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Genetic Alteration of *p16*^{INKA4A} Promoter Region in Endometrial Carcinoma

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Objective : This study was performed to evaluate the status of *p16* tumor suppressor gene in 25 endometrial carcinomas (ECs) and to correlate the loss of heterozygosity (LOH) at *p16* locus, the presence of inactivating mutations, the methylation status of the promotor, and the expression of *p16* protein with clinicopathological parameters.

Methods : Methylation-specific PCR (MSP) distinguishes unmethylated from methylated alleles in a given gene on sequence changes produced after bisulfite treatment of DNA. Allelic losses were determined at two polymorphic dinucleotide repeat microsatellite markers of the *p16* gene on chromosome 9p21 that included D9S974 and D9S1748 at CDKN2A. Mutations were analyzed by exons 1 and 2 of *p16* PCR-SSCP. Immunohistochemical staining for p16 protein was performed. The associations between genetic alterations of the *p16* and the clinicopathological parameters of ECs were evaluated by chi-squared or Fisher's extraction tests.

Results : The median age of the 25 cases was 52 years, ranging from 32 to 72. The median tumor size was 3.6 cm, ranging from 0.8 to 9.5 cm. Histologically, the ECs were 21 endometrioid, 2 adenosquamous, 1 secretory and 1 papillary serous types. Nine cases of p16 protein staining were negative or minimal positive in 25 ECs (36%). Allelic losses were found in 6 loci (66.7%) of 5 ECs without p16 protein expression (Fisher's extraction test, p=0.0029), In this study, only 2 of 25 ECs (8%) disclosed mutations. Non-endometrioid (secretory and adenosquamous) carcinomas showed more frequent mutation and methylation than endometrioid carcinomas (p=0.043) and high grades (G3, p=0.018) showed more frequent mutation and methylation than low grade ECs.

Conclusion : This study suggests that methylation of p16 promoter region seems not to be common (only 9.5% in our present series) and not to be associated with loss of nuclear p16 protein expression. Loss of p16 protein indicates a higher frequency of LOH, which contributes to the development of high grade or aggressive ECs. The mechanism of p16 inactivation is not clear, so other genetic or nongenetic mechanisms for inactivation should be further studied.

Key Words **:** Endometrial carcinoma, p16 promoter

Introduction

The *p16*^{INKA4A} *(p16)* gene is located on chromosomal locus 9p21 and this tumor suppressor gene encodes a nuclear protein participating in the regulation of the cell-cycle through the inhibition of cyclin-dependent kinase (cdks) 4 and 6 interaction with cyclin D1.[1,2] In the absence of p16 protein, cdk 4/6 binds to cyclin D1 and phosphorylates the retinoblastoma protein (pRB). This mechanism leads to the deregulation of the pRB which blocks at the G1/S phases of the cell cylce and, thus, to cell proliferation.[3]

Inactivation of the *p16* has been reported in different human malignancies, and the predominant mode of inactivation appears to be by homozygous deletions and methylation, but mutations of this gene appear to be less common. Salvesen et al described that loss of nuclear p16 protein expression is not associated with promoter methylation but defines a subgroup of aggressive endometrial carcinoma (EC) with poor prognosis.[4]

Germ-line mutations of the *p16* have been demonstrated in kindreds of familiar melanoma and pancreatic adenocarcinoma.[5,6] Chromosomal deletions and point

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mutations of the *p16* locus have also been noted in various tumor types, including pancreatic adenocarcinomas, colon carcinomas, extrahepatic bile duct cancers, and squamous cell carcinomas of the head and neck.[7-9] Point mutations of *p16* in human ECs were detected only in a limited percentage of the cases investigated, suggesting a low incidence of those genetic alterations in malignant uterine tumors.[10-12] Recently, several cell lines of evidence have pointed out that hypermethylation at the promoter region of the *p16* may represent a predominant mechanism of gene silencing in human tumors.[13-15] Aberrant methylation of promoter-associated 5-CpG islands of *p16* was found in the subgroup of ECs, although some studies dealing with this subject significantly differ in the incidence of the methylation status.[4,16] Semczuk indicated that *p16* inactivation plays a role in the tumorigenesis of the subset of sporadic ECs, particularly in cases exhibiting an aggressive clinical behavior.[17]

This study was performed to evaluate the status of *p16* tumor suppressor gene in 25 ECs and to correlate the loss of heterozygosity (LOH) at *p16* locus, the presence of inactivating mutations, the methylation status of the promotor, and the expression of *p16* protein with clinicopathological parameters.

Material and Methods

1. Clinical and morphological features

The clinical and morphological data were summarized in Table 1. When the twenty five ECs were analyzed by modified World Health Organization (WHO) and International Society of Gynecological Pathologists (ISGYP) classification of ECs, 21 endometrioid, 2 adenosquamous, 1 secretory, and 1 papillary serous histological types were obtained from the Department of Pathology in Kyungpook National University Hospital. According to hormonal status, ECs were classified to 17 type I (estrogen-related) and 8 type II (non-estrogen-related).[18]

The tissues were routinely fixed in 10% buffered formalin and processed into paraffin blocks according to the established protocols.[19] The tissue blocks with sufficient tumor tissue were randomly selected for study of immunohistochemistry (IHC) using tissue microarray and genetic study. Grading was based on the architectural pattern, nuclear features, or both. The architectural grade was determined by the extent to which the tumor was composed of solid masses of cells as compared with well-defined glands. Grade 1 is no more than 5% of the tumor, composed of solid masses; Grade 2, 6-50% of the tumor, composed of solid masses; Grade 3, more than 50% of the tumor, composed of solid masses.

2. Tissue microarray (TMA)

TMA technology was used to perform IHC. Before arraying from each hematoxylin and eosin (H&E) stained slide, the three pathologists defined morphologically representative areas of the 25 tumors. For the tissue microarray construction, small tissue cylinders (diameter 2 mm) were punched from 25 formalin-fixed and paraffin-embedded different tissues (the donor blocks) and placed into a single empty recipient master block, using a custom-built precision. After construction of the array block, multiple consecutive tissue samples were cut until all the tissue samples were represented on a single section.[20] One such section was placed on a microscopic slide and was H&E-stained for histological verification of adequacy of the arrayed tumor tissues. Sections were placed on charged polylysine-coated slides for standard IHC techniques.

3. Immunohistochemical staining for p16 protein

Tissue sections with 5 μm in thickness were obtained from tissue microarray blocks. Briefly, the tissue sections were deparaffinized, rehydrated, incubated with preheated Epitope Retrieval Solution (Dako, USA) in the staining jar for 40 minutes (min) at 95-99℃. The slides were allowed to cool for 20 min at room temperature and washed with washing buffer (Dako, USA) for 5 min. Sections were blocked with Peroxidase-Blocking Reagent (Dako, USA) for 5 min, washed for 5 min and incubated with Anti-Human *p16* reagent (1:20, Dako, USA) for 30 min. Sections were rinsed with fresh washing buffer for 5 minutes, incubated with Visualization Reagent (Dako, USA) for 30 minutes. After rinsed with fresh washing buffer two times (each time for 3 minutes), the sections were incubated with Substrate-Chromogen Solution (DAB, Dako, USA) for 10 minutes. The slides were washed and counterstained with Mayers hematoxylin, rinsed slides in

distilled water and were mounted.

Intensity was recorded as 0 to 3. The percentage of nuclear staining was recorded as 0=no tumor cells positive, 1=less than 10% of the tumor cells positive, 2=more than 10% of the tumor cells positive. A cut-off value of less than 10% for nuclear staining cells was used to separate lack of staining.[21] Three pathologists interpreted and counted the IHC slides.

ECs were classified[18] according to the hormonal status of ECs using IHC for estrogen receptor (ER) and progesterone receptor (PR). Immunohistochemical staining was used Cap plus detection kit (Zymed, USA). The slides were incubated with blocking solution (Zymed, USA) for 30 minutes at room temperature to reduce nonspecific background staining. Sections were then incubated overnight at 4℃ with ER Ab-16 polyclonal antibody (1:200, Neomarker, USA) and PR Ab-8 (1:50, Neomarker, USA). After washing 1×PBS for 5 minutes thrice, the sections were incubated with biotinylated secondary antibody for 30 minute at room temperature, rinsed in 1×

Table 1. Summary of Clinicopathologic and Genetic Data in 25 Endometrial Carcinomas

No.	Age	WHO	Two types	Grade	FIGO stage	LOH		Mutation		Methylation	IHC		
						D9S974	D9S1748	p16E1	p16E2		P16	ER	PR
1	61	Endometrioid	1	1	1c	(-)	(+)	(-)	(-)	(-)	0	1	1
2	48	Endometrioid	1	1	1b	(+)	(-)	(-)	(-)	(-)	0	1	0
3	41	Secretory	1	1	1b	(+)	(+)	(-)	(+)	(-)	0	1	1
4	56	Endometrioid	1	1	3c	(-)	(-)	(-)	(-)	N.D.	0	0	1
5	47	Endometrioid	2	2	3c	(-)	(+)	(-)	(-)	(-)	0	0	1
6	53	Endometrioid	1	1	1b	(+)	(-)	(-)	(-)	N.D.	0	0	1
7	32	Adenosquamous	1	1	1c	(-)	(-)	(-)	(-)	(-)	0	1	1
8	47	Endometrioid	1	1	1c	(-)	(-)	(-)	(-)	(-)	0	1	1
9	47	Endometrioid	1	1	3a	(-)	(-)	(-)	(-)	(-)	0	1	1
10	41	Endometrioid	1	1	1b	(-)	(-)	(-)	(-)	(-)	1	1	1
11	59	Endometrioid	1	1	1b	(-)	(-)	(-)	(-)	(-)	1	1	1
12	48	Endometrioid	2	2	1a	(-)	(-)	(-)	(-)	N.D.	1	0	1
13	58	Endometrioid	1	1	1a	(-)	(-)	(-)	(-)	N.D.	2	1	1
14	39	Endometrioid	2	3	1b	(-)	(-)	(-)	(-)	(-)	1	0	1
15	64	Endometrioid	1	1	1a	(-)	(-)	(-)	(-)	(-)	1	1	1
16	48	Endometrioid	2	1	1a	(-)	(-)	(-)	(-)	(-)	1	0	1
17	74	Papillary serous	2	1	1c	(-)	(-)	(-)	(-)	(-)	2	1	1
18	50	Adenosquamous	2	3	1c	(-)	(-)	(-)	(-)	(+)	2	0	1
19	48	Endometrioid	1	1	1b	(-)	(-)	(-)	(-)	(-)	2	1	1
20	51	Endometrioid	1	1	1b	(-)	(-)	(-)	(-)	(-)	1	1	1
21	46	Endometrioid	1	2	1a	(-)	(-)	(-)	(-)	(+)	2	1	1
22	69	Endometrioid	2	1	1c	(-)	(-)	(-)	(-)	(-)	2	0	1
23	55	Endometrioid	2	1	1b	(-)	(-)	(-)	(-)	(-)	2	0	1
24	72	Endometrioid	1	1	3a	(-)	(+)	(-)	(-)	(-)	2	1	1
25	60	Endometrioid	1	3	1c	(-)	(-)	(+)	(-)	(-)	2	1	1

WHO: World Health Organization classification of endometrial carcinoma,
IHC: immunohistochemistry,
Two types: estrogen related ECs and estrogen unrelated ECs,
LOH: loss of heterozygosity,
ER: estrogen recepter,
PR: progesteron recepter

Table 2. Sequences of the primers used for analysis of *p16* genetic alterations

Primer	Sequence (5'-3') Forward	Sequence (5'-3') Reverse	Description
D9S974	GAG CCT GGT CTG GAT CAT AA	AAG CTT ACA GAA CCA GAC AG	LOH
D9S1748	CAC CTC AGA AGT CAG TGA GT	GTG CTT GAA ATA CAC CTT TCC	LOH
p16 E1	GGG AGC AGC ATG GAG CCG	AGT CGC CCG CCA TCC CCT	Mutation
p16 E2	ACA AGC TTC CTT TCC GTC ATG CCG	CCA GGC ATC GCG CAC GTC CA	Mutation
p16-U	TTA TTA GAG GGT GGG GTG GAT TGT	CAA CCC CAA ACC ACA ACC ATA A	Methylation
p16-M	TTA TTA GAG GGT GGG GCG GAT CGC	GAC CCC GAA CCG CGA CCG TAA	Methylation

p16-U: unmethylation specific primer,
p16-M: methylation specific primer,
LOH: loss of heterozygosity

PBS for 5 minutes thrice. The slides were incubated streptavidin- HRP conjugate (Zymed, USA) for 30 minutes at room temperature followed by repeated washing 1× PBS for 5 minutes twice. Next the sections were incubated with 3, 3'-diaminobenzidine substrate (Zymed, USA) for 5 minutes, stop in deionized water, counterstained with Mayer's hematoxylin, rinsed in tap water. The slides were dehydrated in graded dilutions of ethanol (70%, 85%, 95%, 100%) for 1 minute, cleared in xylene for 10 minutes and mounted with malinol. For evaluation of ER and PR staining, each tumor was scored: 0, <10% tumor cell staining or background type staining; 1+, >10% positive nuclear staining.

4. Microdissection and DNA extraction

Ten serial sections with 7 μm thickness were obtained from the paraffin-embedded blocks, and stained with H&E. The target slides were placed on the stage of a microscope preparation of the tumor specimens and were precisely performed under 40x magnification microscopic visualization using surgical blade. Selected, normal uterine cervical and salpingeal cells were used as a source of the control DNA for each case. After scrapping of the non-neoplastic tissue, remained tumor cells accounted for more than 90% of the cells.[19] The microdissected cells, adhering to the blade was carefully submerged in a 1.5 mL eppendorf tube containing 0.5 mL DNA extraction buffer (100 mM Tris-HCL, pH 8.0; 1% Tween 20.0, 1 mM EDTA), and add 0.1 mg/mL proteinase K. The obtained tissue was then incubated at 50℃ for two days. The mixture was then boiled for 5 minutes to inactivate the proteinase K and the DNA was extracted using standard techniques. The extracted genomic DNA was stored at -20℃. DNA from the tumor and normal tissues of each cases were obtained, separately.

5. Bisulfite treatment of genomic DNA

Genomic DNA was subjected to bisulfite modification, as previously described.[22] Briefly, 2 μg of DNA was denatured in 0.2 M NaOH at 37℃ for 15 min. To the denatured DNA, 3M sodium bisulfite (pH 5.0) and 10 mM hydroquinone were added, and the sample was incubated at 50℃ for 16 hours. The sample was desalted with the Wizard DNA cleanup system (Promega, USA) and desulfonated by treatment with NaOH at a final concentration of 1 M. The DNA sample was ethanol-precipitated with ammonium acetate and dissolved in 20 μl of TE buffer.

6. Methylation-specific PCR (MSP)

MSP distinguishes unmethylated from methylated alleles in a given gene on sequence changes produced after bisulfite treatment of DNA, which converts unmethylated, but not methylated, cytosines to uracil. Modified DNA (100-200 ng) was amplified with specific primers designed for methylated and unmethylated DNA. Primer sequences of *p16* for the unmethylated reaction (Table 2) were 5'-TTA TTA GAG GGT GGG GTG GAT TGT-3' (sense) and 5'-CAA CCC CAA ACC ACA ACC ATA A-3' (antisense). Those for the methylated reaction were 5'-TTA TTA GAG GGT GGG GCG GAT CGC-3' (sense) and 5'-GAC CCC GAA CCG CGA CCG TAA-3' (antisense).

Primers were purchased from Cosmogentech (Seoul,

Korea). The PCR mixture, contained 1×PCR buffer, dNTPs (2.5 mM each), primers (10 pmol each), modified DNA and 1.0 U of AmpliTaq Gold (Perkin-Elmer, USA) in a final volume of 20 μl. Amplifications were performed for 40 cycles (35 sec at 95℃, 35 sec at 60℃ and 35 sec at 72℃), after an initial denaturation at 95℃ for 10 min, and followed by a final extension at 72℃ for 10 min. PCR products were electrophoresed in a 2.0% agarose gel, stained with ethidium bromide and visualized under the UV illumination. DNA from the SNU182 and SNU398 cell lines were utilized as a positive control for the *p16* unmethylated and methylated reactions, respectively.

7. LOH study for *p16*

Allelic losses were determined at two polymorphic dinucleotide repeat microsatellite markers of the *p16* gene on chromosome 9p21 that included D9S974 and D9S1748 at CDKN2A.[23] The primers were synthesized in Cosmogentech (Seoul, Korea). The sequences of used primers were as follows (Table 2), D9S974: 5'-GAG CCT GGT CTG GAT CAT AA -3' (sense), 5'-AAG CTT ACA GAA CCA GAC AG-3' (antisense), D9S1748: 5'-CAC CTC AGA AGT CAG TGA GT (sense) -3', 5'-GTG CTT GAA ATA CAC CTT TCC-3' (antisense).

PCR amplification of marker loci was carried out on paired normal-tumor DNAs in 20 μl reaction volume containing 100 ng template DNA, 10 pmol each forward in PCR premix (Bioneer, Korea). Amplifications were performed for 40 cycles (30 sec at 95℃, 30 sec at 52℃ and 30 sec at 72℃), after an initial denaturation at 95 ℃ for 10 min, followed by a final extension at 72 ℃ for 10 min. The PCR products were denatured and then loaded

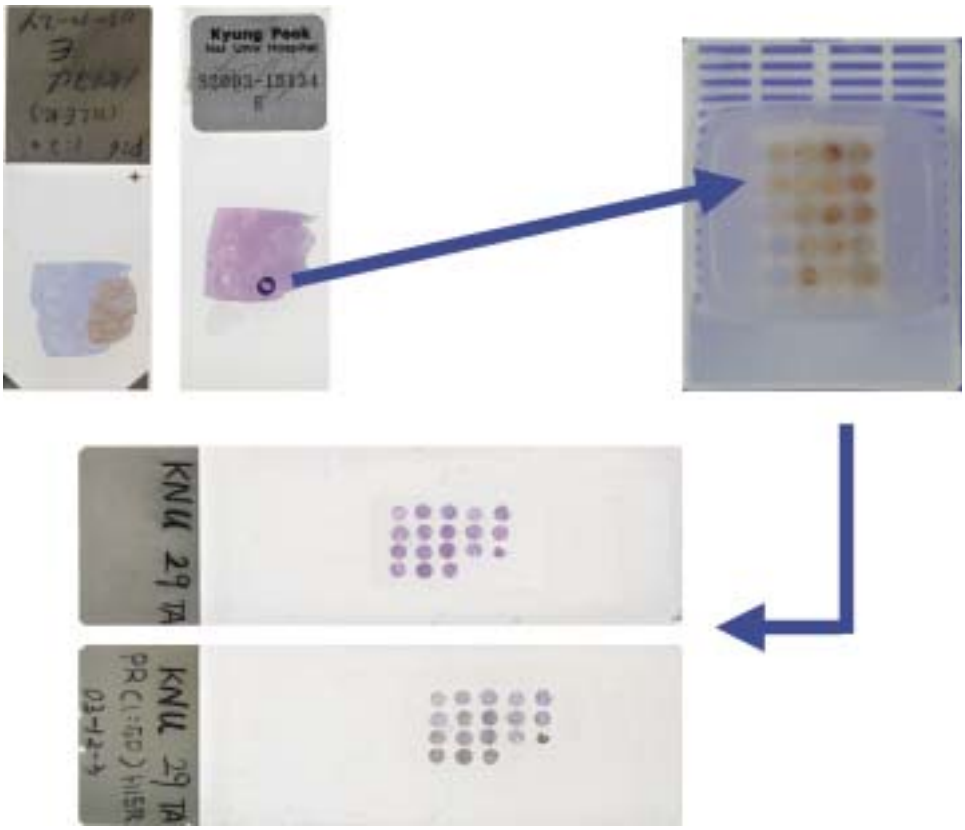

Fig. 1. For the TMA construction, small tissue cylinders (diameter 2 mm) were punched from 25 formalin-fixed and paraffin-embedded different tissues (donor blocks) and placed into a single empty master block, using a custom-built precision. After construction of the array block, multiple consecutive tissue samples were cut until all the tissue samples were represented on a single section. Sections were placed on charged polylysine-coated slides for standard immuno-histochemical techniques.

onto a 7% denaturing polyacrylamide gels containing 7 M Urea, and run at 400 V for 3 hours at room temperature. The gel was stained with silver stain plus kit (Bio-Rad, USA). LOH was defined as a visible reduction of 50% or more in the band intensity of the tumor sample when compared to the corresponding normal band.

8. Mutation analysis of p16

Mutations were analyzed by PCR-SSCP. Exons 1 and 2 of the *p16* were amplified by use of the following primers: exon 1: sense 5'-GGG AGC AGC ATG GAG CCG-3' and antisense 5'-AGT CGC CCG CCA TCC CCT-3', exon 2: sense 5'-ACA AGC TTC CTT TCC GTC ATG CCG-3' and antisense 5'-CCA GGC ATC GCG CAC GTC CA-3' (Table 2). The PCR program was same condition with above LOH method except annealing temperature (58℃). PCR product was boiled for 5 min and chilled in ice. Sample was loaded onto 15% non-denaturing polyacrylamide gels, and run at 200 V for 8 hours at 4℃. The gels were stained with silver nitrate. Briefly, gel was fixed in 10% acetic acid for 30 min, and three times washed by distilled water for 10 min. Stained with 0.1% silver nitrate (with formaldehyde) and then developed with 3% sodium carbonate. The reaction was stop in 10% acetic acid.

9. Data analysis

The associations between genetic alterations of the *p16* and the clinicopathological parameters of ECs were evaluated by chi-squared or Fisher's extraction tests. The statistical difference was considered to be significant if the *p* value was less than 0.05. The data were analyzed with the Statistical Package Service Solution software (SAS version 8.12).

Results

1. Clinicopathological characteristics

The median age of the 25 cases was 52 years, ranging from 32 to 72. The median tumor size was 3.6 cm, ranging from 0.8 to 9.5 cm. Histologically, the ECs were 21 endometrioid, 2 adenosquamous, 1 secretory and 1 papillary serous types. Twenty-one cases (84%) of ECs were in stage I and 4 in stage III. Four patients showed

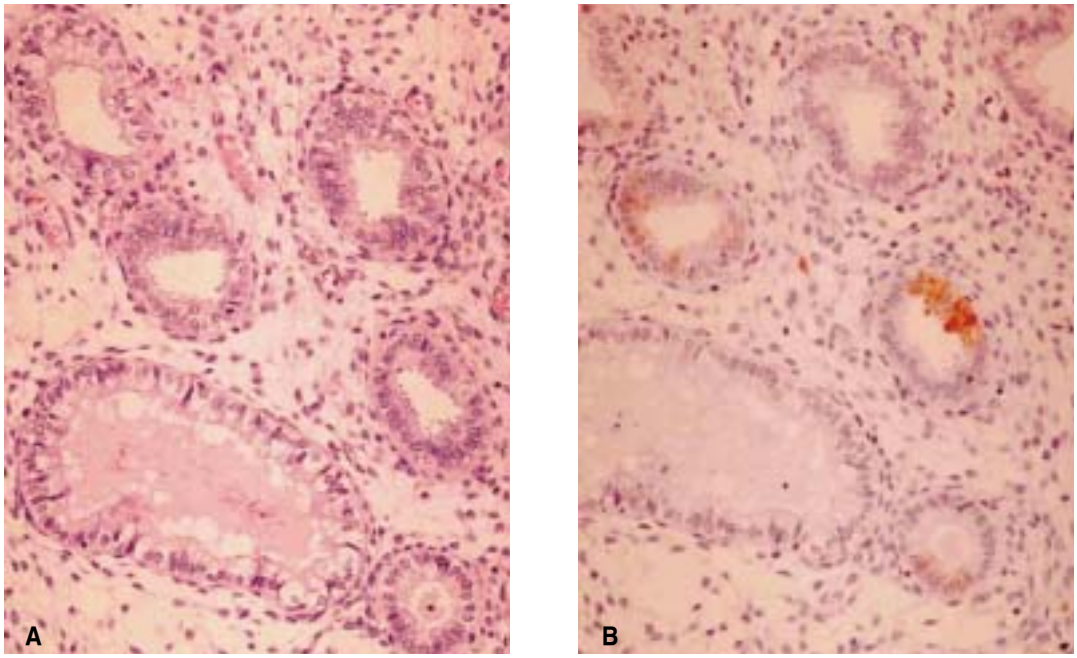

Fig. 2. Normal endometrial secretory endometrium (A) shows focal nuclear and cytoplasmic staining (grade 1) of the p16 protein in glandular epithelial and stromal cells (B).

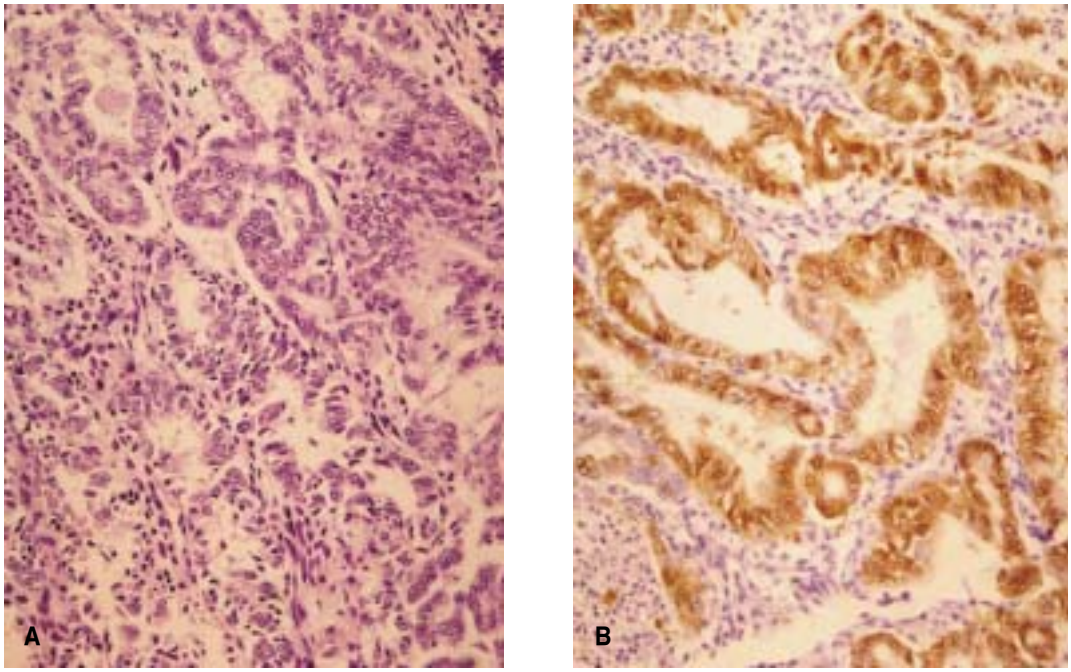

Fig. 3. An endometrioid EC (A) shows diffuse grade 3 immunostaining in the nuclei and cytoplasm of the anaplstic glanddular cells (B).

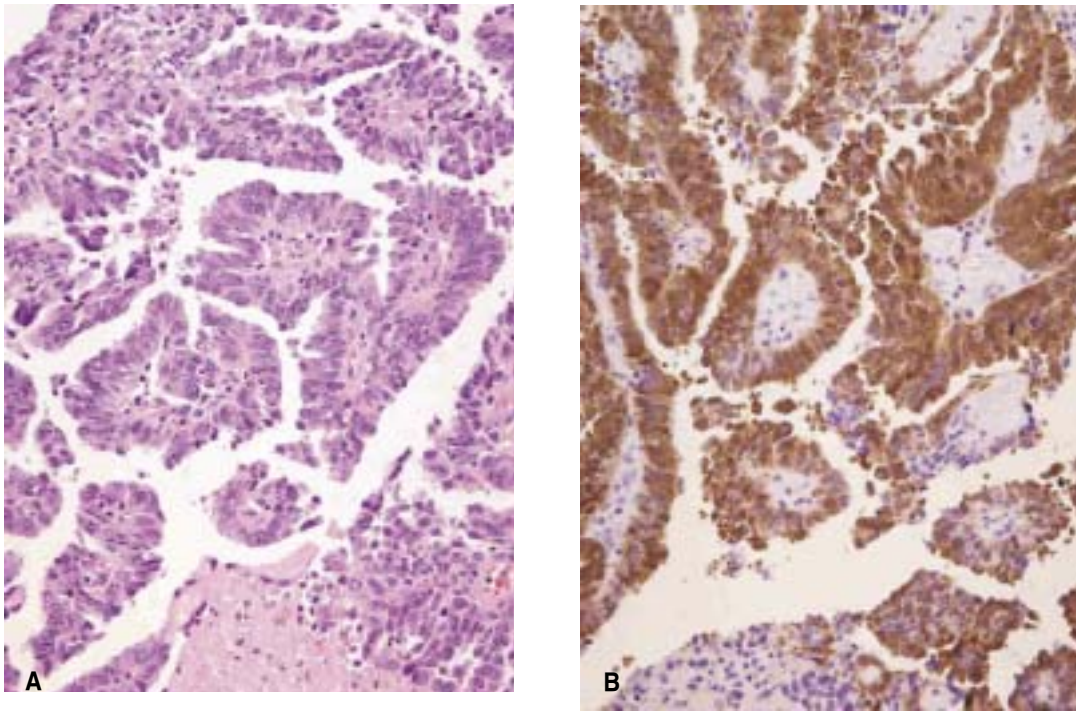

Fig. 4. A serous papillary EC (A) shows especially strong immunoreactivity for p16 protein. Diffuse strong (grade 3) nuclear and cytoplasmic staining is noted (B).

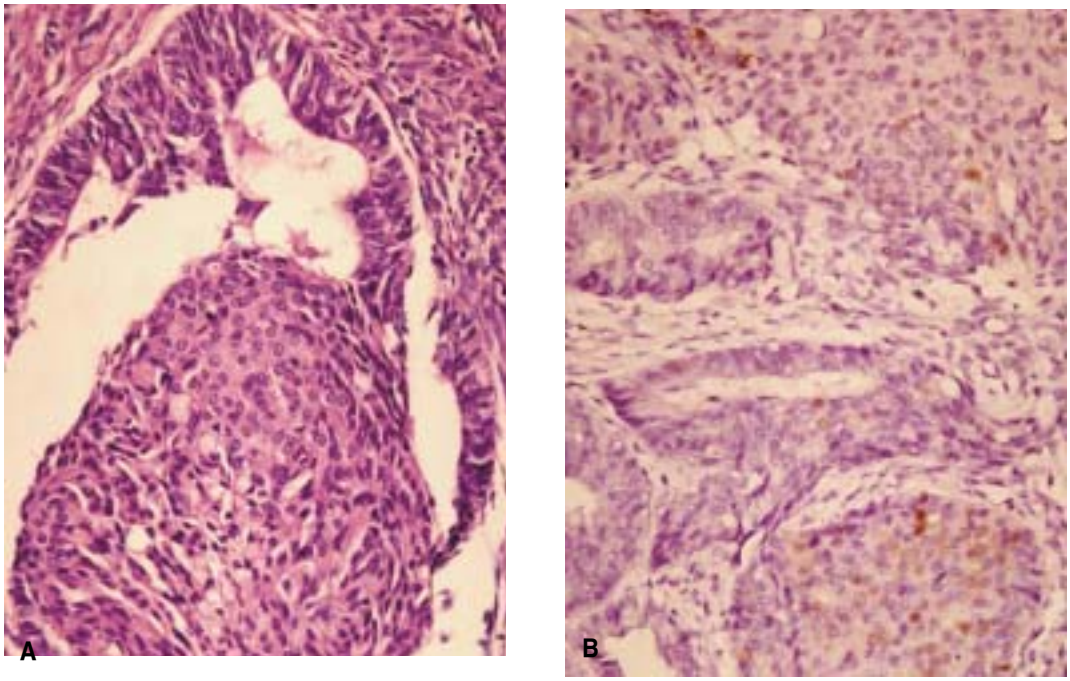

Fig. 5. An adenosquamous type EC (A) shows focal staining for p16 protein in adenocarcinoma portion. But the squamous portion shows absent or minimal staining pattern (B).

parametrial lymph nodes and/or adnexal metastasis. When grading on the architectural pattern and nuclear features, or both, ECs showed 21 Grade1, 3 Grade2 and 3 Grade3. The clinical and morphological data were summarized in Table 1.

2. Correlation of p16 protein with clinicopathological factors

IHC of normal uterine endometrium showed minimal or focal staining (grade 1) in nuclei of the endometrial glands, stroma and myometrial spindle cells. Secretory endometrium revealed focal nuclear and cytoplasmic staining (grade 1) for the p16 protein in glandular epithelial and stromal cells (Fig. 2), but in proliferative endometrium, the glandular nuclear staining was rare. In ECs, the p16 immunostaining pattern was a little different according to WHO histological classification of ECs. Endometrioid ECs usually showed diffuse immunostaining (grade 3) in the nuclei and cytoplasm of the anaplastic glandular cells (Fig. 3). A serous papillary EC shows especially strong nuclear and cytoplasmic immunoreactivity for p16 protein (Fig. 4). Two adenosquamous type of ECs showed focal staining for p16 protein in adenocarcinoma portion, but the squamous portion showed absent or minimal staining pattern (Fig. 5). Myometrial invasion, lymph node metastasis, and lymphatic invasion did not statistically correlate with clinicopathological parameters (Table 3).

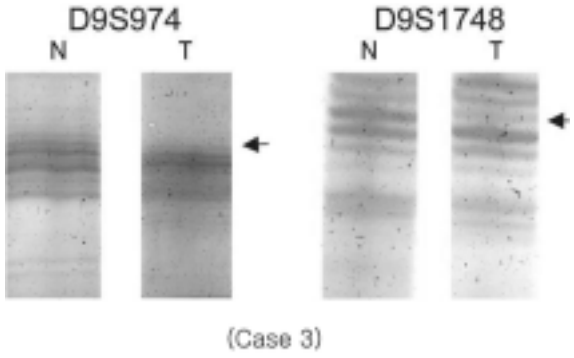

Fig. 6. LOH analysis at D9S974 and D9S1748 markers in ECs (T3) and corresponding normal tissue (N3). Both allelic losses in case 3 are indicated as two arrows.

3. Genetic Alterations of p16 with clinicopathological factors

Clinical and pathological charateristics of ECs in relation to genetic alterations and IHC were presented in Table 3. LOH analyses at D9S974 and D9S1748 loci of

Table 3. Clinical and Pathological Characteristics of Endometrial Carcinoma Patients in Relation to Genetic Alterations and Immunohistochemistry of *p16*

Characteristic	No.	*p16* LOH n(%)	*p16* mutation or methylaion n(%)	*P16* Positive IHC n(%)
Patient age (years)				
≤65	22	5 (22.73)	4 (18.18)	13 (59.09)
>65	3	1 (33.33)	0 (0)	3 (100)
		P=0.6866	P=0.4203	P=0.1661
Clinical stage				
I	21	4 (19.05)	4 (19.05)	15 (71.43)
II-III	4	2 (50.00)	0 (0)	1 (25.00)
		P=0.1840	P=0.3409	P=0.0762
Histological type				
Endometrioid	21	5 (23.81)	2 (9.52)	14 (66.67)
Non-endometrioid	4	1 (25.00)	2 (50.00)	2 (50.00)
		P=0.9592	P=0.0430	P=0.5245
Histological two type				
Type 1	17	5 (29.41)	3 (17.65)	9 (52.94)
Type 2	8	1 (12.50)	1 (12.50)	7 (87.50)
		P=0.3557	P=0.7443	P=0.0931
Histological grading				
G1	19	5 (26.32)	1 (5.26)	11 (57.89)
G2	3	1 (33.33)	1 (33.33)	2 (66.67)
G3	3	0 (0)	2 (66.67)	3 (100)
		P=0.5637	P=0.0180	P=0.3671
Myometrial invasion				
0	8	1 (12.50)	1 (12.50)	7 (87.50)
1	6	1 (16.67)	1 (16.67)	4 (66.67)
2	11	4 (36.36)	2 (18.18)	5 (45.45)
		P=0.4320	P=0.9447	P=0.1671

IHC: immunohistochemistry, Histological two types: estrogen related ECs and estrogen unrelated ECs, LOH: loss of heterozygosity, Architectural grading was determined by the extent of solid masses of cells as grade 1: no more than 5% of solid tumor masses, grade 2: 6-50%, grade 3: more than 50% Myometrial invasion 0: in endometrium, 1: within half of myometrium, 2: above half of myometrial wall

Table 4. Immunohistochemistry and Genetic Alterations of the *p16* in 25 Endometrial Carcinomas

		Genetic Alterations							
		Allelic losses		Mutation		Methylation		Total	
IHC	No.	N (%)	*p*-value[2]	N (%)	*p*-value	N (%)	*p*-value	N (%)	*p*-value[2]
Negative	9	6 (66.7)[1]	0.0029	1 (11.1)	0.999	0 (0.0)	0.520	7 (77.8)	0.0168
Positive	16	1 (6.3)		1 (6.3)		2 (12.5)		4 (25.0)	
Total	25	7		2		2		11	

[1] A secretory carcinoma showed both allelic losses of D9S974 and D9S9748, and methylation of p16E2
[2] Fishers extract test

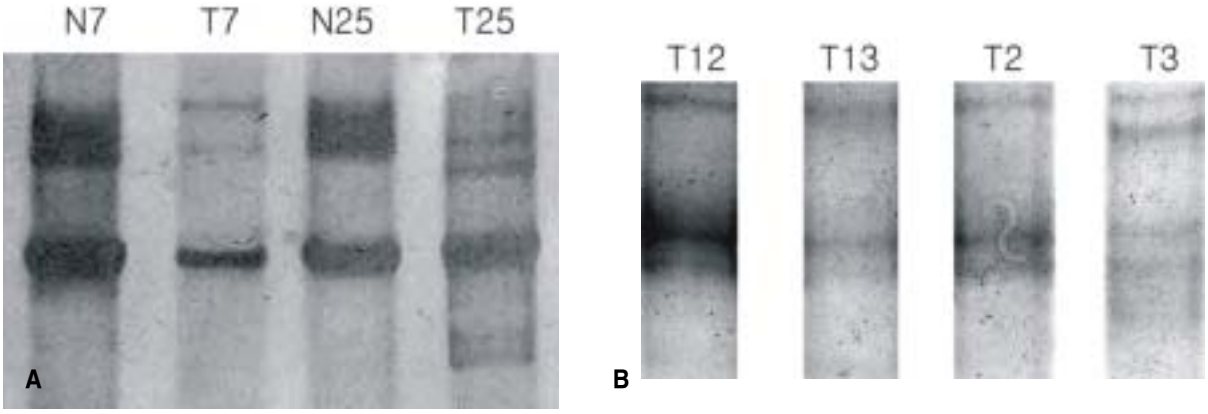

Fig. 7. Mutational analyses by PCR-SSCP of exon 1 (A) and 2 (B) of p16 gene are shown. The shifts of both bands are indicated as arrows in ECs of case 25 and case 3.

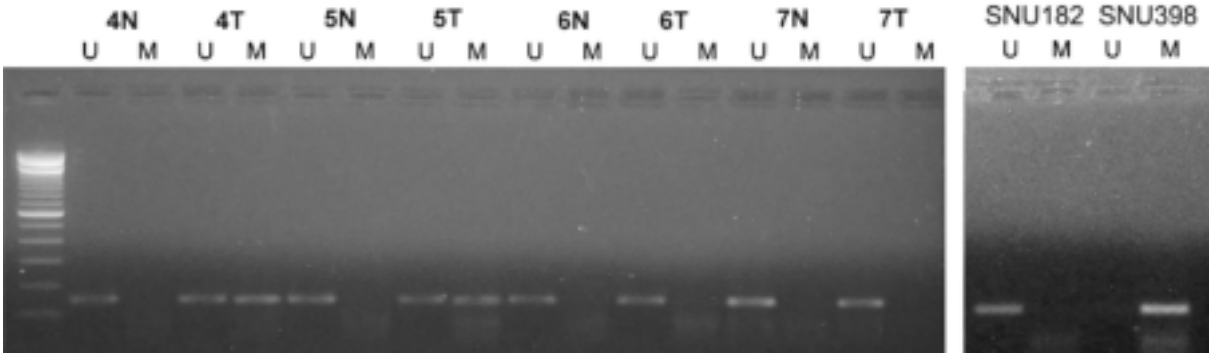

Fig. 8. MSP for p16 gene in 4 cases of ECs. U and M indicate reactions, using p16-U primers specific for unmethylated CpG sites and p16-M primers specific for methylated CpG sites, respectively. Tumor tissue (T) of case 4 & 5 shows a methylated band. N indicates normal tissue. SNU182 and SNU398 cell lines were utilized as a positive control for the p16 unmethylated and methylated reactions, respectively.

p16 gene were performed in 25 ECs and corresponding normal tissue (Fig. 6). Allelic losses of the *p16* were detected in 7 loci of 6 cases (24%). A secretory EC revealed allelic losses of both D9S974 and D9S1748.

PCR-SSCP analyses of *p16* exon 1 and exon 2 were performed in 25 sporadic ECs (Fig. 7). One of 2 tumors was endometrioid and the other was secretory type of ECs. They were represented at the Ib and Ic FIGO stages of disease.

Twenty-one cases (84%) of 25 patients were available for the study of *p16* promoter methylation status by the MSP technique. Only 2 of 25 ECs (8%) exhibited aberrant promoter hypermethylation in 5-CpG islands of the *p16*.

4. Relationship between protein overexpression and genetic alterations of *p16* gene

The comparison for overexperssion and genetic alterations of *p16* gene presented in Table 4. Nine cases of p16 protein staining were negative or minimal positive in 25 ECs (36%). Allelic losses were found in 6 loci (66.7%) of 5 ECs without p16 protein expression. Absent/minimal p16 protein in ECs were statistically related with allelic losses (p=0.0029) of D9S974 and D9S1748 (Table 4). Non-endometrioid (secretory and adenosquamous) carcinomas showed the more frequent mutation and methylation than the endometrioid carcinomas (p=0.043) and high grades (G3, p=0.018) were more frequent than the low grade ECs. A secretory carcinoma revealed multiple *p16* alterations (a mutation of p16 exon 2 and allelic losses of both D9S974 and D9S1748).

According to hormonal status,[20] ECs were classified and the type I (estrogen-related) was 17 and the type II (non-

estrogen-related) was 8 cases. But this study did not show hormonal relation with *p16* status, statistically.

Genetic alterations of $p16^{INKA4A}$ (*p16*) suppressor gene in endometrial adenocarcinomas (ECs) have only rarely been described. We examined loss of heterozygosity (LOH), promoter hypermethylation, and mutation of exon 1 and 2 in 25 sporadic ECs. Promoter hypermethylation was detected by methylation specific PCR (MSP), LOH by PCR of D9S974 and D9S1748, and point mutations by PCR-SSCP (polymerase chain reaction-single stranded conformational polymorphism) analysis for exon 1 and 2. The genetic abnormalities were compared with the p16 protein expression by immunohistochemistry (IHC) using tissue microarray (TMA) and with clinicopathological parameters.

Only 2 of 21 (9.5%) tumors exhibited aberrant promoter hypermethylation. Allelic losses of the p16 were detected in 24% (6/25) and point mutations in 9.5% (2/21). The alterations of the p16 were more frequently found in non-endometrioid (secretory and adenosquamous) (p=0.043), high grade (G3, p=0.018) and FIGO stage II or III (p=0.076) ECs. Nine cases of p16 protein staining were negative or minimally positive in 25 ECs (36%). Allelic losses were found in 6 loci (66.7%) of 5 ECs without p16 protein expression. Absent/minimal p16 protein in ECs were statistically related with allelic losses (p=0.0029).

In conclusion, this study suggest that methylation of p16 promoter region seems not to be common (only 9.5% in our present series) and not to be associated with loss of nuclear p16 protein expression. Loss of p16 protein indicates a higher frequency of LOH, which contributes to the development of high grade or aggressive ECs.

Discussion

Absent or minimal nuclear expression of p16 protein was found in 15% of the ECs in this study, but clearly lower than that was reported in two previous studies.[2,12] Shiozawa et al. and Milde- Langosch et al. found that 66% and 74% of ECs, respectively, revealed absent or minimal staining.[28,29] This contrast could be due to differences in technical methods, criteria for positivity, and patient selection. Furthermore, the differences between various primary antibodies and dilutions and incubation times and temperatures are also important. This study used TMA technology, which solved the above difficult technical problems and increased the reproducibility, because this method can perform a synchronous experiment for immunohistochemical procedure.[20] And the examination of a same tumoral portion can avoid the regional staining changes, which might be appeared because of tumoral heterogeneity.

p16 blocks progression through the cell cycle by binding to either CDK4 or CDK6, thus inhibiting the action of cyclin D.[30] The major function of cyclin D is to drive the cell cycle forward by binding to CDKs and forming a catalytically active complex that phosphorylates the pRb protein, which results in the release of E2F and new transcription of important cell cycle genes. Thus, p16 is considered a tumor suppressor gene, and its major biochemical effect is to halt cell cycle progression at the G1/S boundary.

Loss of p16 function may lead to cancer progression by unregulated cellular proliferation.[2] It is possible that deletions and/or mutations of the p16 gene may play a more significant role in ECs with minimal or low protein expression. However, in ECs, p16 alteration seems to be rare. In this study, only 2 of 25 ECs (8%) disclosed mutations. Peiffer et al. found p16 gene mutations in 2 of 34 tumors (6%). Hatta et al. did not find any alternations in 15 ECs.[10,11]

Because hypermethylation of p16 has been documented in a number of other tumor types and appears to be a common mode of inactivation of this gene,[24,25] we wanted to determine the role of p16 promoter hypermethylation in ECs, particularly in the group with loss of protein expression. In this study, two of 21 ECs (9.5%) exhibited aberrant promoter hypermethylation in 5'-CpG islands of the p16. Recent reports found that no or only 1 of the 138 cases showed hypermethylation for the p16 promoter region (0.7%) in ECs.[4,25] These reports support that hypermethylation of p16 is not the predominant mechanism of p16 inactivation in ECs.

Wong et al. found homozygous deletions in only 1 of 38 tumors (3%) and p16 gene mutaions in 5% of 38 tumors.[26] Although deletions and mutations of *p16* gene in ECs were rare, allelic losses were found in 6 loci (66.7%) of 5 ECs without p16 protein expression. Absent/minimal

nuclear staining of p16 protein was found in 15% of ECs and statistically related with allelic losses (p=0.0029) of D9S974 and D9S1748 (Table 4). Non-endometrioid (secretory and adenosquamous) carcinomas showed the more frequent mutation and methylation than the endometrioid carcinomas (p=0.043) and high grade ECs (G3, p=0.018) were more frequent than the low grade them. El-Rifai et al. found that the tendency of p16 LOH occur more frequent in malignant than in benign gastrointestinal stromal tumors (GISTs) seems to be in accordance with the comparative genomic hybridization study of who found frequent losses in 9p, particularly in malignant GISTs.[27] Moreover, the authors reported that losses in chromosome 9p occur more frequently in metastatic than in nonmetastatic GISTs, suggesting a relation with an aggressive course of the disease.

Two types of ECs had been proposed, according to hormonal status. A first, estrogen-related neoplasm that occurs in the younger, perimenopausal women and ends to be low grade and a second, more aggressive form of ECs, unrelated to estrogenic stimulation, that occurs in older postmenopausal women.[20] According to hormonal status, assisted by IHC for ER and PR, ECs were classified to type I and type II. The type I (estrogen- related) was 17 and the type II (nonestrogen-related) was 8 cases. This study did not show a correlation with the p16 alteration and the hormonal status.

In this study, mutation and methylation of p16 is associated with non-endometrioid (secretory and adeno-squamous) and high grade ECs and absent or minimal nuclear staining was related with frequent LOH and genectic alterations of p16 gene.

These results suggest that methylation of p16 promoter region seems not to be common (only 9.5% in our present series) and not to be associated with loss of nuclear p16 protein expression. Loss of p16 protein indicates a higher frequency of LOH, which contributes to the development of high grade or aggressive ECs. The mechanism of p16 inactivation is not clear, other genetic or nongenetic mechanisms for inactivation should be further studied.

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자궁내막 암종에서 *p16* promoter 부위의 유전적 이상

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목적 : 자궁내막암종에서 p16 억제 유전자의 이상에 대한 연구는 드물다. 자궁내막 암종 25예에서 p16 억제 유전자의 대립유전자 소실, promoter 과메틸화, exon 1, 2번의 변이를 조사하여 임상 및 병리학적인 변수와의 연관성을 비교함으로써 자궁내막암종의 발암과정에서 *p16* 유전자의 역할을 조사하고자 하였다.

연구 방법 : Promoter 과메틸화는 메틸화 특이적 PCR (MSP)을 이용하였고, 대립유전자 소실은 유전자 위치에 있는 D9S974와 D9S1748의 PCR을 통해서 확인하였다. 또한 돌연변이의 확인을 위해 exon 1, 2번의 PCR-SSCP 분석을 시행하였다. p16 단백에 대한 면역조직화학적 검사를 실시하였고, 상기 유전적 이상과 면역염색의 단백질 발현의 소실을 임상적 및 병리학적 매개변수와 비교하였다.

결과 : p16 단백 발현의 소실은 25예 중 9예(36%)에서 관찰되었고, 이 9예 중 대립유전자의 소실이 5예 자궁내막암의 6좌(66.7%)에서 관찰되어 통계학적으로 유의한 상관관계를 보였다(Fisher's extraction test, p=0.0029). 돌연변이는 2예(8%)에서 확인되었고, 21예 중 2예(9.5%)에서 비정상적인 promoter 과메틸화를 관찰하였다. 자궁내막암종에서 *p16*의 돌연변이와 과메칠화를 보이는 경우는 비-자궁내막형(secretory 및 adenosquamous)과 고등급(grade 3) 암종에서 자주 관찰되었다(각각 p=0.043 및 p=0.018). p16 단백 소실을 보이는 자궁내막암종 전체 9예 중에서 *p16* 유전자의 대립유전자 소실, 돌연변이, 과메틸화 3종 중 한가지 이상에서 이상을 보이는 경우는 7예(77.8%)였고, 단백 소실이 없지만 3종 중 한가지에서 이상을 보이는 예는 16예 중 4예 (25%)로써, p16 단백소실은 유전자 이상과 통계적인 유의한 상관 관계가 있었다(p=0.017).

결론 : *p16* promoter의 과메틸화는 자궁내막 선암종의 발암과정에서 중요한 경로는 아니며, 핵 내 p16 단백질의 발현 소실과도 관련은 없었지만, 자궁내막 선암종 중, 침윤성을 나타내는 예에서 관찰될 수 있다는 것을 시사한다.

중심단어 : 자궁내막암, p16 촉진제