miR-200a, miR-200b and miR-429 Are Onco-miRs that Target the *PTEN* Gene in Endometrioid Endometrial Carcinoma

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Abstract. *Endometrioid endometrial carcinoma (EEC) is a common malignancy of the female genital tract. However, no adequate biomarker is currently available for predicting the prognosis of this cancer. Recent studies have revealed dysregulated expression of several microRNAs (miRNAs) in various cancer tissues, and therefore, these cancer-associated miRNAs (also called onco-miRs) could be promising prognostic biomarkers of cancer progression or metastasis. In this study, in order to identify onco-miRs and their possible targets involved in EEC, we performed microarray-based integrative analyses of miRNA and mRNA expression in specimens excised from EEC lesions and adjacent normal endometrial tissues. Using integrated statistical analyses, we identified miR-200a, miR-200b and miR-429 as highly up-regulated onco-miRs in EECs. Conversely, we detected expression of a tumor-suppressor gene, phosphatase and tensin homolog (PTEN), which was predicted in silico using a miRNA-targeting mRNA prediction algorithm, as a target of the three miRNAs and which was down-regulated in EECs. Furthermore, these miRNAs were validated to target PTEN experimentally using luciferase assays and real-time polymerase chain reaction. These results suggest that the occurrence of EEC is, at least in part, mediated by miRNA-induced suppression of PTEN expression.*

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Key Words: Endometrioid endometrial cancer, microRNA, *PTEN*.

Endometrial carcinoma is a common malignancy of the female genital tract. Although the mortality associated with such disease is relatively low compared to other gynaecological cancers (overall 5-year survival is approximately 80% for all stages), certain histological types of this cancer are highly invasive and metastatic; thus, they are associated with poorer survival rates (1, 2). Generally, endometrial carcinoma is categorised into two subtypes: type I and type II. Type I, alternatively called endometrioid endometrial carcinoma (EEC), which accounts for approximately 80% of cases, occurs most frequently in pre- and perimenopausal women and is often accompanied by a history of unopposed oestrogen exposure or endometrial hyperplasia. EEC often exhibits minimal invasion into the uterine wall and thus carries a good prognosis (3). In contrast, type II endometrial carcinoma, which includes uterine papillary serous carcinoma and uterine clear cell carcinoma, occurs mostly in older, post-menopausal women. This type of carcinoma is independent of oestrogen exposure and carries a relatively poor prognosis (4, 5). When EEC is diagnosed at an early stage, surgery typically results in a good prognosis. However, patients with advanced stages of EEC exhibit more aggressive characteristics and have a less favourable prognosis (3) than those with type II endometrial carcinoma. Therefore, there is an urgent need for highly sensitive and specific molecular prognostic biomarkers to better-predict the outcome of EEC.

MicroRNAs (miRNAs) are small (approximately 22 nucleotides) non-coding RNAs involved in post-transcriptional regulation of gene expression. To date, numerous studies have been conducted to identify dysregulated miRNAs, also called 'onco-miRs', in many types of cancers, including endometrial carcinoma (6-9). In the present study, in order to identify onco-miRs and their possible target genes in EEC, we performed microarray-based integrated analyses of miRNA and mRNA expression in clinical specimens excised from patients with EEC.

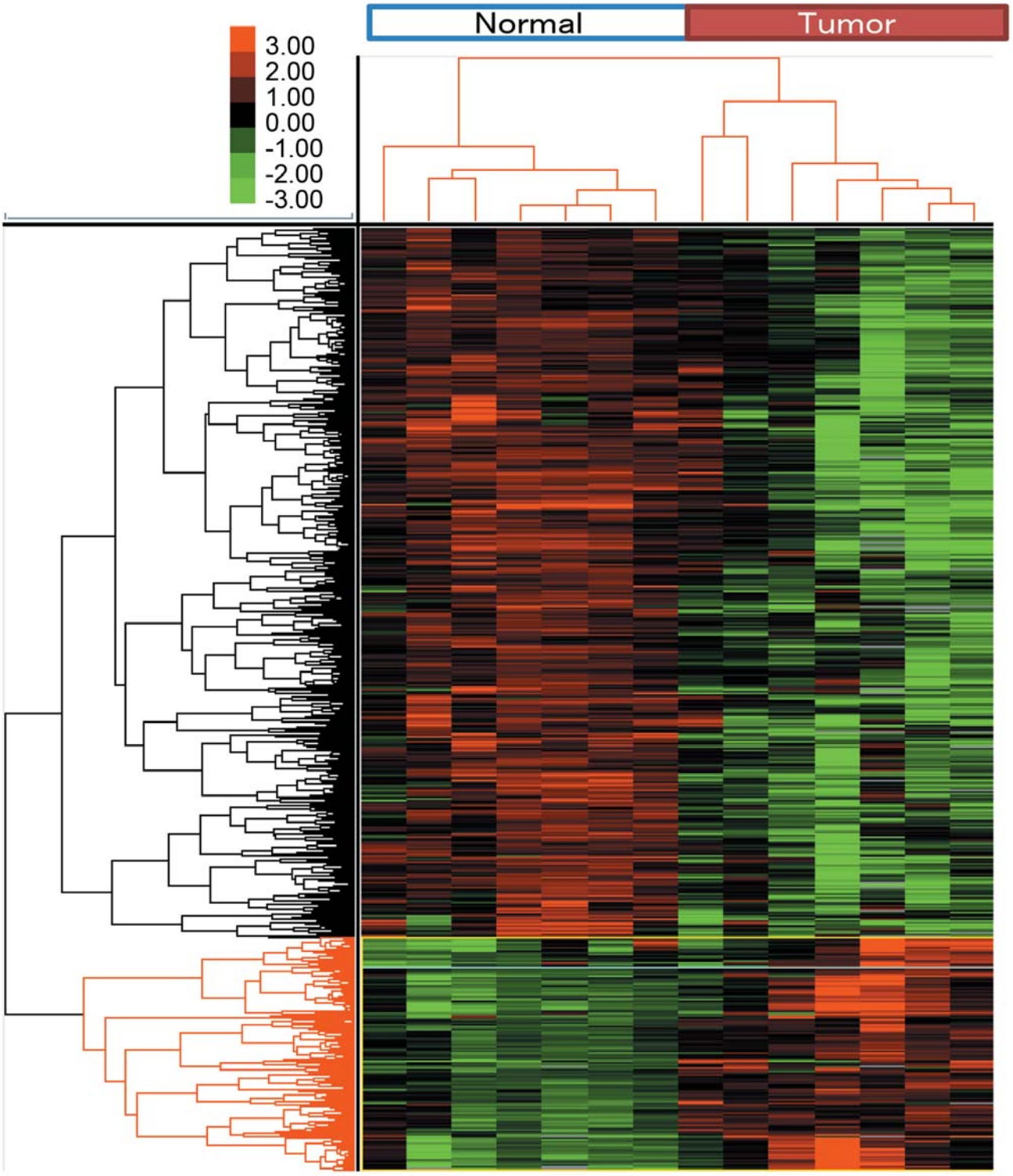

Figure 1. *Heatmap visualising dysregulated miRNA expression in endometrioid endometrial carcinoma. Data for 24 up-regulated and 127 down-regulated miRNAs are shown. Hierarchical clustering of the miRNAs based on expression patterns is also shown. Data were normalised using the global normalisation method.*

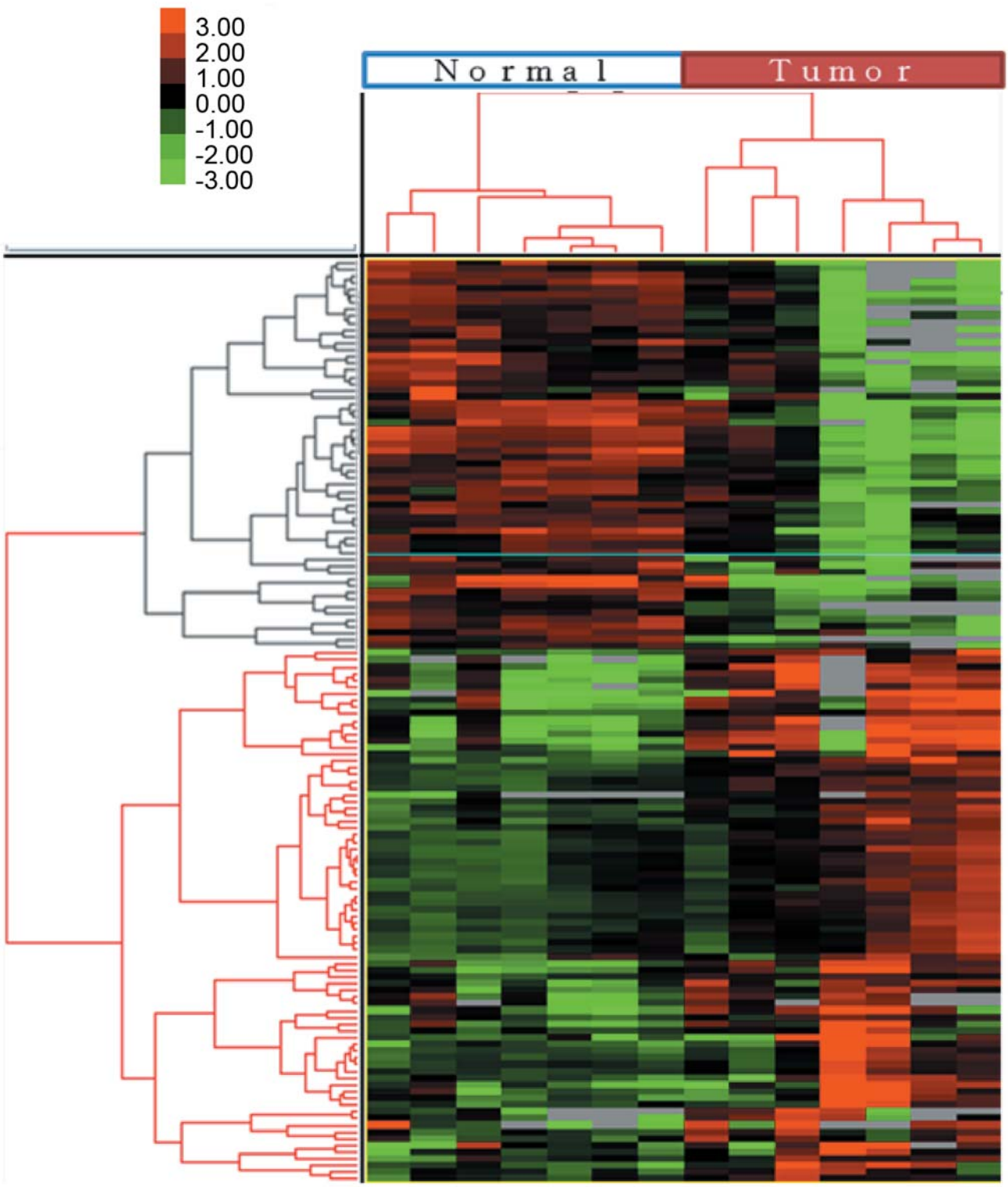

Figure 2. *Heatmap visualising dysregulated mRNA expression in endometrioid endometrial carcinoma. Data for 322 up-regulated and 981 down-regulated mRNAs are shown. Hierarchical clustering of the genes based on expression patterns is also shown. Data were normalised using the quantile normalisation method.*

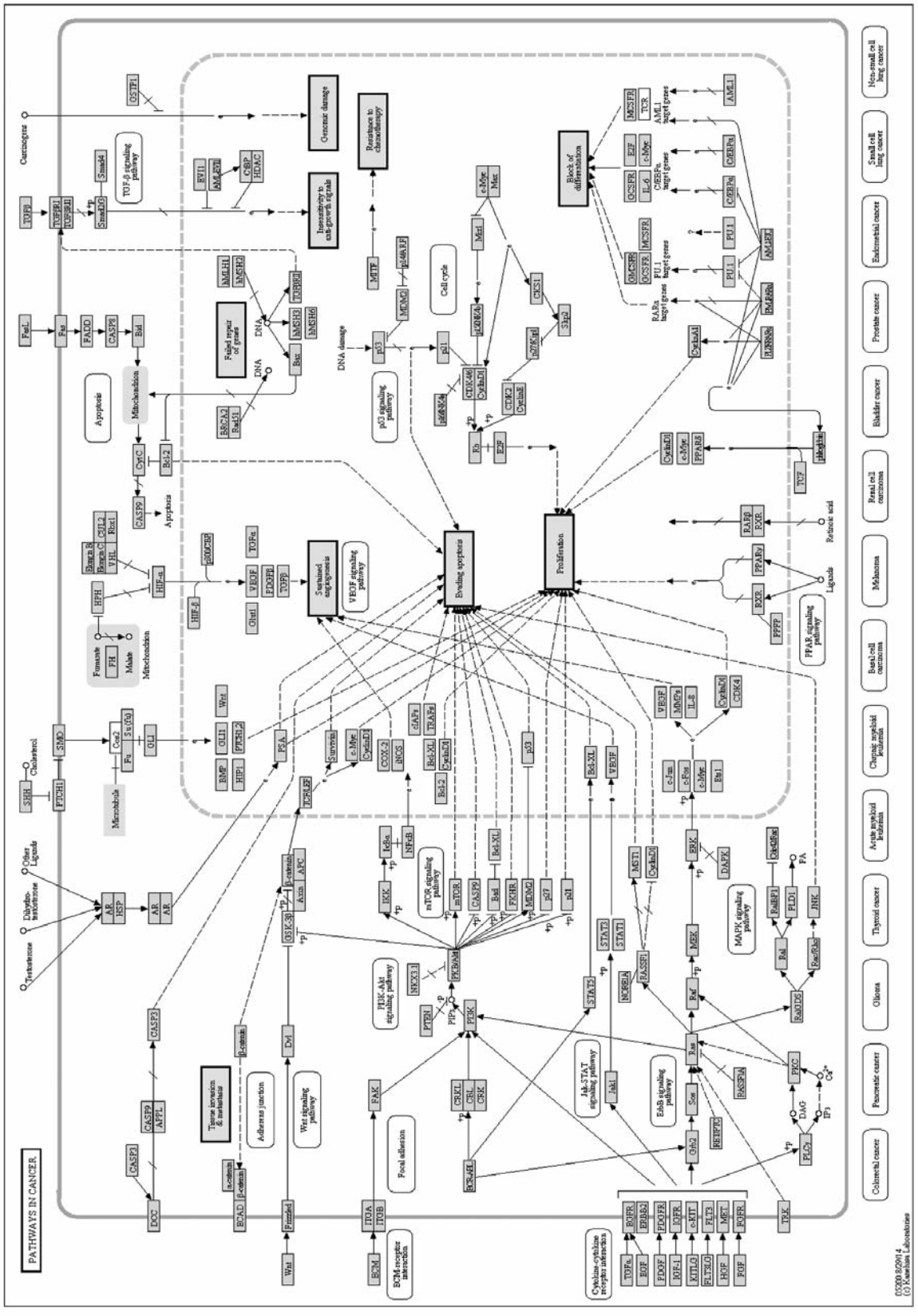

Figure 3. *KEGG Pathway–Pathways in Cancer.*

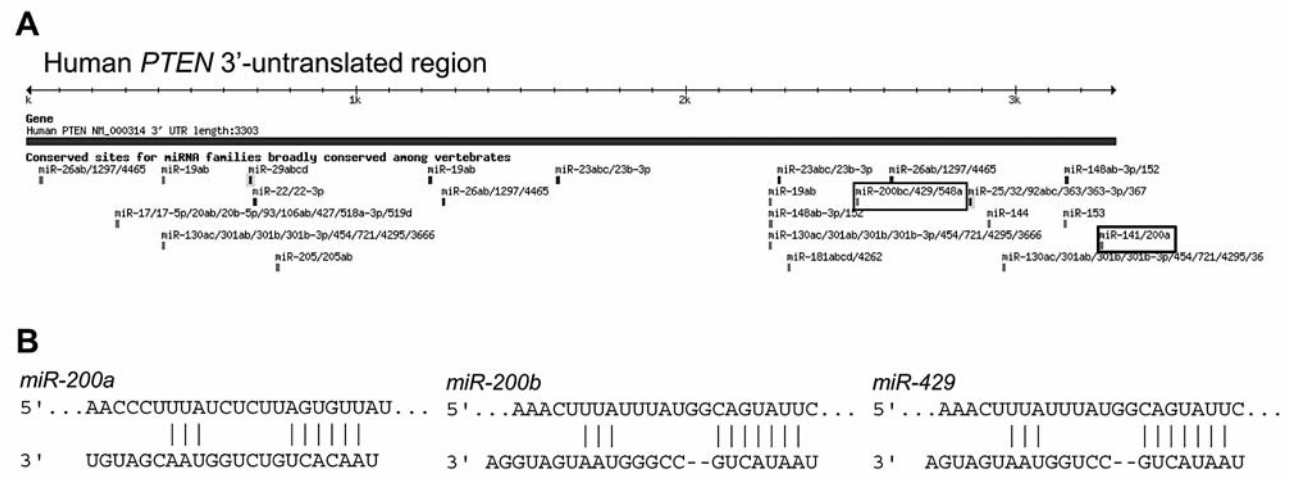

Figure 4. *Putative recognition of miRNAs in the 3'-untranslated region of phosphatase and tensin homolog (PTEN) mRNA. A: Putative human PTEN-targeting miRNAs and their recognition sites were predicted using TargetScan. miRNA families conserved only in mammals are shown. The putative recognition sites of miR-200a, -200b and -429 are boxed. B: Sequence-matching between miRNA and the human PTEN 3'-untranslated region at each site is shown.*

Materials and Methods

Ethics. This study was approved by the Nippon Medical School Ethics Committee, and informed consent was obtained from all participants.

Specimen. Tissue specimen used in this study were obtained surgically from patients with EEC (n=7) recruited from Nippon Medical School Main Hospital. The clinical characteristics of individual patients are listed in Table I. From tissues isolated from the patients, the carcinoma lesions were separated immediately from adjacent normal endometrium, and both groups of tissues were subjected to the RNA isolation procedure described below.

Cell culture. Two human EEC-derived endometrial epithelial cell lines, Ishikawa and HEC-1B, were used in this study. Ishikawa cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and maintained in Eagle's minimum essential medium (MEM) (Wako, Osaka, Japan) supplemented with amino acids (WAKO) and 10% foetal bovine serum (FBS). HEC-1B cells were obtained from the Health Science Research Resources Bank (Sen-nan, Japan) and maintained in MEM supplemented with 10% FBS. These cells were cultured at 37˚C in a humidified incubator with 5% CO_2.

Real-time reverse transcription polymerase chain reaction (qRT-PCR). Total cellular RNAs were isolated using ISOGEN reagent (Wako, Osaka, Japan) according to the manufacturer's protocol. First-strand cDNAs were synthesised from the RNAs using the PrimeScript RT Reagent Kit (Perfect Real Time; Takara-bio, Ohtsu, Japan) and were subjected to quantitative real-time PCR (qPCR) analysis using gene-specific primers (http://bya13131.wix.com/primers) and THUNDERBIRD SYBR qPCR Mix (Toyobo, Tokyo, Japan). Amplification of the PCR products was performed using an ABI7300 real-time PCR system (Life Technologies, Rockville, MD, USA). To normalize mRNA expression levels, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the endogenous internal control.

Table I. *Patients' characteristics*.

Case	Age, years	Stage (FIGO)	Histology	Grade
1	60	IA	Endometrioid	2
2	68	IB	Endometrioid	2
3	60	IB	Endometrioid	1
4	27	IA	Endometrioid	1
5	52	IIIA	Endometrioid	1
6	76	IB	Endometrioid	3
7	41	IIIC	Endometrioid	2

FIGO: The international federation of gynecology and obstetrics.

Array-based mRNA and miRNA expression analyses. To identify mRNAs and miRNAs with dysregulated expression in EECs lesion, we performed microarray analyses using the Whole Human Genome DNA Microarray and Human miRNA Microarray (Agilent Technologies, Santa Clara, CA, USA).

Based on the microarray data, we selected miRNAs that satisfied the following criteria as those dysregulated in EEC: i) an average fold change (FC) in signal intensity between EEC lesions and normal tissues (EEC/normal) >2.0 or <0.5; ii) $p<0.05$ and 3) $q<0.05$.

Data analysis. Microarray data were initially normalised among the samples according to the quantile normalisation method using GeneSpring GX software (Agilent Technologies). mRNAs and miRNAs with dysregulated expression in EEC lesions compared to normal endometrium tissues were identified by combining significance analysis of microarrays (SAM) and the FC methods with the aid of the samr package (http://cran.r-project.org/web/ packages/samr/index.html).

Hierarchical clustering for miRNAs and mRNAs was performed using Cluster 3.0 software (Eisen Lab., Berkeley, CA, USA) and visualized using TreeView software (http://taxonomy.zoology.

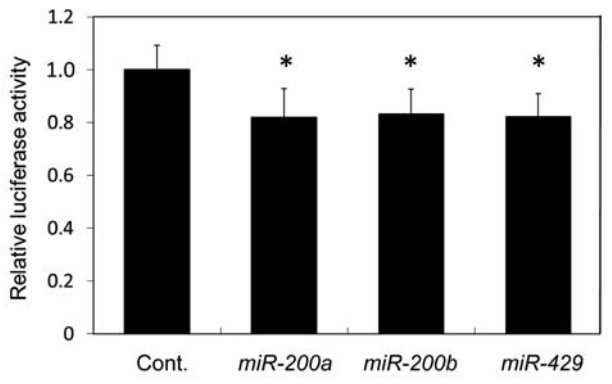

Figure 5. *Validation of miR-200a, miR-200b and miR-429 targeting phosphatase and tensin homolog (PTEN) using the luciferase assay. Human endometrioid endometrial carcinoma-derived Ishikawa cells were transfected with synthetic miRNA mimics or a negative control (Cont.) (20 nM) together with the pEZX-MT01- hPTEN3'UTR construct and pRL-tk. Luciferase activity in the cell lysates 48 h after transfection of the miRNA mimics was measured. Relative luciferase activities normalized to Renilla luciferase activities are shown. The average normalized relative luciferase activity value in the control cells was set at 1. Data are presented as the means±SD (n=3). Statistically significant differences were determined using Student's t-test. *p<0.05 compared with the control.*

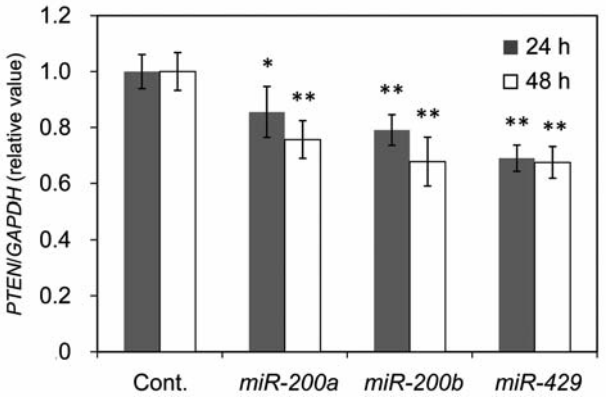

Figure 6. *Phosphatase and tensin homolog (PTEN) silencing by miR-200a, miR-200b and miR-429 at the mRNA level. Human endometrioid endometrial carcinoma-derived Ishikawa cells were transfected with synthetic miRNA mimics or negative control (Cont.) at a final concentration of 20 mM. The relative expression levels of PTEN mRNA 24 and 48 h after transfection are shown. The average normalised PTEN expression value in the control cells was set at 1 (n=3). Data are expressed as the means±SD. Statistically significant differences were determined using Student's t-test. *p<0.05, **p<0.01 compared with the controls.*

gla.ac.uk/rod/rod.html). mRNAs significantly dysregulated in the EEC lesions based on the above bioinformatical analyses were then subjected to gene enrichment analysis using DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/home.jsp).

To determine possible target genes of dysregulated miRNAs in EEC, combinations of miRNAs and mRNAs whose expression levels exhibited inverse correlations in specimens were identified using

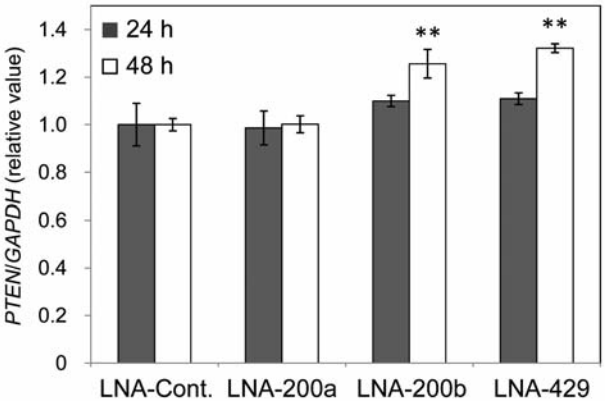

Figure 7. *Increased phosphatase and tensin homolog (PTEN) mRNA expression after inhibition of miR-200b and miR-429. Human EEC-derived HEC-1B cells were transfected with synthetic miRNA inhibitors or negative control (Cont.) at a final concentration of 20 mM. The relative expression levels of PTEN mRNA 24 and 48 h after transfection are shown. The average normalised PTEN expression value in the control cells was set at 1 (n=3). Data are expressed as the means±SD. Statistically significant differences were determined using Student's t-test. **p<0.01 compared to controls.*

correlation analysis. Combinations of miRNAs and mRNAs that satisfied the criteria of correlation coefficient less than −0.8 with $p<0.001$ based on no correlation test were selected as candidates.

miRNA in silico target prediction. Putative miRNA targets were predicted using TargetScan Human 6.2 (http://www.targetscan.org/).

Luciferase assay. For luciferase assays, Ishikawa cells were transfected with pEZX-MT01-hPTEN, a firefly luciferase reporter construct containing the 3'-UTR of *PTEN* (HmiT015535; Genecopoeia, Rockville, MD, USA) and with pRL-tk (a constitutive Renilla luciferase expression plasmid), together with 20 nM pre-miR reagents (synthetic miRNA mimics; Life Technologies) or 20 nM miRCURY LNA miRNA power inhibitors (synthetic miRNA inhibitors; Exiqon, Vedbaek, Denmark). After 24-72 h of transfection, the cells were processed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Luminescence was detected using a TD-20/20 Luminometer (Promega).

Statistics. The data are expressed as the means ± standard deviation (SD). Significant differences between groups were assessed using the Student's t-test.

Results

Microarray-based comparative analysis of miRNA expression in EEC and normal endometrium. We identified 73 and 58 miRNAs that were significantly up-regulated and down-regulated, respectively, in EEC lesions compared to normal tissues (Table II and III). A heatmap representing the dysregulation of these miRNAs in individual specimens is shown in Figure 1.

Table II. *miRNAs up-regulated in endometrioid endometrial carcinoma lesions*.

miRNA	Fold change (EEC/Normal)	*p*	*q*
hsa-miR-449a	16.38	0.0028	0.0000
hsa-miR-200a	10.60	0.0020	0.0000
hsa-miR-429	10.26	0.0020	0.0000
*hsa-miR-200a**	8.95	0.0023	0.0000
hsa-miR-200b	8.29	0.0021	0.0000
hsa-miR-183	8.09	0.0024	0.0000
hsa-miR-141	7.68	0.0033	0.0000
hsa-miR-1290	7.52	0.0022	0.0000
hsa-miR-96	7.29	0.0025	0.0000
hsa-miR-375	6.95	0.0056	0.0032
hsa-miR-203	6.57	0.0022	0.0000
hsa-miR-200c	6.31	0.0043	0.0000
hsa-miR-135b	6.01	0.0037	0.0000
*hsa-miR-141**	5.96	0.0024	0.0000
hsa-miR-494	5.92	0.0027	0.0000
hsa-miR-155	5.77	0.0020	0.0000
*hsa-miR-200b**	5.52	0.0027	0.0000
hsa-miR-363	5.36	0.0092	0.0032
hsa-miR-210	5.00	0.0025	0.0000
hsa-miR-18b	5.00	0.0047	0.0000
hsa-miR-1246	4.73	0.0060	0.0032
hsa-miR-548f	4.31	0.0044	0.0000
hsa-miR-7	4.26	0.0048	0.0000
hsa-miR-196a	4.24	0.0134	0.0097
hsa-miR-301b	3.91	0.0071	0.0032
hsa-miR-885-5p	3.83	0.0049	0.0032
*hsa-miR-135a**	3.65	0.0111	0.0032
hsa-miR-20b	3.45	0.0086	0.0032
hsa-miR-130b	3.35	0.0042	0.0000
hsa-miR-1826	3.35	0.0070	0.0032
hsa-miR-765	3.26	0.0148	0.0097
hsa-miR-182	3.17	0.0048	0.0000
hsa-miR-1181	3.13	0.0277	0.0122
hsa-miR-18a	3.03	0.0094	0.0032
*hsa-miR-629**	3.02	0.0324	0.0122
hsa-miR-17	3.00	0.0126	0.0097
hsa-miR-93	2.97	0.0058	0.0032
hsa-miR-548c-3p	2.93	0.0163	0.0097
hsa-miR-425	2.93	0.0071	0.0032
hsa-miR-663	2.84	0.0169	0.0097
hsa-miR-766	2.80	0.0038	0.0000
hsa-miR-1281	2.71	0.0047	0.0000
hsa-miR-602	2.66	0.0055	0.0032
hsa-miR-1825	2.55	0.0067	0.0032
*hsa-let-7b**	2.54	0.0078	0.0032
hsa-miR-1234	2.53	0.0062	0.0032
*hsa-let-7f-1**	2.52	0.0067	0.0032
hsa-miR-106b	2.49	0.0094	0.0032
hsa-miR-1228	2.48	0.0058	0.0032
hsa-miR-634	2.43	0.0109	0.0032
hsa-miR-142-3p	2.42	0.0189	0.0097
*hsa-miR-744**	2.41	0.0316	0.0122
hsa-miR-1249	2.38	0.0062	0.0032
hsa-miR-1227	2.37	0.0135	0.0097
hsa-miR-296-5p	2.37	0.0080	0.0032
hsa-miR-32	2.37	0.0266	0.0122
hsa-miR-1238	2.34	0.0091	0.0032
hsa-miR-1237	2.28	0.0108	0.0032
*hsa-miR-191**	2.27	0.0134	0.0097
hsa-miR-142-5p	2.25	0.0279	0.0122
hsa-miR-1539	2.24	0.0177	0.0097
hsa-miR-1225-3p	2.23	0.0136	0.0097
hsa-miR-484	2.22	0.0169	0.0097
hsa-miR-181b	2.22	0.0095	0.0032
hsa-miR-19b	2.20	0.0406	0.0166
*hsa-miR-33b**	2.19	0.0186	0.0097
hsa-miR-20a	2.19	0.0294	0.0122
hsa-miR-1280	2.17	0.0260	0.0122
hsa-miR-92b	2.14	0.0362	0.0166
*hsa-miR-129**	2.13	0.0294	0.0122
hsa-miR-15b	2.11	0.0356	0.0166
*hsa-miR-425**	2.09	0.0255	0.0122
hsa-miR-574-5p	2.05	0.0322	0.0122

Microarray-based comparative analysis of mRNA expression in EEC and normal endometrium. We also performed microarray analysis to assess mRNA expression and identified 322 and 979 mRNAs significantly up-regulated and down-regulated, respectively, in tumourous lesions compared to normal tissues. A heatmap representing the dysregulation of these mRNAs in individual specimens is shown in Figure 2.

We then performed bioinformatics-based analyses to link the dysregulated mRNAs (coding genes) with biological functions. Ontological analysis revealed that mRNAs dysregulated in EEC were closely related to cell cycle/mitosis function, as shown in the top 10 ontology hits (Table IV). Furthermore, using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, the pathway termed Cell Cycle was demonstrated as being closely related to the up-regulated mRNAs, which was supported by the extremely small *p*-value (8.41×10^{-6}) and false discovery rate (1.02×10^{-2}) from this analysis (Table V). These results are consistent with the generally accepted knowledge that the cell cycle or mitosis are aberrantly regulated in cancer, including EEC. Notably, 29 of the dysregulated genes are components of the KEGG pathway termed Pathways in Cancer (Figure 3).

Identification of miRNAs and cancer-related mRNAs reciprocally dysregulated in EEC. Recent studies have revealed that mammalian miRNAs suppress target expression predominantly at the mRNA level rather than the protein level (10, 11). Thus, the expression levels of onco-miRs, if any, and their target genes in EEC were hypothesised to be regulated reciprocally. Among the 29 dysregulated cancer-related genes

Table III. *miRNAs down-regulated in endometrioid endometrial carcinoma lesions.*

miRNA	Fold change (EEC/Normal)	*p*	*q*
hsa-miR-1	0.05	0.0020	0.0000
hsa-miR-381	0.08	0.0020	0.0000
hsa-miR-376a	0.09	0.0020	0.0000
*hsa-miR-136**	0.09	0.0020	0.0000
hsa-miR-133b	0.09	0.0020	0.0000
*hsa-miR-145**	0.10	0.0021	0.0000
hsa-miR-139-5p	0.10	0.0020	0.0000
hsa-miR-376c	0.10	0.0020	0.0000
hsa-miR-337-5p	0.11	0.0021	0.0000
hsa-miR-145	0.11	0.0022	0.0000
hsa-miR-204	0.12	0.0024	0.0000
hsa-miR-143	0.12	0.0023	0.0000
hsa-miR-379	0.13	0.0021	0.0000
*hsa-miR-143**	0.14	0.0025	0.0000
hsa-miR-377	0.15	0.0022	0.0000
*hsa-miR-125b-2**	0.16	0.0026	0.0000
hsa-miR-424	0.16	0.0020	0.0000
hsa-miR-127-3p	0.16	0.0021	0.0000
hsa-miR-152	0.16	0.0023	0.0000
hsa-miR-495	0.18	0.0020	0.0000
hsa-miR-542-5p	0.18	0.0025	0.0000
hsa-miR-99a	0.19	0.0022	0.0000
hsa-miR-100	0.19	0.0024	0.0000
hsa-miR-450a	0.19	0.0020	0.0000
hsa-miR-199b-5p	0.20	0.0023	0.0000
*hsa-miR-126**	0.20	0.0027	0.0000
hsa-miR-140-3p	0.20	0.0027	0.0000
hsa-miR-452	0.21	0.0045	0.0000
hsa-miR-154	0.22	0.0023	0.0000
hsa-miR-136	0.22	0.0028	0.0000
hsa-miR-542-3p	0.23	0.0026	0.0000
*hsa-miR-144**	0.23	0.0051	0.0000
hsa-miR-299-3p	0.24	0.0027	0.0000
hsa-miR-487b	0.25	0.0026	0.0000
hsa-miR-654-3p	0.25	0.0031	0.0000
hsa-miR-140-5p	0.25	0.0039	0.0000
hsa-miR-101	0.26	0.0042	0.0000
hsa-miR-410	0.26	0.0036	0.0000
hsa-miR-369-5p	0.26	0.0047	0.0000
hsa-miR-455-5p	0.26	0.0037	0.0000
hsa-miR-299-5p	0.28	0.0031	0.0000
hsa-miR-125b	0.29	0.0031	0.0000
hsa-miR-497	0.30	0.0102	0.0237
*hsa-miR-24-1**	0.30	0.0126	0.0282
hsa-miR-195	0.30	0.0093	0.0206
hsa-miR-29c	0.31	0.0076	0.0122
*hsa-miR-221**	0.31	0.0125	0.0282
hsa-miR-503	0.32	0.0064	0.0097
hsa-miR-126	0.32	0.0063	0.0097
hsa-miR-23b	0.33	0.0167	0.0358
hsa-miR-144	0.36	0.0171	0.0358
*hsa-miR-154**	0.36	0.0072	0.0115
*hsa-miR-29c**	0.37	0.0136	0.0322
hsa-miR-411	0.39	0.0043	0.0000
hsa-miR-382	0.39	0.0073	0.0115
hsa-miR-627	0.40	0.0165	0.0358
hsa-miR-543	0.42	0.0124	0.0282
hsa-miR-1271	0.43	0.0157	0.0358

Table IV. *Gene ontologies (GO) related to the genes dysregulated in endometrioid endometrial carcinoma.*

GO ID	GO Term	Count	*p*-Value	FDR
0007067	Mitosis	46	6.26E-12	1.13E-08
0000280	Nuclear division	46	6.26E-12	1.13E-08
0007049	Cell cycle	103	7.86E-12	1.42E-08
0000087	M Phase of mitotic cell cycle	46	1.20E-11	2.17E-08
0048285	Organelle fission	46	2.63E-11	4.76E-08
0000279	M Phase	57	3.99E-11	7.23E-08
0022403	Cell cycle phase	63	8.23E-10	1.49E-06
0000278	Mitotic cell cycle	57	3.76E-09	6.81E-06
0022402	Cell cycle process	75	8.38E-09	1.52E-05
0051301	Cell division	48	1.36E-08	2.46E-05
0065004	Protein–DNA complex assembly	23	7.26E-08	1.31E-04
0006334	Nucleosome assembly	22	7.70E-08	1.40E-04
0034728	Nucleosome organization	23	1.10E-07	1.99E-04
0006323	DNA packaging	26	1.30E-07	2.35E-04
0031497	Chromatin assembly	22	1.48E-07	2.67E-04
0006333	Chromatin assembly or disassembly	26	6.75E-07	0.001223
0007517	Muscle organ development	34	2.98E-06	0.005399
0007059	Chromosome segregation	18	1.69E-05	0.030524
0007051	Spindle organization	13	2.37E-05	0.042943
0051726	Regulation of cell cycle	43	3.24E-05	0.058611
0008283	Cell proliferation	52	4.53E-05	0.081931
0000226	Microtubule cytoskeleton organization	24	9.11E-05	0.164794
0007017	Microtubule-based process	34	1.31E-04	0.236576
0007346	Regulation of mitotic cell cycle	23	4.00E-04	0.722341
0043627	Response to estrogen stimulus	18	4.84E-04	0.872661

FDR: False discovery rate.

described above, we searched for genes satisfying the following criteria: i) expression inversely correlated with one or more miRNA(s) dysregulated in EEC, and ii) *in silico* targets of one or more miRNA(s) dysregulated in EEC. Bioinformatical analyses revealed that *PTEN* and *phosphoinositide-3-kinase, regulatory subunit 1 (PIK3R1)* fulfilled these criteria. *PTEN* is down-regulated in EEC and putatively targeted by *miR-200a, miR-200b* and *miR-429*, which are all up-regulated in EEC. The putative recognition sites of these miRNAs in the *PTEN* 3'-untranslated region (3'-UTR) and their sequences are shown in Figure 4.

PIK3R1 is also down-regulated in EEC and putatively targeted by *miR-15b*. In the present study we focused on *PTEN* because it has been repeatedly demonstrated (12-15) to be a significant tumor-suppressor gene in EEC, and *miR-200a, miR-200b* and *miR-429* were all found to be highly up-regulated in EEC (10.60-, 10.26- and 8.29-fold, respectively; Table II).

PTEN is a possible target of miR-200a, miR-200b, and miR-429 in EEC cells. To examine whether *PTEN* is a direct

Table V. *Pathways related to the genes dysregulated in endometrioid endometrial carcinoma.*

KEGG ID	Pathway	Count	*p*-Value	FDR
hsa04110	Cell cycle	23	8.41E-06	0.010167
hsa04710	Circadian rhythm	6	8.20E-04	0.986952
hsa04914	Progesterone-mediated oocyte	13	0.00725	8.420136
hsa04114	Oocyte meiosis	15	0.008793	10.12583
hsa04115	p53 signaling pathway	11	0.009581	10.98549
hsa05218	Melanoma	11	0.012874	14.49826
hsa05322	Systemic lupus erythematosus	13	0.021074	22.69858
hsa04510	Focal adhesion	21	0.027448	28.56793
hsa05200	Pathways in cancer	29	0.059877	52.59053
hsa03030	DNA replication	6	0.073364	60.18883
hsa00670	One carbon pool by folate	4	0.075697	61.38362
hsa05212	Pancreatic cancer	9	0.082098	64.495
hsa04270	Vascular smooth muscle contraction	12	0.094189	69.75306
hsa04210	Apoptosis	10	0.096177	70.54571

KEGG: Kyoto encyclopedia of genes and genomes; FDR: false discovery rate.

target of *miR-200a*, *miR-200b*, or *miR-429*, we performed luciferase assays using a construct harbouring a sequence corresponding to the 3'-UTR of human *PTEN* mRNA downstream of the luciferase gene. As shown in Figure 5, overexpression of these miRNAs weakly but significantly reduced the reporter plasmid-derived luciferase activity in Ishikawa cells. We next examined the effects of *miR-200a, miR-200b* and *miR-429* overexpression in Ishikawa cells on *PTEN* mRNA expression. As shown in Figure 6, *PTEN* mRNA expression was down-regulated significantly by these miRNAs at 24 and 48 h following transfection of the miRNA mimics.

Next, we examined the effect of functional inhibition of endogenous *miR-200a, miR-200b* and *miR-429* in EEC-derived cells. Since the endogenous expression levels of these miRNAs are low in Ishikawa cells (data not shown), we used HEC-1B, another human EEC-derived cell line, for this experiment. As shown in Figure 7, transfection of HEC-1B cells with LNA-based *miR-200b* and *miR-429* inhibitors led to a significant increase in *PTEN* mRNA expression, suggesting that these two miRNAs are expressed endogenously in the cells and target *PTEN* mRNA. However, in this experiment, *miR-200a* failed to up-regulate *PTEN* mRNA expression, which may be due to the relatively weak expression of *miR-200a* compared to others in this cell line.

Discussion

In this study, we identified miRNAs and mRNAs significantly dysregulated in EEC based on array-based comprehensive analyses and demonstrated that *miR-200a*, *miR-200b* and *miR-429* are onco-miRs that possibly target *PTEN* in EEC.

Interestingly, the dysregulated miRNAs belong to the *miR-200* family, *i.e. miRs-141, -200a, -200b, -200c* and *-429* were all highly up-regulated in EEC (Table I), which has been demonstrated in previous studies comparing miRNA expression profiles between EEC and normal endometrial tissues (16-18). However, reports describing the target mRNAs of these miRNAs in EEC are limited. To our knowledge, only Park *et al*. have reported an experimentally validated target of the *miR-200* family in EEC: *bromodomain containing 7 (BRD7)* as a target of *miR-200c (19)*.

Based on our *in silico* analysis, using two different algorithms we predicted *PTEN* as a possible target of *miR-200a*, *miR-200b* and *miR-429*, consistent with the reciprocal expression of these miRNAs and the *PTEN* transcript in EEC and normal endometrial tissues. Genetic studies have revealed mutations in *PTEN*, *v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-RAS)*, β-catenin, *p53*, *human epidermal growth factor receptor 2 (HER-2/neu), p16* and E-cadherin genes in EEC (12-15). In particular, the incidence of *PTEN* mutations has been reported to be the highest among all genetic alterations in EEC, existing in 34-55% of cases (12-15). However, considering that these mutations are not present in all patients with EEC universally, transcriptional/posttranscriptional alterations in *PTEN* expression, including epigenetic alteration, are likely involved. Therefore, our results regarding miRNA-mediated post-transcriptional regulation of *PTEN* in EEC facilitate a better understanding of EEC biology, which can lead to the discovery of novel molecular targets for this disease. Herein, only an *in vitro* study using the representative EEC-derived cell lines was conducted; therefore, additional studies are necessary to demonstrate the miRNA-mediated regulation of *PTEN in vivo*.

Competing Interests

The Authors have declared that no competing interests exist in regard to this study.

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