SPANDIDOS PUBLICATIONS

Relationship of the aberrant DNA hypermethylation of cancer-related genes with carcinogenesis of endometrial cancer

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Abstract. Epigenetic abnormalities including the aberrant DNA hypermethylation of the promoter CpG islands play a key role in the mechanism of gene inactivation in cell carcinogenesis. To identify the genes associated with aberrant DNA hypermethylation in endometrial carcinogenesis, we studied the hypermethylation of the promoter regions of five genes: *hMLH1*, *APC*, *E-cadherin*, *RAR-ß* and *p16*. The frequencies of aberrant hypermethylation were 40.4% (21/52) in *hMLH1*, 22% (11/50) in *APC*, 14% (7/50) in *E-cadherin*, and 2.3% (1/44) in *RAR-ß* in endometrial cancer specimens. No aberrant DNA methylation was found in *p16*. In atypical endometrial hyperplasia, the frequencies of aberrant methylation were 14.3% (2/14) in *hMLH1* and 7.3% (1/14) in *APC*, whereas normal endometrial cells showed no aberrant hypermethylation of any of the five genes. The high frequencies of the aberrant DNA hypermethylation of *hMLH1*, *APC* and *E-cadherin* suggest that the methylation of the DNA mismatch repair and Wnt signal-related genes may be associated with endometrial carcinogenesis.

Introduction

The relationship of cellular oncogenic transformation with aberrant DNA hypermethylation in promoter regions (i.e., epigenetic changes) is an area of growing interest. Genes ranging from tumor suppressors to DNA mismatch repair and cell cycle-related genes are known to be inactivated by aberrant DNA methylation in cancer. The DNA mismatch repair genes human MutL homolog-1 (*hMLH1*) and human MutS homolog (*hMSH2*) function in the repair of base-pair mismatches that occur in gene amplification during cell division. The characteristic seen in cancer cells when the DNA mismatch repair system breaks down is referred to as microsatellite instability (MSI). Microsatellites are repeated DNA sequences of ~1 to 5 bases, and DNA replication errors occur frequently at these sites upon the inactivation of the DNA mismatch repair genes. MSI is detected in ~40% of patients with endometrial cancer (1,2), suggesting that mutations of the DNA mismatch repair genes are associated with endometrial carcinogenesis. Therefore, in this study we examined the aberrant DNA methylation of *hMLH1*, a leading candidate in the DNA mismatch repair gene group regarding the production of MSI.

The *ß-catenin* gene codes for a cell adhesion molecule that plays a key role in the Wnt signaling pathway and is generally localized in the cell membrane, where it binds to *E-cadherin*, an adhesion molecule. Free *ß-catenin* forms a complex with *adenomatous polyposis coli* (*APC*) and axin is phosphorylated by GSK-3ß and degraded via the proteosome pathway. The mutation of the *ß-catenin* gene increases the level of undegraded *ß-catenin* in the cells and causes the transition of *ß-catenin* into the nucleus, which induces the activation of the Wnt signaling pathway and enhances the transcriptional activity of target genes including *cyclin D*, leading to cell cycle aberrations. The activation of the Wnt signaling pathway is also thought to be important in endometrial carcinogenesis (3), and therefore *E-cadherin* and *APC*, which are components of the Wnt signaling pathway, are also candidate genes for aberrant DNA methylation in endometrial cancer.

p16 is a tumor suppressor gene that codes for a protein that binds to CDK4 and CDK6 and inhibits the phosphorylation of the RB/E2F complex by the CDK-Cyclin D. *p16*-knockout mice develop multiple cancers in different organs, and therefore *p16* inactivation is thought to play an important role in cell carcinogenesis. The frequencies of *p16* mutation and deletion in endometrial cancer are only 5-6% and 3%, respectively (4,5), but reduced protein levels have been found in 19% of cases (5), and this may be associated with aberrant DNA methylation.

Type I endometrial cancer is also estrogen-dependent; estrogen increases the risk of endometrial cancer through a mechanism that has yet to be fully explained. Estrogen acts in a receptor-specific manner as a molecular switch to regulate transcription factor function. Estrogen receptors have highly differentiated structures, and aberrant methylation of the *estrogen receptor* (*ER*) gene in endometrial cancer has

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Table I. Primer sequences used in MSP analysis and RT-PCR.

Gene name	PCR analysis	Sense	Antisense	Size (bp)	Ann[a] temp (˚C)
hMLH1	Methylated	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	112	60
	Unmethylated	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	124	60
APC	Methylated	TATTGCGGAGTGCGGGTC	TCGACGAACTCCCGACGA	100	68
	Unmethylated	GTGTTTTATTGTGGAGTGTGGGTT	CCAATCAACAAACTCCCAACAA	110	67
RAR-ß	Methylated	GGTTAGTAGTTCGGGTAGGGTTTATC	CCGAATCCTACCCCGACG	235	59
	Unmethylated	TTAGTAGTTTGGGTAGGGTTTATT	CCAAATCCTACCCCAACA	233	59
p16	Methylated	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150	67
	Unmethylated	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151	66

[a]Annealing temperature.

been reported (6). The *ER* protein shares a common fold with glucocorticoid and retinoic acid receptors (these receptors all belong to the nuclear receptor superfamily), but the frequency of the aberrant methylation of the *retinoic acid receptor-ß* (*RAR-ß*) gene in endometrial cancer has not been determined. However, studies of cancers in other organs (7,8) suggest a relationship between those cancers and the aberrant DNA methylation of *RAR-ß*.

To identify genes associated with aberrant DNA methylation in endometrial carcinogenesis, we studied the aberrant DNA methylation of the promoter regions of five genes (*hMLH1*, *APC*, *E-cadherin*, *RAR-ß* and *p16*) that show high frequencies of aberrant DNA methylation in different cancers and may be important in endometrial carcinogenesis.

Materials and methods

Clinical specimens. The subjects were 93 patients who gave informed consent to the collection of endometrial specimens (27 normal endometria, 14 atypical endometrial hyperplasia, and 52 endometrial cancers). The cells obtained from the tissue specimens were examined by liquid-based cytology using the ThinPrep system (Cytyc Corporation, Boxborough, MA, USA) with preservation fluid (PreservCyt Solution, Cytyc Corporation) (9). A pathological diagnosis of the endometrial tissue was consistent with the cytology results for all the 93 subjects. Of the 27 patients with a normal endometrium, 16 were in the secretory phase and 11 were in the proliferative phase. Of the 52 patients with endometrial cancer, 44 had ovarian endometrioid adenocarcinoma (G1, 24; G2, 10; G3, 10) and 8 had adenosquamous carcinoma. The grade of histological differentiation (G1 to G3) and the cancer stage at surgery were determined based on the Guidelines for Endometrial Cancer published by the Japan Society of Obstetrics and Gynecology.

DNA extraction and methylation-specific PCR (MSP) analysis. DNA was extracted from 93 endometrial specimens using liquid-based cytology with a Get Pure DNA kit (Dojindo Molecular Technologies Inc., Kumamoto, Japan). Distilled water was added to 1 µg of the extracted DNA up to a volume of 50 µl, 5.5 µl 3 N NaOH solution was added, and, after mixing, the solution was incubated at 37˚C for 15 min. Following this, 520 µl 3 M sodium bisulfite (Sigma, St. Louis, MO, USA), which was prepared at pH 5.5 with 30 µl 10 mM hydroquinone (Sigma) and 10 N NaOH, was added to the solution. After mixing in an upturned position to prevent vaporization, the solution was overlaid with mineral oil and incubated at 50˚C overnight. Next, 1 ml clean-up resin (Promega Corporation, Madison, WI, USA) was added to the lower layer, and the resulting solution was mixed in an upturned position and then injected into a column. After rinsing with 2 ml 80% isopropanol, the column was centrifuged at 15,000 rpm for 3 min to remove the isopropanol completely, after which 50 µl distilled water (70˚C) was added directly to the column, and the column was centrifuged at 15,000 rpm for 2 min to extract the DNA adsorbed in the column. Then, 5.5 µl 2 N NaOH was added to the resulting DNA solution, and, after mixing, the solution was incubated at 37˚C for 20 min, after which 66 µl 5 N ammonium acetate solution and 243 µl 95% ethanol were added, and the solution was incubated at 80˚C for 1 h and centrifuged at 15,000 rpm for 30 min to precipitate the DNA. Approximately 50 µl of the supernatant was left in the tube, and the rest of the supernatant was collected, mixed with 1 ml 70% ethanol, and then centrifuged at 15,000 rpm for 30 min to rinse the DNA. The precipitated DNA was air-dried and dissolved in 20 µl distilled water; 2 µl of this solution was used as the MSP template solution. AmpliTaq Gold & 10X PCR buffer/$MgCl_2$ with dNTP (Applied Biosystems, Foster City, CA, USA) was used in the PCR analysis, and the DNA was analyzed using a GeneAmp PCR 9700 system (Applied Biosystems). A CpG WIZ *E-cadherin* amplification kit (Chemicon, Temecula, CA, USA) was used as the MSP for the *E-cadherin* gene. The PCR conditions and primer sequences for the other genes are shown in Table I.

Immunohistochemical analysis of endometrial cancer tissues. Twenty surgical endometrial specimens from 52 patients were examined using liquid-based cytology. Formalin-fixed,

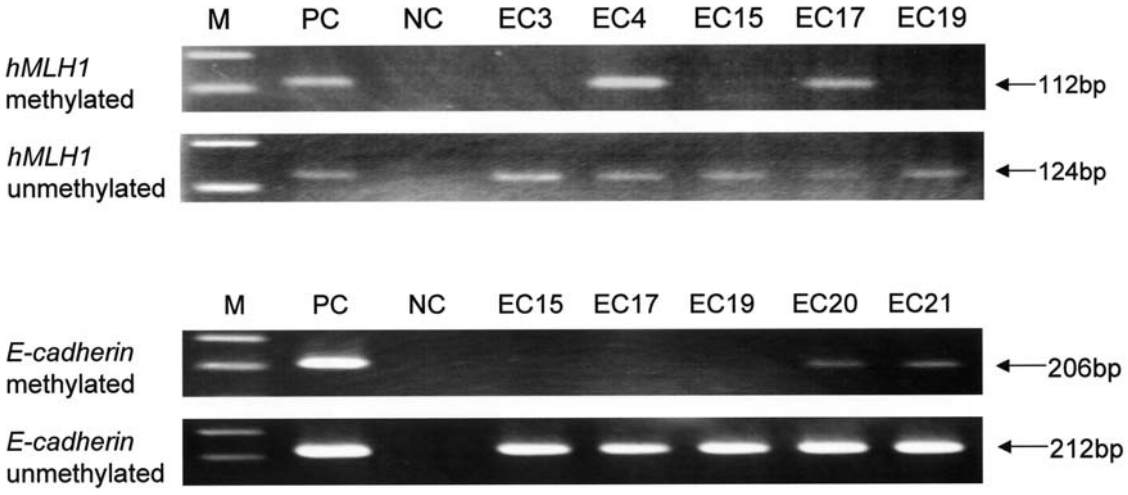

Figure 1. MSP analysis of the *hMLH1* and *E-cadherin* genes in endometrial cancer specimens. MSP analysis was conducted using DNA extracted from endometrial cancer specimens. The results for *hMLH1* and *E-cadherin* are shown in the upper and lower panels, respectively. For *hMLH1* the aberrant methylation band is shown in lanes EC4 and EC17, and for *E-cadherin* this band is shown in lanes EC20 and EC21. M, marker; PC, positive control; NC, negative control; EC, endometrial cancer.

paraffin-embedded specimens were prepared and the slices were stained in a silane-coated slide using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). After deparaffinizing, the slides were heated in 10 mM citric acid buffer solution (pH 7.0) at 120˚C for 10 min in an autoclave for antigen retrieval. After allowing the slides to cool to room temperature, intrinsic peroxidase activity was eliminated by treating the slides with 3% H_2O_2 in phosphate-buffered saline (PBS) for 5 min. The slides were rinsed twice with PBS and blocked with normal goat serum, and then rinsed twice again with PBS and diluted with 1% bovine serum albumin (BSA) in PBS and incubated with the primary antibody at 4˚C overnight. The primary antibodies were the 50-fold diluted anti-*hMLH1* antibody (BD Bioscience Pharmingen, San Diego, CA, USA) and the 500-fold diluted anti-*E-cadherin* antibody (Takara, Tokyo, Japan). After rinsing three times with PBS, the slides were incubated with the secondary antibody (biotin-labeled anti-mouse IgG) at room temperature for 30 min. After rinsing three more times with PBS, the slides were incubated with ABC (avidin-biotin peroxidase) complex at room temperature for 30 min. After further rinsing three times with PBS, the slides were treated with 0.2 mg/ml diaminobenzidine (DAB) for about 5 min for coloring. After rinsing twice with PBS, the slides were treated with hematoxylin solution for nuclear staining, and then dehydrated and observed microscopically. For judging the immunohistochemical staining intensity of the *hMLH1* protein, the nuclei of endometrial stromal cells were used as an internal control; if the nuclei of the tumor cells containing the protein showed a stronger staining intensity than the control nuclei, the specimen was considered positive, whereas a specimen was considered negative if the tumor cell nuclei showed a lower staining intensity than the control nuclei (10). Regarding the *E-cadherin* staining, the protein is localized in the cell membrane in normal epithelial cells, and the immunohistochemical analysis was conducted in accordance with the criteria of Wu *et al*: Specimens with ≥25% of the tumor cells that stained for *E-cadherin* in the cell membrane were considered positive, and specimens with <25% of the tumor cells giving this staining result were considered negative (11).

Statistical analysis. The correlation of the aberrant DNA methylation of the *hMLH1*, *APC* and *E-cadherin* genes with the clinicopathological factors, grade of histological differentiation and cancer stage at surgery was analyzed using Mann-Whitney tests. The correlation of the aberrant DNA methylation of each of these genes with the patients' age was also examined, after confirming that the groups of patients with and without aberrant methylation showed a normal age distribution based on a normal distribution test. An F test was used to confirm that the population variances of the two independent groups were equal to each other, and then the differences in the population means were examined using the Student's t-test. The correlation of the aberrant DNA methylation level of *hMLH1* with those of *APC*, *E-cadherin* and *RAR-β*, respectively, was calculated using Fisher's exact test, and the correlations of the aberrant DNA methylation of *hMLH1* and *E-cadherin* with the immunohistochemical staining data were also examined using Fisher's exact test.

Results

Aberrant DNA methylation of cancer-related genes in endometrial specimens. Fig. 1 shows partial results of the MSP analysis of the endometrial cancer cells obtained using liquid-based cytology. A band due to the aberrant methylation of the *hMLH1* gene was present in samples EC4 and EC17 (size, 112 bp), and bands due to the aberrant methylation of *E-cadherin* were found in samples EC20 and EC21 (206 bp). MSP analysis of the endometrial cancer specimens indicated that the frequencies of the aberrant methylation of the promoter regions were 40.4% (21/52) for *hMLH1*, 22% (11/50) for *APC*, 14% (7/50) for *E-cadherin*, and 2.3% (1/44) for *RAR-β*. No aberrant methylation was found in the promoter region of the

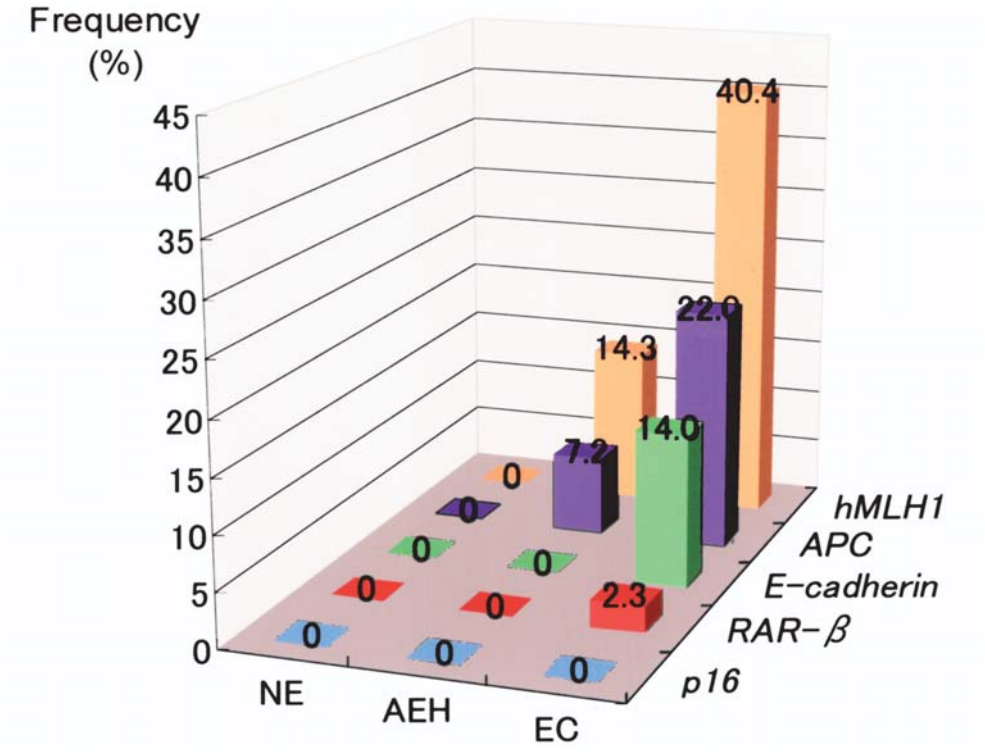

Figure 2. Frequencies of aberrant methylation of cancer-related genes in specimens from normal endometria, atypical endometrial hyperplasia and endometrial cancer. In the endometrial cancer specimens, *hMLH1* exhibited the highest frequency of aberrant methylation, followed by *APC* and *E-cadherin*. Aberrant methylation of *hMLH1* was also found in atypical endometrial hyperplasia, whereas normal endometrial cells showed no aberrant methylation of the five genes. NE, normal endometrium; AEH, atypical endometrial hyperplasia; EC, endometrial cancer.

Case	Age	Tissue type	Cancer stage	Differentiation grade	*hMLH1*	*E-cad*	*APC*	*RAR-β*	*p16*
EC1	52	endometrioid adenocarcinoma	Ⅰb	G3	■				
EC2	50	endometrioid adenocarcinoma	Ⅰa	G1					
EC3	51	endometrioid adenocarcinoma	Ⅲc	G1					
EC4	54	adenosquamous carcinoma	Ⅲc	G3	■				
EC5	51	endometrioid adenocarcinoma	Ⅰa	G1		■			
EC6	61	endometrioid adenocarcinoma	Ⅰb	G1	■				
EC7	70	endometrioid adenocarcinoma	Ⅲc	G2					
EC8	61	endometrioid adenocarcinoma	Ⅱb	G1		■			
EC9	62	adenosquamous carcinoma	Ⅲa	G2					
EC10	40	endometrioid adenocarcinoma	Ⅱa	G1					
EC11	59	endometrioid adenocarcinoma	Ⅱa	G3					
EC12	57	endometrioid adenocarcinoma	Ⅰb	G3		■	■		
EC13	80	endometrioid adenocarcinoma	Ⅲc	G3		■			
EC14	54	adenosquamous carcinoma	Ⅰb	G1					
EC15	53	endometrioid adenocarcinoma	Ⅰb	G3			■		
EC16	42	endometrioid adenocarcinoma	Ⅱb	G1	■		■		
EC17	71	endometrioid adenocarcinoma	Ⅲc	G3					
EC18	60	endometrioid adenocarcinoma	Ⅰb	G1		■			
EC19	57	endometrioid adenocarcinoma	Ⅲa	G2					
EC20	71	endometrioid adenocarcinoma	Ⅱa	G1		■			
EC21	37	endometrioid adenocarcinoma	Ⅱa	G2	■	■			
EC22	47	endometrioid adenocarcinoma	Ⅲb	G1					
EC23	67	endometrioid adenocarcinoma	Ⅰc	G2					
EC24	53	endometrioid adenocarcinoma	Ⅰa	G1	■				
EC25	69	endometrioid adenocarcinoma	Ⅲc	G2	■				
EC26	55	endometrioid adenocarcinoma	Ⅲc	G2	■		■		
EC27	54	endometrioid adenocarcinoma	Ⅰa	G1					
EC28	63	endometrioid adenocarcinoma	Ⅰa	G1	■		■		
EC29	41	endometrioid adenocarcinoma	Ⅰb	G1	■				

Case	Age	Tissue type	Cancer stage	Differentiation grade	*hMLH1*	*E-cad*	*APC*	*RAR-β*	*p16*
EC30	62	adenosquamous carcinoma	Ⅰb	G1	■		■		
EC31	58	endometrioid adenocarcinoma	Ⅰb	G2	■		■		
EC32	56	endometrioid adenocarcinoma	Ⅲc	G3					
EC33	71	endometrioid adenocarcinoma	Ⅰb	G2					
EC34	53	adenosquamous carcinoma	Ⅰb	G3					
EC35	50	endometrioid adenocarcinoma	Ⅲa	G3					
EC36	42	adenosquamous carcinoma	Ⅲc	G3					
EC37	55	endometrioid adenocarcinoma	Ⅰc	G3				■	
EC38	34	adenosquamous carcinoma	Ⅲc	G1					
EC39	61	endometrioid adenocarcinoma	Ⅰc	G1					
EC40	61	endometrioid adenocarcinoma	Ⅰc	G1			■		
EC41	61	endometrioid adenocarcinoma	Ⅰb	G1	■				
EC42	59	endometrioid adenocarcinoma	Ⅰb	G1					
EC43	55	adenosquamous carcinoma	Ⅳb	G2	■				
EC44	54	endometrioid adenocarcinoma	Ⅱa	G1	■				
EC45	57	endometrioid adenocarcinoma	Ⅱa	G1	■		■		
EC46	56	endometrioid adenocarcinoma	Ⅰb	G2					
EC47	78	endometrioid adenocarcinoma	Ⅰb	G3			■		
EC48	65	endometrioid adenocarcinoma	Ⅰb	G2	■				
EC49	37	endometrioid adenocarcinoma	Ⅰa	G1	■				
EC50	36	endometrioid adenocarcinoma	Ⅰa	G1					
EC51	26	endometrioid adenocarcinoma	Ⅰa	G1	■				
EC52	18	endometrioid adenocarcinoma	Ⅰa	G1					

■ Methylated □ Unmethylated ▒ Not done

Figure 3. Aberrant methylation of the promoter regions of cancer-related genes in endometrial cancer. G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated; EC, endometrial cancer; *E-cad*, *E-cadherin*.

p16 gene. In the atypical endometrial hyperplasia samples, the frequencies of the aberrant methylation of the promoter regions were 14.3% (2/14) for *hMLH1* and 7.3% (1/14) for *APC*. Normal endometrial cells in the proliferative and secretory phases showed no aberrant methylation of the promoter regions of the five examined genes (Figs. 2-4).

Case	Age	Tissue type	hMLH1	E-cad	APC	RAR-β	p16
NE1	37	sec					
NE2	43	sec					
NE3	51	sec					
NE4	35	sec					
NE5	39	sec					
NE6	41	sec					
NE7	47	sec					
NE8	40	sec					
NE9	36	sec					
NE10	49	sec					
NE11	51	sec					
NE12	52	sec					
NE13	44	sec					
NE14	47	sec					
NE15	23	sec					
NE16	34	sec					
NE17	37	pro					
NE18	37	pro					
NE19	51	pro					
NE20	49	pro					
NE21	43	pro					
NE22	36	pro					
NE23	43	pro					
NE24	42	pro					
NE25	27	pro					
NE26	44	pro					
NE27	32	pro					

Case	Age	hMLH1	E-cad	APC	RAR-β	p16
AEH1	34					
AEH2	30					
AEH3	32					
AEH4	35					
AEH5	35					
AEH6	46					
AEH7	41					
AEH8	33					
AEH9	41					
AEH10	50					
AEH11	45					
AEH12	47					
AEH13	45					
AEH14	33					

Methylated Unmethylated

Not done

Figure 4. Aberrant methylation of the promoter regions of cancer-related genes in atypical endometrial hyperplasia. AEH, atypical endometrial hyperplasia; *E-cad, E-cadherin*.

Immunohistochemical analysis of hMLH1 and E-cadherin protein expression. The relationship of the aberrant DNA methylation of the promoter regions of the *hMLH1* and *E-cadherin* genes with protein expression was determined immunohistochemically. Of the 20 surgical specimens of endometrial cancer showing aberrant methylation, most showed negative protein staining (*hMLH1*, $p<0.01$; *E-cadherin*, $p<0.05$) (Fig. 5) (Table II).

Correlation of aberrant DNA methylation of cancer-related genes with clinicopathological factors. The correlations of the aberrant DNA methylation of the promoter regions of *hMLH1*, *APC* and *E-cadherin* with the clinicopathological factors were examined in endometrial cancer patients. For the *hMLH1*, APC and *E-cadherin* genes, no correlation was found between aberrant methylation and the grade of histological differentiation or with cancer stage at surgery. Aberrant DNA methylation is generally thought to increase with age, but no significant difference was found in the mean age between patients with and without aberrant methylation of *hMLH1*, *APC* and *E-cadherin*, respectively. Therefore, these data do not indicate that aberrant methylation occurs more frequently in elderly patients with endometrial cancer (Table IV).

The relationship of the aberrant methylation of the promoter region of *hMLH1*, which showed the highest frequency in the endometrial cancer samples, was also examined with that of other genes, but no correlation was found with the methylation of *APC*, *E-cadherin* or *RAR-β*.

Discussion

Of the five endometrial cancer-related genes examined, the aberrant methylation of *hMLH1*, a DNA mismatch repair gene, was found most frequently (40.4%). The frequencies of the aberrant methylation of *hMLH1* have been reported as 14% to 26% in gastric cancer (12,13) and 7% to 32% in lung cancer (14,15); therefore, the frequency of the aberrant methylation of this gene in endometrial cancer is higher than in other cancers. After *hMLH1*, the second most likely genes to show aberrant methylation were *APC* and *E-cadherin*, which are Wnt-related genes. Collectively, these data suggest that abnormal DNA mismatch repair and aberrant Wnt signaling are associated with endometrial carcinogenesis. However, patients with an aberrant methylation of *hMLH1* rarely corresponded to those with an aberrant methylation of *E-cadherin*, and therefore carcinogenesis due to the aberrant methylation of these respective genes may occur through independent mechanisms.

The aberrant methylation of *hMLH1* may cause a reduced protein expression that leads to abnormal DNA mismatch repair and MSI. However, although MSI has been found in ~40% of patients with endometrial cancer, the *hMLH1*

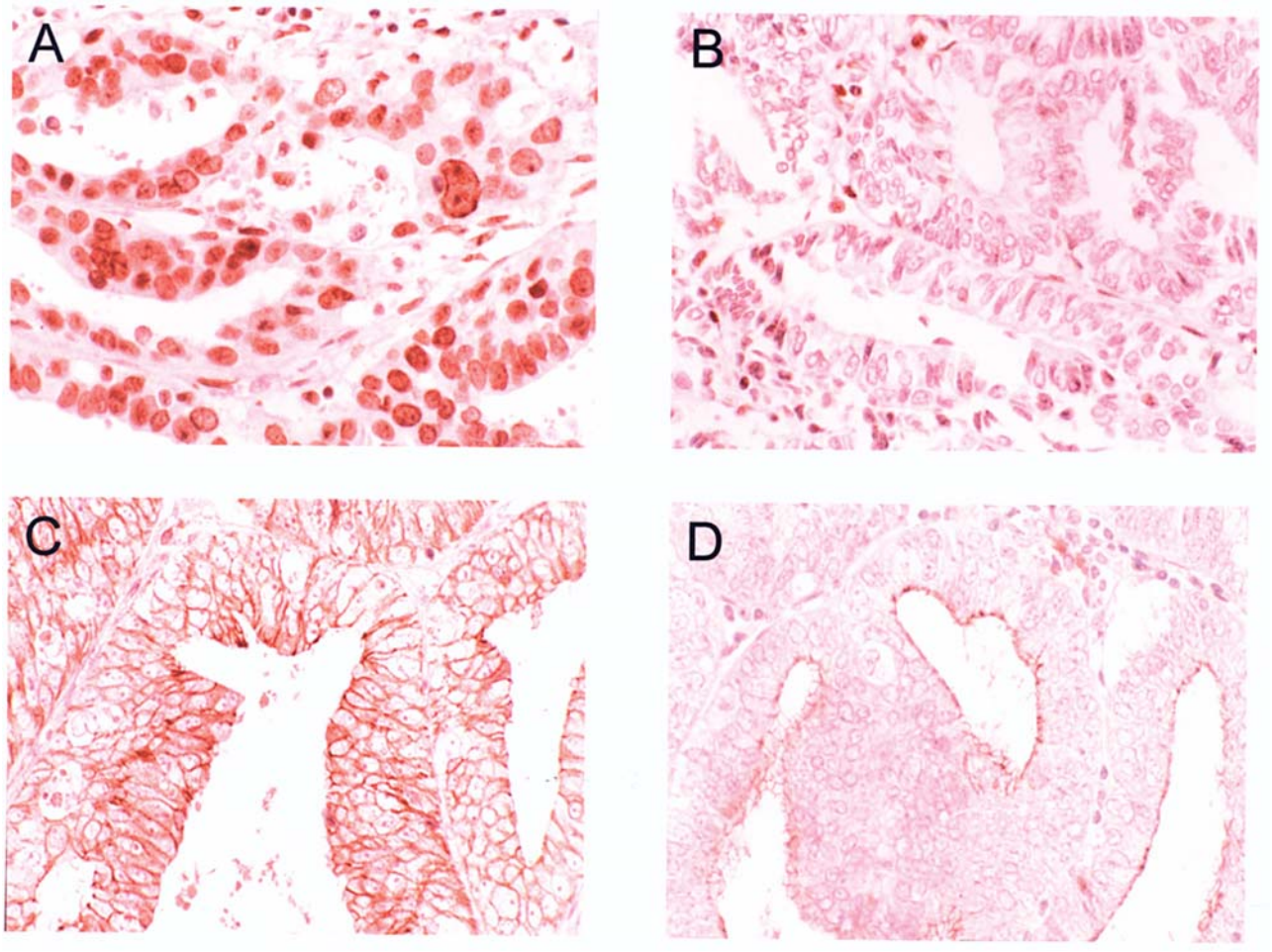

Figure 5. (A and B) Immunohistochemical analysis of the endometrial cancer specimens using the anti-*hMLH1* antibody. (A) In patients with an unmethylated *hMLH1* gene (EC23), the nuclei of the cancer cells were strongly stained. (B) In patients with aberrant methylation of *hMLH1* (EC31), the cell nuclei were less strongly stained. (C and D) Immunohistochemical analysis of the endometrial cancer specimens using the anti-*E-cadherin* antibody. (C) In patients with unmethylated *E-cadherin* (EC29), the cell membranes of the cancer cells were strongly stained. (D) In patients with aberrant methylation of *E-cadherin* (EC8), the cell membranes were less strongly stained. EC, endometrial cancer.

Table II. Relationship of the aberrant DNA methylation of the *hMLH1* and *E-cadherin* genes with reduced protein expression.

	Expressed	Not expressed	
hMLH1			
methylated	3	9	p<0.01
unmethylated	8	0	
E-cadherin			
methylated	2	2	p<0.05
unmethylated	15	1	

mutation frequency in MSI-positive endometrial cancer patients is extremely low (16,17), suggesting that MSI may occur due to the aberrant methylation of the promoter regions, and not due to the *hMLH1* mutation. Furthermore, the aberrant methylation of *hMLH1* has been found in atypical endometrial hyperplasia, but is not observed in the normal endometrium;

Table III. Correlation of the aberrant DNA methylation of cancer-related genes with the grade of histological differentiation and clinical stage at surgery.

	G1	G2	G3	Stage			
				I	II	III	IV
hMLH1							
M	12	6	3	11	4	5	1
U	15	6	10	19	4	8	0
E-cadherin							
M	4	1	2	3	3	1	0
U	22	11	10	24	5	13	1
APC							
M	5	2	4	7	2	2	0
U	21	10	8	20	6	12	1

G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated; M, methylated; U, unmethylated.

Correlation of the aberrant DNA methylation of cancer-related genes with the mean onset age of endometrial cancer.

	hMLH1		*E-cadherin*		*APC*		*RAR-ß*
Methylated	53.3±11.46	NS	59.6±13.78	NS	59.7±9.37	NS	55
Unmethylated	55.7±12.78		54.9± 10.84		54.3±11.57		56.26±9.76

NS, not significant.

therefore, aberrant methylation occurs in the early stage of carcinogenesis. Since such aberrant methylation can be detected in a small amount of cytological material by using minimally invasive procedures, the determination of the methylation levels of genes such as *hMLH1* is a potential supplementary diagnostic method for endometrial cancer.

An accumulation of *ß-catenin* in the nucleus, which indicates aberrant Wnt-signaling, has been observed in 23.8% of patients with endometrial cancer (18), and is thought to be one of the causes of endometrial cancer. In contrast, the *ß-catenin* mutation frequency is only 11%, significantly lower than the frequency of the accumulation of *ß-catenin* in the nucleus (18). Furthermore, the accumulation of *ß-catenin* in the nucleus has been observed in patients without *ß-catenin* mutations; therefore, the transition and accumulation of *ß-catenin* in the nucleus are dependent on a mechanism other than gene mutation. The aberrant methylation of the promoter region of *E-cadherin*, which codes for a scaffolding protein that binds to *ß-catenin* and is present in the cell membrane as a cell adhesion molecule, was found in 14% of the patients with endometrial cancer in our patient population. Reduced levels of the *E-cadherin* protein were frequently observed in the patients with aberrant methylation of *E-cadherin*, suggesting that the inactivation of *E-cadherin* by aberrant methylation could be associated with changes in the localization of *ß-catenin* in endometrial cancer. The aberrant methylation of *E-cadherin* has also been found in G3 adenocarcinoma (19), but no correlation with the localization of *ß-catenin* has been investigated. The aberrant methylation of *E-cadherin* was not detected in the patients with atypical endometrial hyperplasia, which is considered pathologically to be Stage 0 endometrial cancer, but was found in the patients with Stage Ia or higher endometrial cancer. This suggests that the aberrant methylation of *E-cadherin* is not involved in early-stage carcinogenesis, in contrast to *hMLH1*.

Similar to the aberrant methylation of the *hMLH1* gene, the aberrant methylation of *APC*, a component of the Wnt signaling pathway, was observed in 7.3% of the patients with atypical endometrial hyperplasia and 22% of the patients with endometrial cancer; however, no reduction in the levels of the *APC* protein was observed. Therefore, our results indicate that there is no relationship between the inactivation of *APC* by aberrant methylation and the onset of endometrial cancer.

The frequencies of the aberrant methylation of *RAR-ß* and *p16* were 2.3% and 0% in the endometrial cancer patients, respectively, which are significantly lower than those in cancers of other organs. These results suggest that the type and frequency of genes undergoing aberrant methylation in endometrial cancer are specific and differ from those in other cancers. The aberrant methylation of the promoter region of *p16* has been reported in 20% of non-Japanese patients with endometrial cancer (20); the difference between this result and our study suggests that the frequencies of aberrant DNA methylation in endometrial cancer may also vary between races. Furthermore, aging is generally thought to be an important factor for aberrant DNA methylation, but we found no tendency for increased aberrant methylation in elderly patients with endometrial cancer. The mechanism of the induction of aberrant DNA methylation may also differ widely between organs and tissues (21), and this may account for the differences in results between the studies.

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