-183* trended higher in cancer cells, but differences were insignificant. Based on these results, we selected *miR-96* and *miR-143* for subsequent analyses. We confirmed that similar results could be obtained using tissue specimens histologically diagnosed as uterine endometrial cancer or normal endometrium (Fig. 2).

We next examined *miR-96* and *miR-143* expression levels using tissues with equivocal diagnoses. The relative expression of *miR-96* was significantly higher in false-positive specimens, which were later histologically

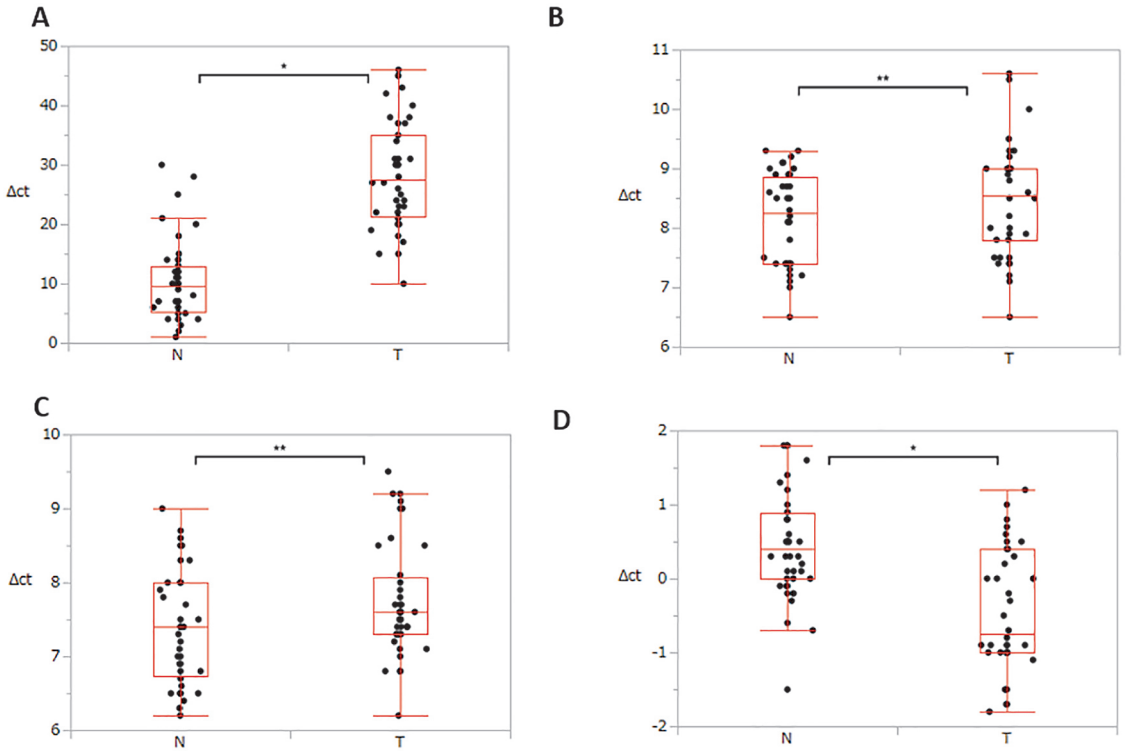

Figure 1 Relative *miR-96* (**A**), *miR-182* (**B**), *miR-183* (**C**), and *miR-143* (**D**) expression in endometrial cancer and normal endometrium. *MiR-96* was significantly upregulated and *miR-143* was significantly downregulated in cancer tissues. *$P < .05$; **$P > .05$

Ct, cycle threshold; N, non-cancerous cases; T, cancerous cases

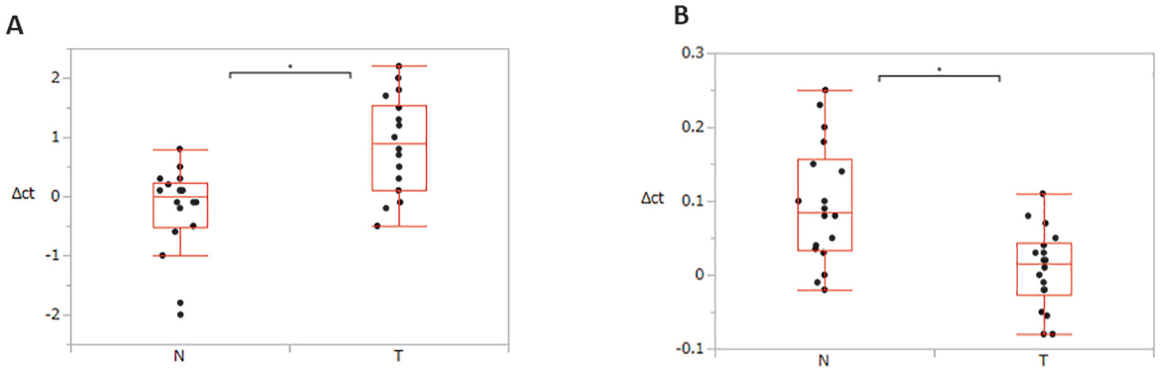

Figure 2 Relative expression of *miR-96* (**A**) and *miR-143* (**B**) in endometrial cancer and normal endometrium. *MiR-96* was significantly upregulated and *miR-143* was significantly downregulated in cancer tissues. *$P < .05$
Ct, cycle threshold; N, cases diagnosed as normal tissue; T, cases diagnosed as cancer.

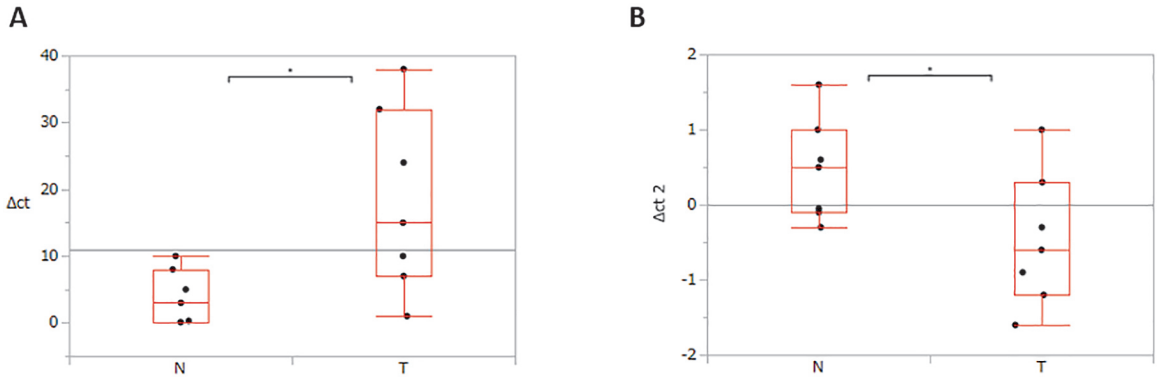

Figure 3 Relative expression of *miR-96* (**A**) and *miR-143* (**B**) in samples with equivocal results. *MiR-96* was significantly upregulated, and *miR-143* was significantly downregulated in endometrial cancer cases. *$P < .05$
Ct, cycle threshold; N, noncancerous cases; T, cancerous cases

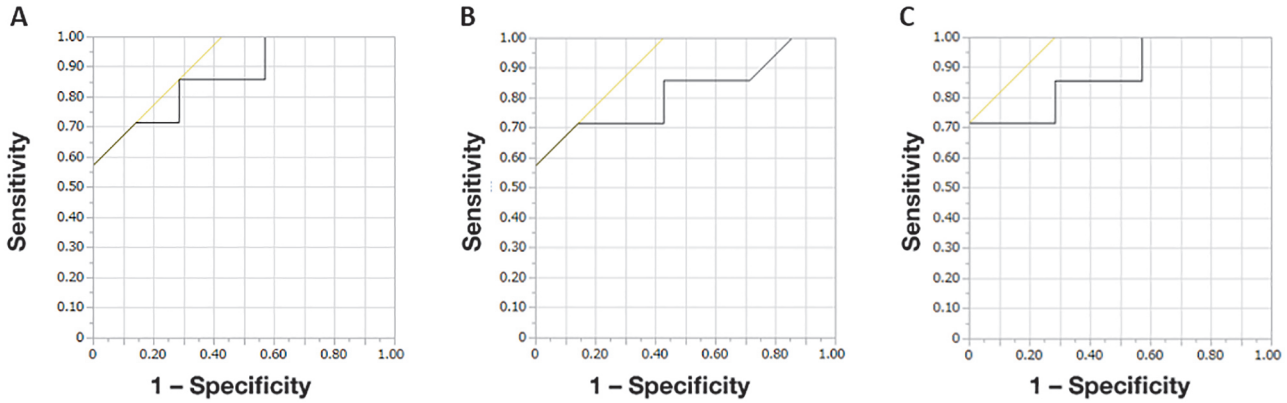

Figure 4 Received operating characteristic (ROC) curve analysis of diagnostic value in cytological samples with equivocal result. ROC curve with its corresponding area under the ROC curve (AUC) for *miR-96* (AUC = 0.867) (**A**) and *miR-143* (AUC = 0.816) (**B**) in liquid biopsy cytology (LBC) of cancer and noncancerous cells. ROC curve with corresponding AUC for two combined miRNA expression levels in LBC from cancer and noncancerous cells (AUC = 0.878) (**C**).

diagnosed as uterine endometrial cancer, than in noncancerous cells, and relative *miR-143* expression was significantly lower in cancer cells than in noncancerous cells (Fig. 3). ROC curve analysis was performed to evaluate the diagnostic value of miRNA expression in false positive specimens. The diagnostic values for *miR-96* and *miR-143* were 0.867 and 0.816, respectively. The combined diagnostic value for the expression of both miRNAs was 0.878 (Fig. 4).

DISCUSSION

This study confirmed that *miR-96* expression was significantly upregulated in uterine endometrial cancer cytological samples compared to non-cancerous cells. The expression of *miR-143* was significantly downregulated in cytological samples of uterine endometrial cancer compared to non-cancerous cells. We found that respective *miR-96* and *miR-143* expression levels were significantly elevated and depressed in specimens with

equivocal results than in non-cancerous cells.

We previously analyzed miRNA expression as an ancillary test in biliary cytology specimens obtained by endoscopic retrograde cholangiopancreatography. We found that *miR-182-5p* expression could discriminate carcinoma cells from non-cancerous cells initially judged to be indeterminate. The expression level of bile-derived *miR-182-5p* is a potential biomarker for discriminating clear cell adenocarcinoma from chronic cholangitis[21]. We also studied miRNA expression profiles using LBC samples in lung cancer and showed that four miRNAs (*miR-21*, *miR-31*, *miR-182*, and *miR-183*) were significantly upregulated in cancerous lung tissue compared to noncancerous lung tissue; furthermore, the expression of these four miRNAs was higher in cytological samples of lung cancer obtained by bronchial brushing than in the samples of normal lung cells[1].

To the best of our knowledge, no reports detail the evaluation of miRNAs using LBC samples in uterine endometrial cancer screening. Notably, *miR-129-2* and *miR-152* are involved in EC development in endometrial cancer via DNA methylation. Many miRNAs, including *miR-125b*, *miR-30c*, *miR-194*, and *miR-34b*, are expressed in endometrial cancer tissues and regulate proliferative, metastatic, and invasive activities[2,3,10,17,20]. miRNAs involved in carcinogenesis are targets for early detection and cancer prevention and may be highly specific diagnostic biomarkers. Other miRNAs, including *miR-125b*, *miR-30c*, *miR-200b/c*, and *miR-429* are associated with cisplatin resistance and may serve as prognostic biomarkers for endometrial cancer[3,5,11,22,25].

One limitation of our study is that the sample size was insufficient to precisely estimate the diagnostic value of the miRNAs. Therefore, conducting a large-scale study with more samples is necessary.

In summary, we demonstrated that *miR-96* and *miR-143* extracted from LBC samples showed different expression patterns in uterine endometrial cancer than in normal endometrium. We also showed that diagnostic accuracy could be increased by analyzing *miR-96* and *miR-143* expression levels in extracts from LBC samples with equivocal results. These results indicate that miRNA analysis is useful for screening for uterine endometrial cancer.

Disclosure

The authors declare that they have no conflict of interest.

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