

## Promoter Methylation of p16 Gene in Cervical Cancer

Dae Hoon Jeong, M.D.<sup>1,2</sup>, Mi Young Youm<sup>1,2</sup>, Hyun Kyung Park, M.D.<sup>1</sup>,  
Young Nam Kim, M.D.<sup>1,2</sup>, Kyung Bok Lee, M.D.<sup>1</sup>, Moon Su Sung, M.D.<sup>1</sup>,  
Ki Tae Kim, M.D.<sup>1,2</sup>, Hyun Chan KIM, M.D.<sup>1</sup>

*Department of Obstetrics and Gynecology<sup>1</sup>, Paik Institute for Clinical Research<sup>2</sup>,  
Busan Paik Hospital, College of Medicine, InJe University, Busan, Korea*

**Objective :** To investigate the promoter methylation status in the p16 gene in primary cervical cancer and to analyze the relationships between the clinicopathologic parameters and the methylation status of p16 gene.

**Methods :** Promoter methylation was evaluated by using a methylation-specific polymerase chain reaction in 78 cervical cancer tissues and 24 control non-neoplastic cervical tissues. Clinicopathologic parameters were obtained from medical records and the relationships between the discrete variables and the methylation status were evaluated.

**Results :** The frequency of the promoter methylation of p16 in cervical cancer was 57.0% (45/78). Primary cervical cancer had a significantly higher methylation frequency for p16 gene as compared to a control non-neoplastic cervix ( $p < 0.0001$ ). Higher stage cancers exhibited an increased promoter methylation frequency for p16 (45.6% in stage Ib, 58.3% in stage IIa, 88.2% in stage IIb, and 66.7% in stage IIIb,  $p = 0.0125$ ).

**Conclusion :** Our results suggest that promoter methylation of p16 is a frequent event in cervical carcinogenesis and has a potential clinical application as markers for cancer progression and prediction of prognosis.

**Key words :** Promoter, Methylation, p16, Cervical cancer

### Introduction

Cervical cancer is the second most common cancer and an important cause of death in women worldwide.<sup>1,2</sup> Multiple epidemiological and molecular biological studies indicate that the human papillomavirus (HPV) is the major causative agent in the development of cervical cancer.<sup>3-6</sup> In addition to HPV infection, it is clear that other factors are also involved in cervical carcinogenesis because the majority of patients with HPV-associated lesions such as cervical intraepithelial neoplasia do not progress to invasive cancer and remain stable or spontaneously regress over time.<sup>7</sup> Therefore, it is likely that host genetic and epigenetic events play an important role in cervical

carcinogenesis.

The term "epigenetic" refers to a heritable change in the pattern of gene expression that is mediated by mechanisms other than alterations in the primary nucleotide sequence of a gene.<sup>8,9</sup> DNA methylation is a main epigenetic modification in human that affects cell function by altering gene expression and refers to the covalent addition of a methyl group to a cytosine that precedes a guanosine in DNA sequence (CpG dinucleotide).<sup>10,11</sup> Approximately 70 to 80% of all CpG dinucleotides are methylated and occur in non-transcribed, repetitive DNA regions.<sup>10</sup> CpG dinucleotides also occur in transcribed portions of the genome, clustered in regions termed CpG islands that are located in the proximal promoter of more than half of all human genes.<sup>12,13</sup> In normal cells, most CpG islands are unmethylated and are associated with active genes or genes capable of active transcription. But, in cancer, methylation of CpG gene promoter regions are associated with inappropriate transcriptional repression and gene inactivation.<sup>14,15</sup> Significantly, many of the inactivated

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교신저자 : 김기태, 614-735 부산시 부산진구 개금동 633-165  
인제대학교 부산백병원 산부인과  
전화 : (051) 890-6428 · 전송 : (051) 897-6380  
E-mail : obgynjeong@hanmail.net

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genes are tumor suppressor genes.<sup>14,15</sup> Inhibition of tumor suppressor genes by methylation is implicated in cancer initiation, development, and progression. Therefore, it is an important mechanism for inactivating of tumor suppressor genes as an alternative to gene mutation or deletion in tumorigenesis.<sup>11,14-16</sup> In all types of cancer CpG island methylation and gene inactivation may occur. However, the target genes that are inactivated by CpG methylation are different for each cancer type.<sup>17</sup>

Recently, aberrant methylation of CpG islands within the promoter regions of several tumor suppressor genes has been reported in cervical cancer.<sup>18-27</sup> However, studies on the relationships between clinicopathologic prognostic factors and methylation status of tumor suppressor genes in cervical cancer are rare.

We investigated the promoter methylation status in tumor suppressor genes which related to the cell cycle (p16) in primary cervical cancer. We also analyzed the relationships between clinicopathologic parameters and methylation status of p16 gene in primary cervical cancer.

## Materials and Methods

### 1. Sample collection

Primary cervical cancer samples were obtained by punch biopsy from 78 patients (age range, 24-79 years; mean, 48.9 years) who were treated at Busan Paik Hospital of Inje University (Busan, Korea) between December 2001 and March 2003. None received any treatment prior to biopsy. Control non-neoplastic cervical samples were obtained from 24 patients who were treated by hysterectomy for benign gynecologic diseases (uterine leiomyoma, 20 cases; adenomyosis, 1 case; ovarian tumor, 1 case; uterine prolapse, 2 cases). A histological confirmation of diagnosis was obtained for all cases and the proportion of malignant cells in all cancer tissues used for the present study was more than 50%. The histological types of cervical cancer were classified according to the criteria of the World Health Organization (WHO). 66 patients were classified as squamous cell carcinoma and 12 patients were classified as adenocarcinoma. The stage for each cancer was established according to International Federation of Gynecology and Obstetrics (FIGO) criteria. 46 patients had a cervical cancer classified as FIGO stage

Ib, 12 had FIGO stage IIa, 17 had FIGO stage IIb, and 3 had FIGO stage IIIb. The treatment modalities for the cervical cancer patients consisted of either operation (n=33), neoadjuvant chemotherapy plus operation (n=34), concurrent chemoradiation (n=7), radiation (n=2), or loss of follow up (n=2). All cervical samples were fresh samples and were stored at -70°C until DNA isolation.

### 2. DNA extraction

Genomic DNA from the samples was isolated using the QIAmp DNA Mini kit (Qiagen, Hilden, Germany) following the instructions provided by the manufacturer.

### 3. Bisulfite modification

Bisulfite conversion of the genomic DNA was performed using the reagents provided with a CpGenome™ DNA Modification Kit (CHEMICON international, Inc, Temecula, CA, USA) according to the manufacturer's protocols. The modified DNA was eluted into 20 l of TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5), and was either used immediately as a template for methylation-specific PCR or stored at -20°C. Briefly, 1 µg of genomic DNA was denatured by sodium hydroxide, and then chemically modified by sodium bisulfite at 50°C for 16 hours.<sup>28</sup> The modified DNA was purified, desulfonated with NaOH, ethanol precipitated, and resuspended in water. After this modification procedure, all unmethylated cytosines are converted to uracil, whereas methylated cytosines remain unchanged. Uracil is recognized as thymine by Taq polymerase.

### 4. Methylation-specific Polymerase Chain Reaction (MSP)

Specific primers were used to amplify the regions of interest. One pair recognized a sequence in which CpG sites are unmethylated (bisulfite modified to UpG), and the other recognized a sequence in which CpG sites are methylated (unmodified by bisulfite treatment). PCR amplification was done with specific primer sequences for the methylated and unmethylated forms of p16 gene. Primer sequences and annealing temperatures are listed in Table 1. Each 20 µl PCR contained 1 µl of bisulfite modified genomic DNA, 1 X PCR buffer, 3.25 mM of MgCl<sub>2</sub>, 0.5 µM of each primer, 500 µM (each) dNTP mix,

**Table 1.** Primer sequences and annealing temperatures

Gene		Sequence	Ta* (°C)
p16 <sup>281</sup>	Unmethylated	5'-TTATTAGAGGGTGGGGTGGATTGT-3' (sense)	60
		5'-CAACCCCAAACCACAACCATAA-3' (antisense)	
	Methylated	5'-TTATTAGAGGGTGGGGCGGATCGC-3' (sense)	65
		5'-GACCCCGAACC GCGACCGTAA-3' (antisense)	

Ta\*: Annealing temperature

and 0.5 Units of FastStart Taq DNA Polymerase (Roche Applied Science, Penzberg, Germany). Thermal cycling was initiated at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at the specific temperature for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 10 min. Human placental DNA was treated in vitro using SssI methylase (New England Biolabs, Inc, Beverly, MA, USA) which methylate every cytosine at all CpGs and was used as a positive control for methylated alleles of each gene. PCR products were run on 2% agarose gels and visualized after ethidium bromide staining.

#### 5. Clinicopathologic parameters and statistical analysis

Clinicopathologic parameters were obtained from medical records which included the patient's age, smoking, histologic type, SCC antigen, tumor size, stage, invasion depth, and lymph node metastasis. The relationship between clinicopathologic parameters and methylation status of p16 gene were evaluated using a Chi-Square test and Fisher's Exact test. Differences were considered statistically significant for  $p < 0.05$ .

### Results

#### 1. Frequency of promoter methylation in cervical cancer

The status of promoter methylation for p16 gene in 78 primary cervical cancer tissue specimens and 24 control non-neoplastic cervical tissue specimens was examined. The promoter methylation frequency for p16 gene in cervical cancer was 57.0% (45/78) (Table 2) (Fig. 1). The promoter methylation frequency for p16 gene in control

non-neoplastic cervix was 8.3% (2/24). Cervical cancer showed a significantly higher methylation frequency for the p16 gene as compared to control non-neoplastic cervix ( $p < 0.0001$ ) (Table 2).

**Table 2.** Promoter methylation frequency in primary cervical cancer

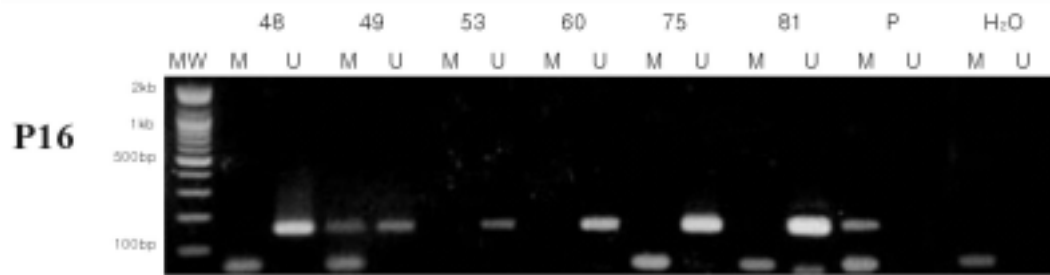
Gene	Cervical cancer (n=78)	Control cervix (n=24)	P value
p16	45 (57.0%)	2 (8.3%)	<0.0001

#### 2. The Relationship between promoter methylation and clinicopathologic parameters

Table 3 shows the methylation distribution for the four genes according to various clinicopathological parameters including age, smoking, histologic type, SCC antigen, tumor size, stage, invasion depth, and lymph node metastasis. Data on invasion depth and lymph node metastasis were obtained from patients who were treated by operation as an initial treatment, and included neither neoadjuvant chemotherapy plus operation group, concurrent chemoradiation group, radiation group, nor loss of follow up group.

A statistically significant correlation was observed between the methylation frequency of p16 and the cervical cancer stage. Higher stage cancers exhibited an increased promoter methylation frequency for p16 (45.6% for stage Ib, 58.3% for stage IIa, 88.2% for stage IIb, and 66.7% for stage IIIb,  $p = 0.0125$ ) (Table 3).

However, there were no methylation frequency associations for p16 gene with regard to age, smoking, histologic type, SCC antigen, tumor size, invasion depth, and lymph node metastasis ( $p > 0.05$ ) (Table 3).



**Fig. 1.** Methylation-specific PCR analysis of promoter methylation for p16 gene in primary cervical cancer. MW, molecular weight marker; Lane M, PCR product indicates the presence of methylated gene promoters; Lane U, PCR product indicates the presence of unmethylated gene promoters; P, positive control for methylation; H<sub>2</sub>O, negative PCR control.

**Table 3.** The relationship between promoter methylation and clinicopathologic parameters of cervical cancers

Characteristic	p16 methylation			P value
	Overall	Present	Absent	
Median age (n=78)	48.9	49.7	47.8	0.4916
<50	43	23 (62.9%)	20 (37.1%)	
≥50	35	22 (53.5%)	13 (46.5%)	
Smoking (n=78)				0.6335
Smoker	4	3 (75.0%)	1 (25.0%)	
Nonsmoker	74	42 (56.8%)	32 (43.2%)	
Histologic types (n=78)				0.2210
Squamous cell carcinoma	66	36 (54.5%)	30 (45.5%)	
Adenocarcinoma	12	9 (75.0%)	3 (25.0%)	
SCC antigen (n=78)				1.0000
<2 ng/mL	43	25 (58.1%)	18 (41.9%)	
≥2 ng/mL	35	20 (57.1%)	15 (42.9%)	
Tumor size (n=78)				0.6364
<4 cm	52	31 (59.6%)	21 (40.4%)	
≥4 cm	26	14 (53.8%)	12 (46.2%)	
Stage (n=78)				0.0125
Ib	46	21 (45.6%)	25 (54.4%)	
IIa	12	7 (58.3%)	5 (41.7%)	
IIb	17	15 (88.2%)	2 (11.8%)	
IIIb	3	2 (66.7%)	1 (33.3%)	
Invasion depth (n=33)				0.4566
<1 cm	21	12 (57.1%)	9 (42.9%)	
≥1 cm	12	9 (75.0%)	3 (25.0%)	
LN metastasis (n=33)				0.2303
No metastasis	24	17 (70.8%)	7 (28.6%)	
≥1 metastasis	9	4 (44.4%)	5 (55.6%)	

## Discussion

Methylation plays an important role in tumorigenesis. In particular, aberrant methylation of normally unmethylated CpG islands for many tumor suppressor genes is associated with transcriptional inactivation and, hence, loss of expression.<sup>11,16</sup> In the present study, I examined the methylation state for p16 gene in primary cervical cancer using methylation-specific PCR. The p16 protein plays a key role in controlling cell growth by inhibiting cyclin-dependent kinase 4 and preventing phosphorylation of the retinoblastoma protein, which maintains the G1 check point.<sup>29,30</sup>

In previous studies, the frequency of promoter methylation of p16 gene in cervical cancer was reported for 28.2-53%,<sup>18-20,23,24</sup> In the present study, the promoter methylation frequency for p16 gene in cervical cancer was 57.0% (45/78) are similar to results from previous studies. Cervical cancer had a significantly higher methylation frequency for the p16 gene as compared to control non-neoplastic cervix. The overall findings suggest that p16 methylation occur frequently and may be used as markers for cervical cancer detection. Although most studies for methylation of tumor suppressor genes have not found methylation in normal samples, the present study showed that the promoter methylation frequency for the p16 in control non-neoplastic cervix was 8.3%. The reasons for these differences is currently unclear.

In the present study, the results for the relationship between promoter methylation and clinicopathologic parameters showed that a significant correlation was observed between the methylation frequency of p16 and the stage. Higher stage cancers exhibited an increased p16 promoter methylation frequency. Wong et al.<sup>24</sup> found that p16 promoter methylation in cervical cancer was more common in tumors at advanced stages and with poorer outcome than in those at early stage and with better clinical outcome. It suggests that p16 promoter methylation may be involved in cancer progression rather than in cancer initiation for cervical cancer. However, there were no methylation frequency associations for p16 gene with regard to age, smoking, histologic type, SCC antigen, tumor size, invasion depth, and lymph node metastasis ( $p > 0.05$ ).

In conclusion, DNA methylation in cancer is rapidly evolving field that can lead to advances in the understanding of the molecular basis of cancer etiology and progression. The present study showed that promoter methylation of p16 genes is a frequent event in cervical carcinogenesis and has a potential clinical application as markers for cancer progression and prediction of prognosis. Further Larger prospective studies are necessary to establish the clinical applicability of these observations.

## References

1. NIH. Cervical cancer. NIH Consensus Statement 1996; 14: 1-38.
2. Pisani P, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer* 1999; 83: 18-29.
3. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* 1999; 189: 12-9.
4. Schiffman MH, Castle P. Epidemiologic studies of a necessary causal risk factor: human papillomavirus infection and cervical neoplasia. *J Natl Cancer Inst* 2003; 95: E2.
5. Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) study group. *J Natl Cancer Inst* 1995; 87: 796-802.
6. Waggoner SE. Cervical cancer. *Lancet* 2003; 361: 2217-25.
7. Holowaty P, Miller AB, Rohan T, To T. Natural history of dysplasia of the uterine cervix. *J Natl Cancer Inst* 1999; 91: 252-8.
8. Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev* 2002; 16: 6-21.
9. Russo VEA, Martienssen RA, Riggs AD, eds. Epigenetic mechanisms of gene regulation. Plainview, N.Y.: Cold Spring Harbor Laboratory Press, 1996.
10. Yoder JA, Walsh CP, Bestor TH. Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet* 1997; 13: 335-40.
11. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res* 1998; 72: 141-96.
12. Bird AP. CpG-rich islands and the function of DNA methylation. *Nature* 1986; 321: 209-13.
13. Gardiner-Garden M, Frommer M. CpG islands in vertebrate genomes. *J Mol Biol* 1987; 196: 261-82.
14. Li LC, Okino ST, Dahiya R. DNA methylation in prostate cancer. *Biochim Biophys Acta* 2004; 1704: 87-102.
15. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter methylation. *N Engl J Med* 2003; 349: 2042-54.
16. Jones PA, Laird PW. Cancer epigenetic comes of age. *Nat Genet* 1999; 21: 163-7.
17. Esteller M, Corn PG, Baylin SB, Herman JG. A gene methylation profile of human cancer. *Cancer Res* 2001; 61: 3225-9.
18. Yang HJ, Liu VW, Wang Y, Chan KY, Tsang PC, Khoo US, et al. Detection of hypermethylated genes in tumor and plasma of cervical cancer patients. *Gynecol Oncol* 2004; 93: 435-40.
19. Dong SM, Kim HS, Rha SH, Sidransky D. Promoter methylation of multiple genes in carcinoma of the uterine cervix. *Clin Cancer Res* 2001; 7: 1982-6.

20. Virmani AK, Muller C, Rath A, Zochbauer-Mueller S, Mathis M, Gazdar AF. Aberrant methylation during cervical carcinogenesis. *Clin Cancer Res* 2001; 7: 584-9.
21. Narayan G, Arias-Pulido H, Koul S, Vargas H, Zhang FF, Vilella J, et al. Frequent promoter methylation of CDH1, DAPK, RARB, and HIC1 genes in carcinoma of cervix uteri: Its relationship to clinical outcome. *Mol Cancer* 2003; 2: 24.
22. Widschwendter A, Gattlinger C, Ivarsson L, Fiegl H, Schneitter A, Ramoni A, et al. Analysis of aberrant DNA methylation and human papillomavirus DNA in cervicovaginal specimens to detect invasive cervical cancer and its precursors. *Clin Cancer Res* 2004; 10: 3396-400.
23. Lea JS, Coleman R, Kurien A, Schorge JO, Miller DS, Minna JD, et al. Aberrant p16 methylation is a biomarker for tobacco exposure in cervical squamous cell carcinogenesis. *Am J Obstet Gynecol* 2004; 190: 674-9.
24. Wong YF, Chung TK, Cheung TH, Nobori T, Yu AL, Yu J, et al. Methylation of p16<sup>INK4A</sup> in primary gynecologic malignancy. *Cancer Lett* 1999; 136: 231-5.
25. Widschwendter A, Muller HM, Fiegl H, Ivarsson L, Muller-Holzner E, Goebel G, et al. DNA methylation in serum and tumors of cervical cancer patients. *Clin Cancer Res* 2004; 10: 565-71.
26. Ivanova T, Vinokurova S, Petrenko A, Eshilev E, Solovyova N, Kisselov F, et al. Frequent methylation of 5' flanking region of TIMP-2 gene in cervical cancer. *Int J Cancer* 2004; 108: 882-6.
27. Widschwendter A, Ivarsson L, Blassnig A, Muller HM, Fiegl H, Wiedemair A, et al. CDH1 and CDH13 methylation in serum is an independent prognostic marker in cervical cancer patients. *Int J Cancer* 2004; 109: 163-6.
28. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl Acad Sci USA* 1996; 93: 9821-6.
29. Sherr CJ. G1 phase progression; cycling on cue. *Cell* 1994; 79: 551-5.
30. Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell* 1995; 81: 323-30.

## 자궁경부암에서 p16 유전자 촉진제의 메틸화

인제대학교 의과대학 부산백병원 산부인과학교실<sup>1</sup>, 백인제기념임상의학연구소<sup>2</sup>  
정대훈<sup>1,2</sup>, 염미영<sup>1,2</sup>, 박현경<sup>1</sup>, 김영남<sup>1,2</sup>, 이경복<sup>1</sup>, 성문수<sup>1</sup>, 김기태<sup>1,2</sup>, 김현찬<sup>1</sup>

**목적** : 자궁경부암에서 종양억제유전자인 p16 유전자 촉진제의 메틸화를 알아보고 자궁경부암의 임상병리학적 인자와의 연관성을 분석하고자 하였다.

**연구 방법** : 자궁경부암 조직 78예와 대조군으로 비신생물 자궁경부 조직 24예를 대상으로 methylation-specific PCR 방법을 이용하여 유전자 촉진제의 메틸화를 조사하였고, 자궁경부암 환자의 의무기록지를 검토하여 메틸화와 임상병리학적 인자와의 연관성을 분석하였다.

**결과** : 자궁경부암에서 p16 유전자 촉진제의 메틸화는 57.0% (45/78)로 관찰되었다. 대조군 비신생물 자궁경부 조직보다 자궁경부암 조직에서 p16 유전자 촉진제의 메틸화의 빈도가 더 높았다( $p < 0.0001$ ). 자궁경부암의 병기에 따른 p16 유전자 촉진제의 메틸화는 Ib기 45.6%, IIa기 58.3%, IIb기 88.2%, IIIb기 66.7%로 자궁경부암의 병기가 높을수록 p16 유전자 촉진제의 메틸화의 빈도는 증가하였다( $p = 0.0125$ ).

**결론** : p16과 유전자 촉진제의 메틸화는 자궁경부암의 암화과정에서 자주 일어나며 암의 진행과 예후를 예측하기 위한 표지자로서 잠재적인 임상적 이용가치가 있을 것으로 생각된다.

**중심단어** : 촉진제, 메틸화, p16, 자궁경부암