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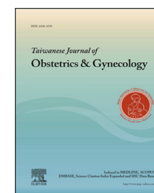

Original Article

Quantitative DNA methylation analysis of selected genes in endometrial carcinogenesis

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A R T I C L E I N F O

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A B S T R A C T

Objective: Most endometrial carcinomas appear to develop from precursors (e.g., endometrial hyperplasia) that progress for several years. Patients who are ultimately diagnosed with carcinoma often present clinically with complaints of abnormal vaginal bleeding years before diagnosis, which offers an opportunity for early diagnosis and curative treatment. The analysis of DNA methylation may be used as a method for detecting endometrial cancer (EC). To test the potential clinical application of this method, we used quantitative methylation analysis of five genes in a full spectrum of endometrial lesions.
Materials and methods: This hospital-based, prospective, case-controlled study was conducted on 68 patients, which included patients who had a normal endometrium ($n = 18$), hyperplasia of the endometrium ($n = 24$), and EC ($n = 26$). Methylation levels of the following genes were determined by using real-time methylation-specific polymerase chain reaction (PCR) amplification: zinc finger protein 177 (*ZNF177*), collagen type XIV α1 (*COL14A1*), dihydropyrimidinase-like 4 (*DPYSL4*), homeobox A9 (*HOXA9*), transmembrane protein with epidermal growth factor-like and two follistatin-like domains 2 (*TMEFF2*). The methylation index (MI) cutoff values for the different diagnoses were determined to test the sensitivity and specificity of the method and to generate the receiver operating characteristic (ROC) curves. The Mann–Whitney *U* test was used to test between-group differences in the MI.
Results: The MI of the five genes was significantly higher in EC than the MIs in specimens of hyperplasia of endometrium and normal appearance ($p < 0.001$). The ROC analysis demonstrated that the sensitivity, specificity, and accuracy for detecting EC were 92.3%, 94.4%, and 95.1%, respectively, for *ZNF177*; 92.3%, 94.4%, and 95.7%, respectively, for *COL14A1*; 80.8%, 94.4%, and 81.4%, respectively, for *HOXA9*; 65.4%, 94.4%, and 89.5%, respectively, for *TMEFF2*; and 61.5%, 94.4%, and 63.3%, respectively, for *DPYSL4*. The combined testing of *ZNF177* and *COL14A1* had the best specificity (100%), but compromised sensitivity (88.5%).
Conclusion: Promoter methylation of *ZNF177*, *COL14A1*, *HOXA9*, *DPYSL4*, and *TMEFF2* genes is a frequent epigenetic event in EC. Furthermore, the epigenetic hypermethylation of *TMEFF2* may be a valuable marker for identifying undetected EC within endometrial hyperplasia.
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Introduction

Endometrial cancer (EC) is a very common gynecological malignancy, and its frequency is increasing in the developed world; it is the fourth most common cancer in women in Europe. However, its mortality rate is the lowest among all gynecological malignancies (20–24/100,000 women), which indicates a good prognostic outcome when it is detected early [1,2]. Endometrial cancer is conventionally classified into two types. Type I is associated with an endocrine milieu of estrogen predominance and develops from endometrial hyperplasia. The tumor is positive for the estrogen receptor and progesterone receptor, shows well-differentiated endometrioid adenocarcinoma, has a lower frequency of lymph node metastasis, shows little muscular invasion, and often has a relatively more favorable prognosis than Type 2 cancer [3–5]. By contrast, Type 2 EC tends to develop in postmenopausal women in an estrogen-independent manner, and may result from *de novo* carcinogenesis that develops directly from the normal endometrium, rather than via endometrial hyperplasia or undiagnosed precancerous lesions. Type 2 cancer is the aggressive histological subtype of EC and represents less than 10% of all ECs, but accounts for more than 50% of recurrences and deaths attributable to EC [6]. The transition from a normal endometrium to a malignant tumor may involve a stepwise accumulation of alterations in cellular mechanisms leading to dysfunctional cell growth [7].

Like many cancers, EC is a complex disease affected by genetic, epigenetic, and environmental factors. One type of epigenetic modification is DNA methylation. Aberrant hypermethylation of cytosine-phosphate-guanine (CpG) islands present in the promoters of genes is associated with gene silencing. Tumorigenesis may be initiated by the epigenetic silencing of genes belonging to different classes of activity such as cell-cycle control, detoxification, differentiation, DNA repair, signal transduction, and apoptosis [8,9]. An overall reduction in the total 5-methylcytosine level and focal hypermethylation in CpG islands near the tumor-suppressor gene transcriptional start sites occur in many different types of cancers, including EC [10].

Most ECs appear to develop from precursors, termed "endometrial hyperplasia," which progress for several years. Patients who are ultimately diagnosed with EC often present clinically with complaints of abnormal vaginal bleeding years before diagnosis, which offers an opportunity for early diagnosis and curative treatment [11]. However, many such patients undergo repeated evaluations, inconvenience, and stress because abnormal vaginal bleeding is an extremely common complaint that is unrelated to EC in a substantial number of women [12]. Thus, it may be challenging to distinguish the minority of women who require management for carcinoma precursors or carcinoma from women with benign causes of abnormal bleeding. It is important to discover the hidden lesions of carcinoma precursors in the endometrium.

In the current study, we used the Illumina Methylation 450K array (Illumina, San Diego, CA, USA) to analyze the global methylation profiling of EC and normal endometrial tissue in patients with cancer versus endometrial tissue from healthy individuals. After analysis of the methylation differences in combination with independent gene expression data, a set of genes that are deregulated by aberrant DNA methylation in EC was identified. We then focused on five genes with aberrant DNA methylation, which included the genes for zinc finger protein 177 (*ZNF177*), collagen type XIV α1 (*COL14A1*), dihydropyrimidinase-like 4 (*DPYSL4*), homeobox A9 (*HOXA9*), and transmembrane protein with epidermal growth factor-like and two follistatin-like domains 2 (*TMEFF2*). We used a quantitative methylation polymerase chain reaction (qMSP) with validation analysis using additional EC tissues, tissues with hyperplasia of the endometrium, and tissues from normal controls.

Materials and methods

Tissue collection

This was a prospective study that initially enrolled 68 participants who were referred to the Department of Obstetrics and Gynecology at the Tri-Service General Hospital of the National Defense Medical Center (Taipei, Taiwan). The study included women who had endometrioid-type EC ($n = 26$; 6 G1 cases, 9 G2 cases, 11 G3 cases). Endometrial biopsy of tissues of patients with hyperplasia ($n = 24$) and with a normal endometrium ($n = 18$) were collected for methylation analysis. All patients underwent a hysterectomy within 3 months of endometrial biopsy. The final diagnosis was based on the worst pathologic finding from the endometrial sampling or hysterectomy. Exclusion criteria were women who had a history of previous endometrial surgery, history of a previous gynecological malignancy, synchronous malignancy, and patients with an endometrial malignancy other than endometrioid adenocarcinoma (e.g., serous or clear cell type). The endometrial biopsies underwent immediate processing for DNA extraction or were fresh frozen and stored at −80°C.

Ethics approval

The study was approved by the ethics committee of the Institutional Review Board of the Tri-Service General Hospital (Taipei, Taiwan). Informed consent was obtained from all patients and control participants.

Genomic DNA extraction

Genomic DNA was extracted from tissue samples using the commercial DNA extraction kit QIAmp Tissue Kit (Qiagen, Hilden, Germany). Tumor DNA was prepared as described previously [8].

Bisulfite conversion

The DNA from the tumor specimens was subjected to bisulfite methylation analysis. We treated DNA with bisulfite using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA), as described previously [13]. In brief, 1 μg of genomic DNA was denatured by incubation with 0.2M sodium hydroxide (NaOH). Aliquots of 10mM hydroquinone and 3M sodium bisulfite (pH 5.0) were added, and the solution was incubated at 50°C for 16 hours. The treated DNA was purified on a Zymo-Spin I column (Zymo Research), desulfonated with 0.3M NaOH, repurified on a Zymo-Spin I column, and resuspended in 20 μL elution buffer. After the bisulfite treatment, all DNA samples were stored at −80°C [13].

Methylation-specific polymerase chain reaction

Methylation-specific polymerase chain reaction (MS-PCR) was conducted in a total volume of 20 μL, which contained 0.8 μL of 2.5μM primers, 1 μL of the sodium bisulfite-treated DNA, and 10 μL of 2× RBC SensiZyme HotStart Taq Mastermix (RBC Bioscience Corp, Taipei, Taiwan). The primers for MS-PCR were designed to detect methylated sequences. Their options were the most G-C-rich regions closest to the transcription start site. Methylation of these regions of CpG islands is associated with transcriptional silencing. Table 1 summarizes the characteristics of the MS-PCR reactions and the primer sequence for each gene. The PCR products were analyzed by electrophoresis on 2.5% agarose gels and visualized after staining with ethidium bromide. All MS-PCR data were obtained from at least three independent modifications of DNA to ensure reproducibility [8,13].

Quantitative methylation polymerase chain reaction

SYBR Green-based quantitative methylation polymerase chain reaction (qMSP; SuperArray Bioscience, Frederick, USA) was performed after the bisulfite treatment on the denatured genomic DNA. The primers and probes for each gene were *ZNF177*, *COL14A1*, *HOXA9*, and *TMEFF2* (the sequences will be provided on request). The gene for the collagen type II a1 gene (*COL2A*) was used as the internal reference gene by amplifying non-CpG sequences. Each sample was analyzed in duplicate.

To generate a positive control and a negative control for MSP/qMSP, the CpG methylated human genomic DNA (Thermo Fisher Scientific, Waltham, USA) and DNA from normal peripheral blood lymphocytes were modified by sodium bisulfite. The SYBR Green-based qMSP method was used to determine the methylation level of each gene in the tissue samples. We used *COL2A* as an internal reference gene by amplifying the non-CpG sequences. Quantitative methylation polymerase chain reaction was conducted in a total volume of 20 μL that contained 1 μL of 10μM primers, 2 μL of bisulfate-treated DNA, and 10 μL of RT^2 SYBR green qPCR Mastermix (Qiagen, Hilden, Germany). The reactions were detected using a LightCycler 480 Real-Time PCR system (Roche, Basel, Switzerland). Each sample was analyzed in triplicate. Test results with a quantification cycle (Cq) value of *COL2A* >38 were defined as a detection failure. After PCR analysis, results with a Cq value of the target gene >45 were defined as undetermined. The DNA methylation level was assessed as the methylation index (MI) by using the formula: $100 \times 2^{[(\text{Cq of } COL2A) - (\text{Cq of gene})]}$ [14].

Statistical analysis

The age and body mass index (BMI) differences between the controls and the patients were compared using independent sample Student *t* tests. The differences in DNA methylation between the three groups were analyzed by the Chi-square test or by the Fisher's exact test. Mann–Whitney *U* tests were used to test differences in MI between groups. Receiver operating characteristic (ROC) curves were generated to confirm the accuracy of diagnosis for each gene, and the sensitivity and specificity were computed for each combination. Each group was required to achieve an adequate power (0.90) with a level <0.05 to test for a 0.35 difference in the area under the ROC curve from 0.5 (i.e., a straight line from bottom left to top right corners, which implies a decision rule that is no better than pure chance). All differences were considered statistically significant at $p < 0.05$. All analyses were performed using IBM SPSS Statistics software, version 22 (IBM Corp, Armonk, USA).

Table 2
The patients' characteristics.

Histopathology	Age (y), mean ± SD	BMI (mean ± SD)
Normal endometrium	45.5 ± 3.4	24.3 ± 3.2
Hyperplasia of endometrium	50.1 ± 3.9[a]	23.4 ± 3.0
Endometrial cancer	61.5 ± 8.3[b]	25.9 ± 6.4

BMI = body mass index; SD = standard deviation.
[a] The difference between hyperplasia of the endometrium and a normal endometrium, as determined by the independent sample Student *t* test ($p = 0.0003$).
[b] The difference between endometrial cancer and hyperplasia of the endometrium, as determined by the independent sample Student *t* test ($p < 0.0001$).

Results

Table 2 shows the characteristics of the enrolled patients. The mean patient age increased in accordance with disease severity ($p < 0.001$), which was expected. Quantitative assessment of DNA methylation for each gene, based on disease severity, is shown in Figure 1 and Table 3. The median MI was low in tissue samples from the normal endometrium for all genes (5.6%). The median MI was increased in tissue samples with endometrial hyperplasia for all genes (16.7% for *ZNF177*, 33.3% for *COL14A1*, 20.8% for *HOXA9*, 50% for *TMEFF2*, and 8.3% for *DPYSL4*). There are significant differences of the median MI in *TMEFF2* between the normal endometrium and endometrial hyperplasia. In patients with EC, the methylation rates were 92.3% for *ZNF177*, 92.3% for *COL12A1*, 80.8% for *HOXA9*, 65.4% for *TMEFF2*, and 61.5% for *DPYSL4*. These values differed significantly from the values in patients with endometrial hyperplasia (16.7%, 33.3%, 20.8%, 50%, and 8.3%, respectively) or with a normal endometrium (5.6%, 5.6%, 5.6%, 5.6%, and 5.6%, respectively; for all comparisons between the 3 groups, $p < 0.001$). To validate the results of qMSP, MS-PCR and its product sequencing were performed with random sampling of EC, hyperplasia of endometrium, and normal controls. The results are shown in Figures 2 and 3. The trends of methylation status of MS-PCR are consistent with the results of qMSP. The result of the sequencing of the MS-PCR product was validated.

To assess the clinical application of the method, the ROC curves were generated, and the areas under the ROC curve were calculated to discriminate between the normal endometrium and the EC group (Figure 4). The sensitivity and specificity were calculated by the best cutoff MI values for each gene (Figure 4). The sensitivity ranged from 61.5% to 92.3% and specificity was 94.4% for the normal endometrium group, and the sensitivity and specificity were both 94.4% for the EC group. The *COL14A1* gene conferred the best performance for the detection of EC with a sensitivity, specificity, and

Table 1
The primer list of the tested genes and the characteristics of methylation-specific polymerase chain reaction.

Gene name		Sense	Antisense	T_m (°C)
ZNF177	M	GGAAGTGGGCGTTCGTCGTTTC	CCCTTCCCTCCCGATTCCG	60
	U	TGGAAGTGGGTGTTTGTTGTTTTGTTA	CTTCCCTCCCAATTCCACCAACC	60
COL14A1	M	TTTTCGTTTTATAATTGGTTGTCGGC	CCCTCTCCTTCTACCGCTACGCT	60
	U	TTTTTTGTTTTATAATTGGTTGTTGGTGGG	CTCCCTCTCCTTCTACCACTACA	60
HOXA9	M	CGGGCGTTTTTCGTTTTAGGC	AAATCCGTCCCAAACGAAACCG	60
	U	TTGGGTGTTTTTTGTTTTAGGTGGG	AAAAATCAAATTCAACACAAAATCCATC	62
TMEFF2	M	AGGAGAGGGATTTAAATTTGCGAAC	CGAAACAACTCAACCATCCCGAC	60
	U	GAGGAGAGGGATTTAAATTTGTGAATG	CCAAAACAACTCAACCATCCCAAC	60
DPYSL4	M	CGTTTCGGTTTTTGGGGAGC	CCGAACCAAAATCCGAACGC	60
	U	TGTGTTTTGGTTTTTGGGGAGTG	CAAACCAAAATCCAAACACCAAACTAT	60
COL2A		GGGAAGATGGGATAGAAGGGAATAT	TCTAACAATTATAAACTCCAACCACCAA	58

COL14A1 = collagen type XIV α1; *DPYSL4* = dihydropyrimidinase-like 4; *HOXA9* = homeobox A9; *TMEFF2* = transmembrane protein with epidermal growth factor-like and two follistatin-like domains 2; *ZNF177* = zinc finger protein 177.

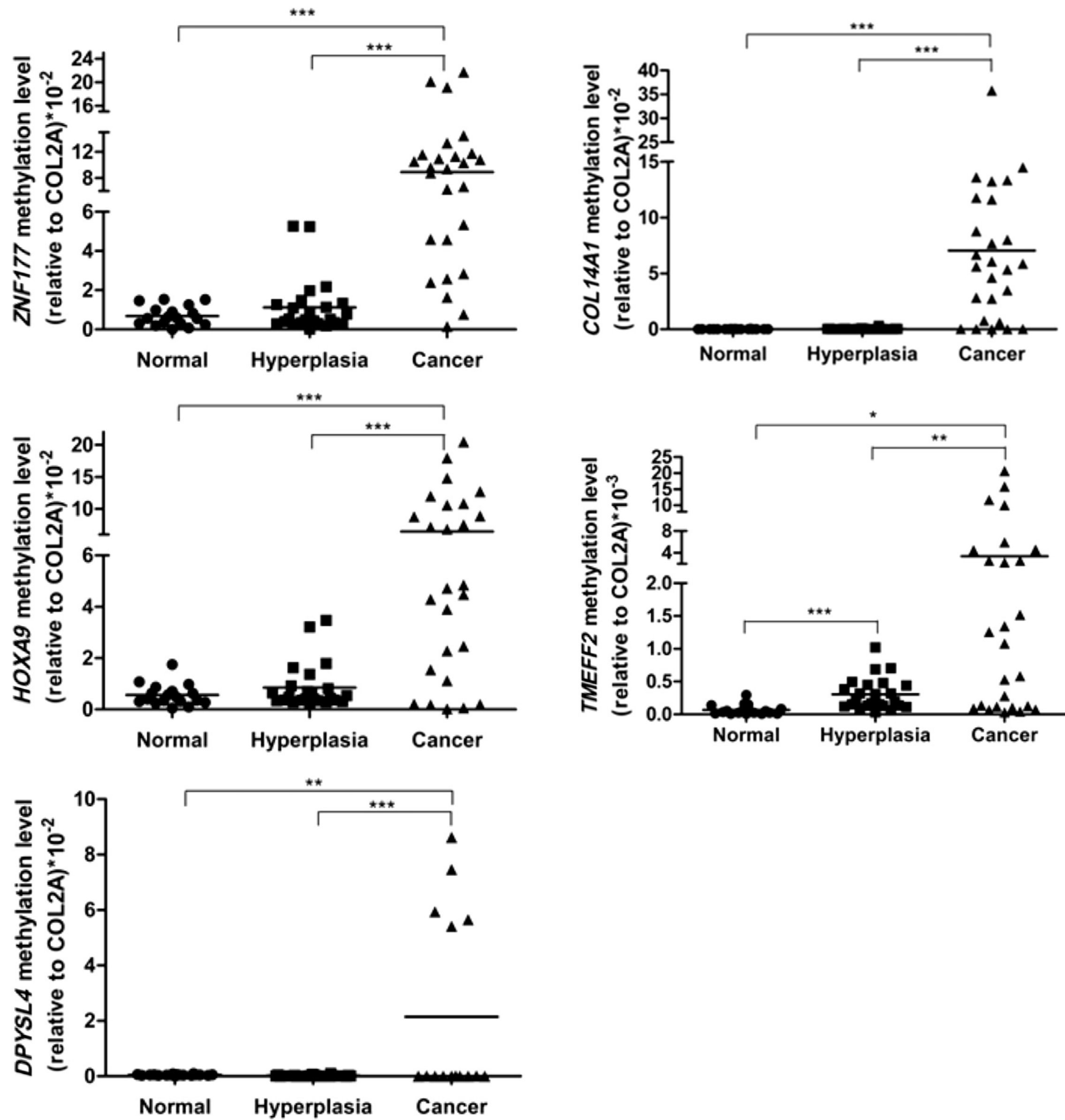

Figure 1. The methylation index (MI) values for the genes *ZNF177*, *COL14A1*, *DPYSL4*, *HOXA9*, and *TMEFF2*, as determined from scrapings of the normal endometrium and tissues of endometrial hyperplasia, and endometrial cancer. They are presented according to histology. Each round, square, or triangle black mark represents the test results of one patient. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; based on the Mann–Whitney *U* test. *COL14A1* = collagen type XIV α1; *DPYSL4* = dihydropyrimidinase-like 4; *HOXA9* = homeobox A9; *TMEFF2* = transmembrane protein with epidermal growth factor-like and two follistatin-like domains 2; *ZNF177* = zinc finger protein 177.

accuracy of 92.3%, 94.4%, and 95.7%, respectively. The performance of combined testing was also calculated (Table 4). Combined testing of either *ZNF177* or *COL14A1* had greater sensitivity (92.3%) and compromised specificity (94.4%). Combined testing of both *ZNF177* and *COL14A1* also had greater specificity (100%) and compromised sensitivity (88.5%). The relationship between the clinicopathological parameters and DNA methylation patterns in the test group patients was explored.

Table 3
The methylation index (MI) for each gene, according to disease severity.

Gene	The number of methylated samples/total (%)		
	Normal endometrium ($n = 18$), n (%)	Hyperplasia of the endometrium ($n = 24$), n (%)	Endometrial cancer ($n = 26$), n (%)
ZNF177	1/18 (5.6)	4/24 (16.7)	24/26 (92.3)[a]
COL14A1	1/18 (5.6)	8/24 (33.3)	24/26 (92.3)[a]
HOXA9	1/18 (5.6)	5/24 (20.8)	21/27 (80.8)[a]
TMEFF2	1/18 (5.6)	12/24 (50)	17/26 (65.4)[a]
DPYSL4	1/18 (5.6)	2/24 (8.3)	16/26 (61.5)[a]

COL14A1 = collagen type XIV α1; *DPYSL4* = dihydropyrimidinase-like 4; *HOXA9* = homeobox A9; *TMEFF2* = transmembrane protein with epidermal growth factor-like and two follistatin-like domains 2; *ZNF177* = zinc finger protein 177.

[a] The value of the comparisons between the three groups ($p < 0.001$). All results have been determined by the Chi-square test or by the Fisher's exact test.

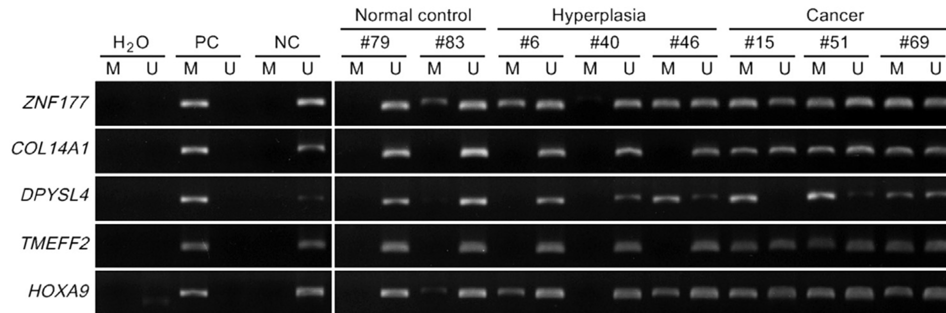

Figure 2. The methylation-specific polymerase chain reaction results for the random sampling of endometrial cancer (case #15, #51, and #69), hyperplasia of endometrium (case #6, #83, and #46), and the normal endometrium (case #79 and #83). *COL14A1* = collagen type XIV α1; *DPYSL4* = dihydropyrimidinase-like 4; *HOXA9* = homeobox A9; H_2O = reaction without template DNA; PC = positive control with commercial methylated DNA; NC = negative control with DNA from male whole blood; M = methylation; *TMEFF2* = transmembrane protein with epidermal growth factor-like and two follistatin-like domains 2; U = unmethylation; *ZNF177* = zinc finger protein 177.

Table 5 shows the correlation between DNA hypermethylation and the clinicopathological parameters of patients with EC. We found that the methylation of *TMEFF2* was higher in well-differentiated tumors than in moderately or poorly differentiated tumors (p = 0.0057). Furthermore, no significant correlations existed between the methylation of any gene in regard to age, BMI, stage, or grade.

Discussion

Endometrial carcinogenesis is a multistep process involving a precursor lesion with underlying genetic and epigenetic events. Therefore, molecular diagnostic methods have been proposed as new ancillary tools for the detection of cancers and for the differential diagnosis of premalignant and malignant lesions. An

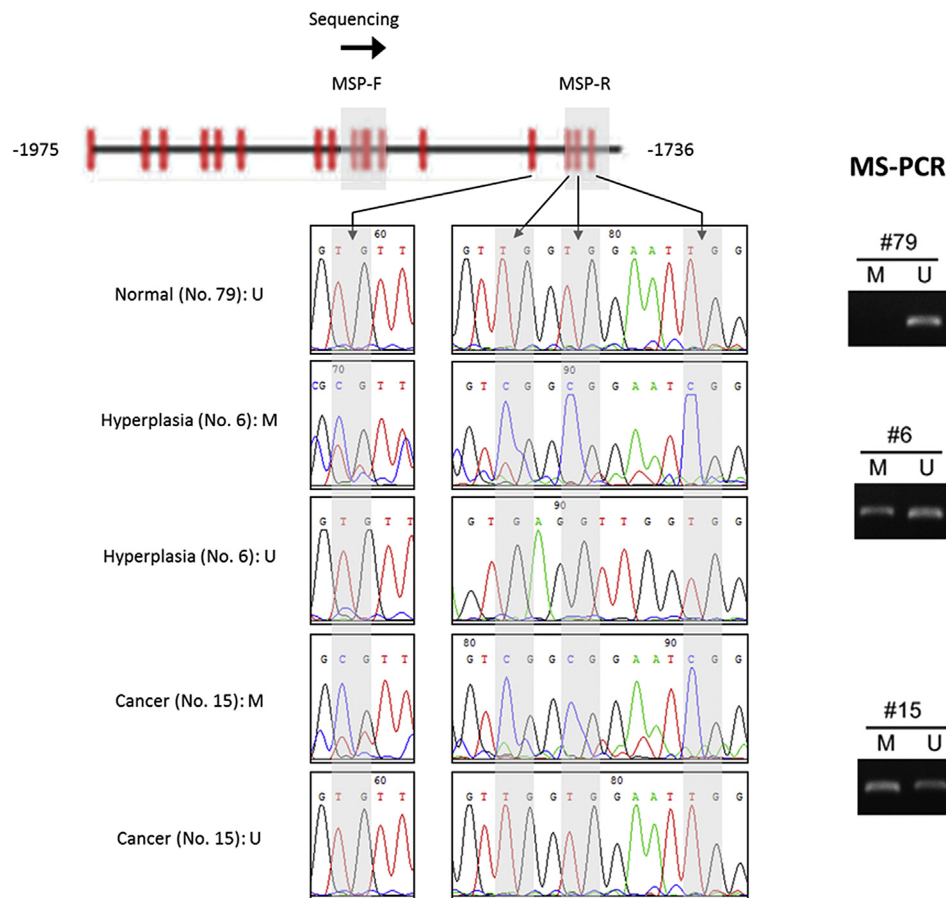

Figure 3. The sequencing of the methylation-specific polymerase chain reaction (MS-PCR) product, compared to MS-PCR. The samples are from the normal endometrium (case #79), hyperplasia of endometrium (case #6), and endometrial cancer (case #15). The MS-PCR shows full methylation in case #79, and shows hemimethylation in cases #6 and #15. The sequence "TG" in MS-PCR-reverse indicates unmethylation, the sequence "CG" in MS-PCR-reverse indicates methylation. M = methylation; MS-PCR = methylation-specific polymerase chain reaction; U = unmethylation.

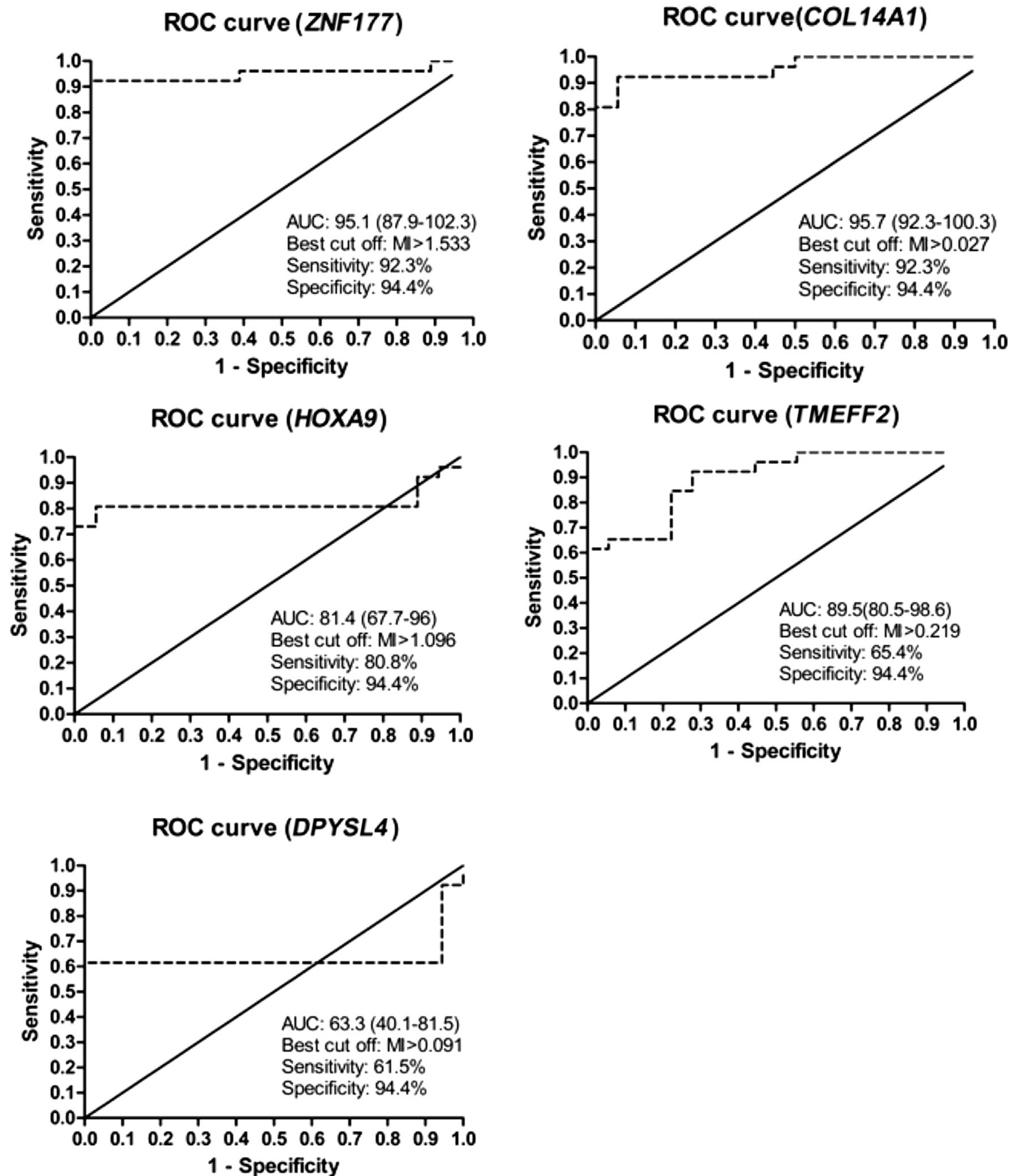

Figure 4. The receiver operating characteristic (ROC) curve analysis of the genes *ZNF177*, *COL14A1*, *DPYSL4*, *HOXA9*, and *TMEFF2*. The area under the ROC curve (AUC) of each gene is calculated for the diagnosis of a normal endometrium and endometrial cancer. AUC = area under the curve; *COL14A1* = collagen type XIV α1; *DPYSL4* = dihydropyrimidinase-like 4; *HOXA9* = homeobox A9; M = methylation; MSP-F = methylation polymerase chain reaction-forward; MSP-R = methylation polymerase chain reaction-reverse; ROC = receiver operating characteristic; *TMEFF2* = transmembrane protein with epidermal growth factor-like and two follistatin-like domains 2; U = unmethylation; *ZNF177* = zinc finger protein 177.

epigenetic mechanism has been proposed for development of type I EC, based on DNA mismatch repair (MMR) deficiency, which is a typical genetic defect in this cancer [15]. The DNA MMR system corrects errors in bases that arise when genes are replicated during cell division. Silencing of the DNA MMR genes reduces the ability to repair gene mutations. Microsatellite instability (MSI) occurs when the MMR system is damaged. Abnormalities in the MMR system may cause replication errors in the repeating unit, leading to changes in length, which is referred to as MSI. This results in an accumulation of cancer-related gene mutations and leads to carcinogenesis, which includes EC [15,16]. The human mutL homolog 1 (*hMLH1*) gene is a typical gene for MMR that is silenced by DNA methylation. In EC, *hMLH1* silencing occurs in approximately 40% of patients and is an important step in the early stages of carcinogenesis in which the loss of DNA MMR function may lead to mutation of genes such as *PTEN* [15,17].

In our present study, our five selected genes, *ZNF177*, *COL14A1*, *HOXA9*, *DPYSL4*, and *TMEFF2*, were identified using the Illumina Methylation 450K Array System with the analysis of the methylation differences alone and in combination with independent gene

Table 4
Combined testing of DNA methylation in the detection of endometrial cancer.

Gene set	Any one gene (methylated)		Both genes (methylated)	
	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
ZNF177/COL14A1	96.2	88.9	88.5	100
ZNF177/HOXA9	96.2	88.9	76.9	100
ZNF177/TMEFF2	96.2	88.9	61.5	100
COL14A1/HOXA9	96.2	88.9	76.9	100
COL14A1/TMEFF2	96.2	88.9	61.5	100
ZNF177/COL14A1/HOXA9	96.2	83.3	73.1	100

COL14A1 = collagen type XIV α1; *DPYSL4* = dihydropyrimidinase-like 4; *HOXA9* = homeobox A9; *TMEFF2* = transmembrane protein with epidermal growth factor-like and two follistatin-like domains 2; *ZNF177* = zinc finger protein 177.

Table 5
The correlation between DNA hypermethylation and the clinicopathological parameters of patients with endometrial cancer.

	ZNF177 (MI > 1.533)			*COL14A1* (MI > 0.027)			*HOXA9* (MI > 1.096)			*TMEFF2* (MI > 0.219)			*DPYSL4* (MI > 0.091)		
	U	M	*p*	U	M	*p*	U	M	*p*	U	M	*p*	U	M	*p*
Age (y)															
≤61	1	14	> 0.99	0	15	0.17	3	12	> 0.99	4	11	0.42	5	10	0.69
>61	1	10		2	9		2	9		5	6		5	6	
BMI															
≤25.9	2	13	0.49	1	14	> 0.99	4	11	0.36	4	11	0.42	5	10	0.69
>25.9	0	11		1	10		1	10		5	6		5	6	
Stage															
I + II	0	19	0.06	1	18	0.47	2	17	0.10	5	14	0.19	7	12	> 0.99
III + IV	2	5		1	6		3	4		4	3		3	4	
Grade															
1	0	6	0.13	1	5	0.72	0	6	0.27	0	6	0.0057	3	3	0.58
2	0	9		0	9		3	6		2	7		1	8	
3	2	9		1	10		3	8		7	4		6	5	

All results were determined by the Chi-square test or by Fisher's exact test.
BMI = body mass index; *COL14A1* = collagen type XIV α1; *DPYSL4* = dihydropyrimidinase-like 4; *HOXA9* = homeobox A9; M = methylation; MI = methylation index; *TMEFF2* = transmembrane protein with epidermal growth factor-like and two follistatin-like domains 2; U = unmethylation; *ZNF177* = zinc finger protein 177.

expression data. These genes are novel and have rarely been reported [18], especially in EC. They are neither MMR genes nor MSI genes. In patients with EC, we found aberrant hypermethylation of *ZNF177*, *COL14A1*, *HOXA9*, *TMEFF2*, and *DPYSL4* in 92.3%, 92.3%, 80.8%, 65.4%, and 61.5% of patients, respectively. A significant decrease in gene expression was present in patients with aberrant methylation of many of these genes ($p < 0.01$). Aberrant methylation of *TMEFF2* was also present in 50% of patients with hyperplasia of the endometrium ($p < 0.001$). However, very low aberrant methylation of the five cancer-related genes was found in patients with a normal endometrium. These results indicated that the normal endometrium does not have aberrant methylation of specific genes (most frequently *ZNF177* and *COL14A1*) that are associated with carcinogenesis in EC. The aberrant methylation of *TMEFF2* in hyperplasia of the endometrium, which occurs in precancerous lesions of EC, supports the hypothesis that aberrant methylation of *TMEFF2* is an important event in carcinogenesis in EC.

Endometrial cancer is the most common malignancy of the female genital tract; however, routine screening is not recommended—the rationale being that symptoms due to these malignancies develop at an early stage in 85% of cases [19–21]. Conventional screening modalities for EC include measuring endometrial thickness with transvaginal sonography and endometrial sampling with cytological examination. Molecular diagnostic methods have been proposed as new ancillary tools for detecting previously undetected cancers and for the differential diagnosis of premalignant and malignant lesions. Abnormal patterns of DNA methylation have been recognized in various cancers. An increase in DNA methylation in gene promoter regions often precedes apparent malignant changes, which suggests that the assessment of DNA methylation could be used for the early diagnosis of cancer [8,12,22]. Our results demonstrate the usefulness of assessing DNA methylation for detecting EC in endometrial hyperplasia. Patients with high methylation levels of *TMEFF2* should receive a thorough assessment, which includes a detailed magnetic resonance imaging evaluation before any major operation, or they should be checked by an intraoperative frozen section because *TMEFF2* has a high methylation rate in hyperplasia of the endometrium and in EC.

Despite these promising results, the current study has some limitations. All patients in the current study were Asian, the sample size was small, and the cutoff value for each gene was defined by the criteria of a hospital-based case-controlled study that used a research platform, which may not be directly applicable to the clinical setting or to wider populations. The application of these new biomarkers may provide a new molecular method of management for ambiguous cases of endometrial sampling and warrants further validation in a larger, population-based study. In addition, the extent to which the current results can be applied to populations with other ethnicities remains to be determined.

In conclusion, promoter methylation of *ZNF177*, *COL14A1*, *HOXA9*, *DPYSL4*, and *TMEFF2* is a frequent epigenetic event in EC. Furthermore, the epigenetic hypermethylation of *TMEFF2* is potentially a valuable marker for identifying undetected EC within endometrial hyperplasia.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

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