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The Relationship DNA Methylation of *p16^{INK4a}* and Colorectal Cancer

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Abstract

Promoter hypermethylation of the *p16^{INK4a}* gene was investigated in 52 sets of samples of tumor tissue and adjacent normal tissue from Korean patients with colorectal cancer, using the proposed modified the Real-time PCR/SYBR Green detection method presented in this study. In normal tissue, 29 of 52 patients (56%) were methylated and in tumor tissue, 23 of 52 patients (44%) were methylated. The 34 cases (65.4%) showed a concordant DNA methylation pattern in both normal tissue and tumor tissue. Analyzing the association between the clinicopathologic features and DNA methylation status of the *p16^{INK4a}* gene, the DNA methylation status according to by Duke's stage was different while other clinicopathological characteristics, including the age, sex, tumor stage, and histologic type of the patient were not found to be correlated with *p16^{INK4a}* methylation. With multivariate logistic regression, it was observed that the DNA methylation status of *p16^{INK4a}* gene in normal tissue was correlated with the DNA methylation status of the *p16^{INK4a}* gene in tumor tissue (P= 0.026). According to a Kaplan-Meier survival analysis, a difference in the survival rate by DNA methylation status was found, but it was not significant.

Keywords: Hypermethylation, *p16^{INK4a}*, Colorectal cancer

Colorectal cancer is the third most common type of cancer and the second leading cause of cancer-related death in the United States[1]. In Korea, the incidence of colorectal cancer has been increasing due to the change in the lifestyle of Koreans, including a more westernized diet (low fiber and high fat diet), as well as a high incidence of smoking and increasing obesity levels[2]. Colorectal cancer is properly screened, as it is a common malignancy with a long preclinical course and a high survival rate if detected in its early stage[3]. Colonoscopy is widely used as a screening test, but is not a perfect test due to its high false negative rate for small lesions (5 mm or less)[4]. Therefore, newer methods have been actively investigated[5].

Gene silencing by DNA methylation in a gene promoter as an epigenetic alteration has garnered attention as early major mechanism involved in carcinogenesis of colorectal cancer[6]. Genes associated with colorectal cancer involved with epigenetic changes include hMLH1, MGMT, GSTP1, APC and *p16^{INK4a}*[7]. It is known the hMLH1 and *p16^{INK4a}* genes are closely associated with colorectal cancer that originates in proximal sites that are difficult to detect via colonoscopy and are a more frequent cancer type in older people[8,9]. Studies related to the *p16^{INK4a}* gene are relatively insufficient compared with those of the hMLH1 gene. The *p16^{INK4a}* gene product is an inhibitor of the cyclin-dependent kinase 4 and 6 complex, preventing Rb protein phosphorylation and thereby arresting cells in the quiescent G1 phase[7,10]. If DNA methylation occurs in the promoter area of the *p16^{INK4a}* gene, the cell cycle becomes out of control due to transcriptional silencing and cancer then occurs[7,10]. The aims of the present study are to evaluate the methylation status of the promoter region of *p16^{INK4a}* in colorectal cancer tissue and to analyze the relationships between the methylation status and various clinicopathological parameters using the proposed modified the Real-time PCR/SYBR Green detection method[10].

Methylation Status of Study Population

In order to examine the methylation status of the *p16^{INK4a}* promoter region, samples of tumor and normal adjacent tissues from 52 colorectal cancer patients were analyzed. In normal tissue, DNA methylation of the promoter area in *p16^{INK4a}* gene was detected in 29 cases (56%) while in tumor tissue, 23 cases (44%) were methylated (Table 1). The methylation status in the *p16^{INK4a}* gene of the study population is described in Figure 1. With Kendall's tau B, it was observed

that the concordance of the DNA methylation status between normal tissue and tumor tissue was 0.325 (P=0.012).

The Association between Clinicopathologic Features and DNA Methylation of the $p16^{INK4a}$ Gene in Tumor Tissue

The age of the patients in the methylated group (61.96±9.57) and the unmethylated group (64.69±10.02) was not a significant factor (Table 2). In addition, a difference in the DNA methylation status according to smoking behavior and drinking behavior was not observed. Survival and tumor location had no association with DNA methylation of the $p16^{INK4a}$ gene. However, it was observed that Duke's stage showed statistical significance depending on the DNA methylation status (P=0.016). In a multivariate logistic regression test, it was observed that DNA methylation of the $p16^{INK4a}$ gene in tumor tissue correlated with DNA methylation of the $p16^{INK4a}$ gene in normal tissue. However, it was not correlated with differences in gender, total cholesterol or Duke's stage (Table 3).

Table 1. Frequency of $p16^{INK4a}$ gene methylation in normal tissue and tumor tissue.

		Tumor tissue	
		Methylation	Unmethylation
Normal tissue	Methylation	17 (32.7%)	12 (23.1%)
	Unmethylation	6 (11.5%)	17 (32.7%)

Survival Analysis by DNA Methylation of The $p16^{INK4a}$ Gene in Tumor Tissue

In the Kaplan-Meier analysis involving a log rank test, the survival rate of the methylated group was lower than that of the unmethylated group; however this difference was not significant (P=0298; Figure 2).

Discussion

This study showed that the DNA methylation rate of the $p16^{INK4a}$ gene in normal tissue (56%) and tumor tissue (43%) was higher than that found in previous studies that reported that the extent of the $p16^{INK4a}$ gene methylation ranged from 18% to 42%[11-14]. In the present study, DNA methylation status was determined quantitatively by measuring the fluorescent strength emitted from the exponential phase of PCR amplification using real-time PCR, in which more specific and precise PCR reaction was occurred compared to plateau phase measurement using a traditional PCR method. In addition, the DNA methylation levels of four points were measured quantitatively by real-time PCR; in earier studies, the DNA methylation status

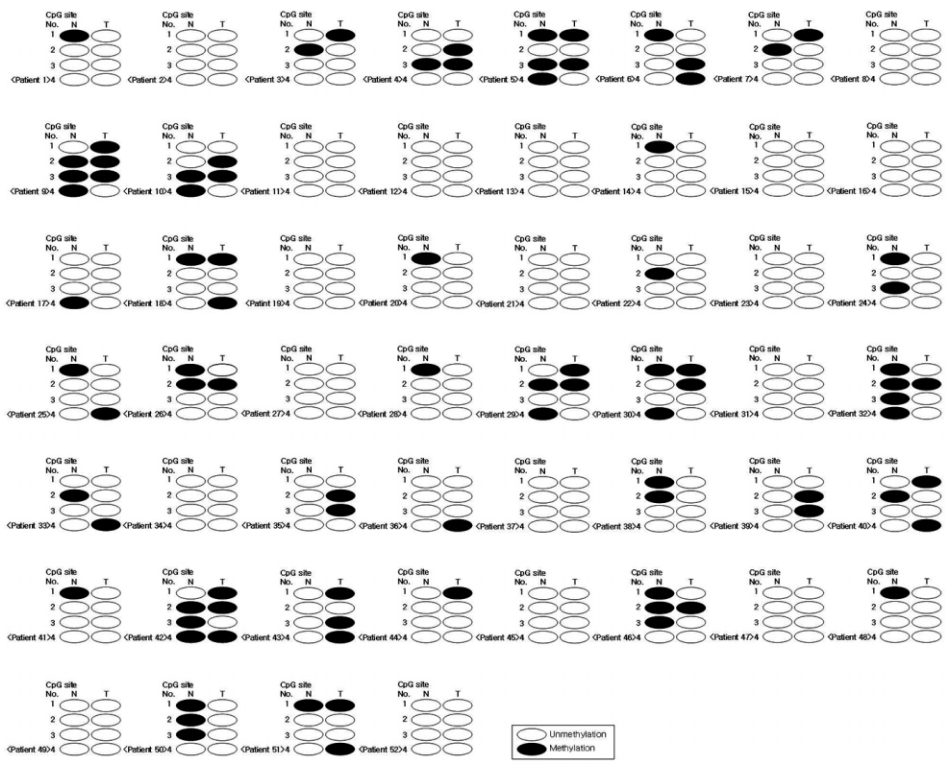

Figure 1. Methylation status of the $p16^{INK4a}$ gene prompter in normal and tumor tissues. The black circles denotes methylation positivity, whereas the open circles indicates that a sample is negative for methylation. *N: normal tissue, T: tumor tissue.

Table 2. The relationship between clincopathologic features and DNA methylation of the *p16^{INK4a}* gene in tumor tissue.

		Methylation	Unmethylation	*P*-value
Age †		61.96±9.59	64.69±10.02	0.324
Gender ‡	Male	13 (25.0%)	12 (23.1%)	0.278
	Female	10 (19.2%)	17 (32.7%)	
Smoking ‡	Non-smoker	12 (23.1%)	17 (32.7%)	0.642
	Smoker	11 (21.2%)	12 (23.1%)	
Amount of smoking (pack/years) †		35.27±14.06	30.67±9.33	0.361
Drinking ‡	Not current drinking	11 (21.2%)	14 (26.9%)	0.974
	Current drinking	12 (23.1%)	15 (28.8%)	
Amount of drinking (gram/week) †		106.56±114.14	130.45±96.14	0.600
Location ‡	Proximal	6 (11.5%)	5 (9.6%)	0.438
	Distal	17 (32.7%)	24 (46.2%)	
Total cholesterol †		201.78±94.68	171.76±30.33	0.114
Body mass index †		22.73±2.55	22.44±2.49	0.687
Survival ‡	Survival	13 (25.0%)	19 (36.5%)	0.508
	Death	10 (19.2%)	10 (19.2%)	
Survival time (month) †		27.45±12.77	29.22±11.21	0.603
Stage ‡	A	2 (3.8%)	0 (0%)	0.016*
	B	11 (21.2%)	18 (34.6%)	
	C	5 (9.6%)	11 (21.2%)	
	D	5 (9.6%)	0 (0%)	

*: P<0.05, †: *P* value was calculated by a t-test, ‡: *P* value was calculated by a Chi-square test

Table 3. The correlation between *p16^{INK4a}* gene methylation and clinicopathological features in a logistic regression test.

Variables	Odd ratio	95% confidence interval	*P*-value
Gender	0.554	1.274-26.627	0.364
Stage	0.404	0.867-1.007	0.115
DNA Methylation in normal tissue	5.46	1.230-24.279	0.026*
Total cholesterol	0.994	0.979-1.009	0.405

*: *P*<0.05

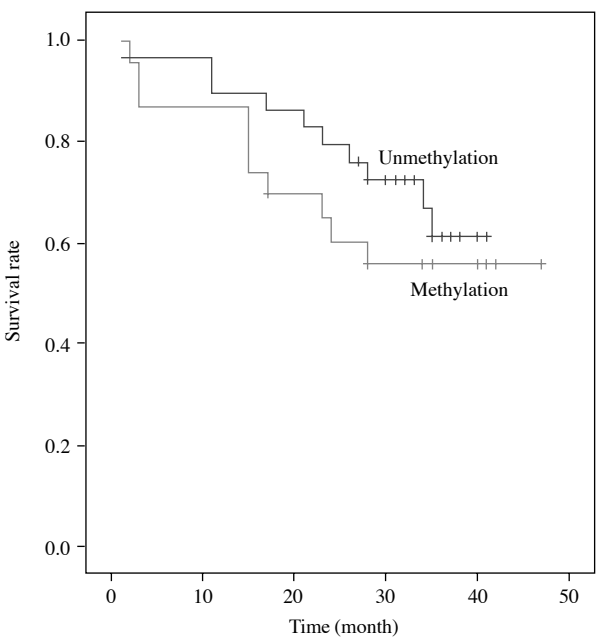

Figure 2. Kaplan-Meier's survival analysis according to DNA methylation status of the *p16^{INK4a}* gene in tumor tissue.

was identified qualitatively using one point. Therefore, the present has a great likelihood of detecting DNA methylation of the *p16^{INK4a}* gene and then showed a more prevalent DNA methylation rate compared to other DNA methylation studies using the methylation-specific PCR (MSP) method.

The strongest feature of the Real-time PCR/SYBR Green detection method is the continuous optical monitoring of the progress of fluorescent PCR reactions. Fluorescence intensity increases proportionally to the amount of PCR products. Therefore, the modified method allows conclusions to be drawn concerning the methylation status relative to a control reaction. The number of Colorectal cancer patients with proximal colon cancer in the present study was relatively low (21.1%), however, DNA methylation of the *p16^{INK4a}* gene was found more frequently in prox-

-492 ACGCACTCAA AC**ACGCCTTT GCTGGCAGGC GGG**GGAGCGC GGCTGGGAGC AGGGAGGCCG
-432 GAGGGCGGTG TGGGGGGCAG GTGGGGAGGA GCCCAGTCCT CCTTC**CTTGC CAACGCTGGC**
-372 **TCTGGCG**AGG GCTGCTTCCG GCTGGTGCCC CCGGGGGAGA CCCAACCTGG GGCGACTTCA
-312 GGGGTGCCAC ATTCGCTAAG TGCTCGGAGT TAATAGCACC TCCTCCGAGC ACTCGCTCAC
-252 AGCGTCCCCT TGCCTGGAAA **GATACCGCGG TCCCTCCAGA GG**ATTTGAGG GACAGGGTCG
-192 GAGGGGGCTC TTCCGCCAGC ACCGGAGGAA GAAAGAGGAG GGGCTGGCTG GTCACCAGAG
-132 GGTGGGGCGG ACCG**AGTGCG CTCGGCGGCT GCGGAG**AGGG GTAGAGCAGG CAGCGGGCGG
- 72 CGGGGAGCAG CATGGAGCCG GCGGCGGGGA GCAG**CATGGA GCCTTCGGCT GACTGGC**TGG
- 12 CCACGGCCGC GG

Figure 3. Methylation analysis of the *p16INK4a* promoter sequencing (Genebank accession number X94154). The positions of the CCGG sites are underlined. Each primer is shown in bold text.

imal colorectal cancer cases[8], and the rate of colorectal cancer occurring in proximal site is increasing in Korea[12]. If a greater number proximal colorectal cancer patients had participated in this study, the possibility of detecting DNA methylation of the *p16INK4a* gene would have likely increased. It is suggested that DNA methylation of the *p16INK4a* gene can offset the existing limitation of colonoscopy related to the difficulty of the detection of small lesions located in the proximal colon. Analyzing the relationship between clinicopathological features and the DNA methylation status of the *p16INK4a* gene, a significant difference was not observed in terms of age[8], tumor location[13], smoking behavior[15] or drinking behavior[16]. However, the DNA methylation status of the *p16INK4a* gene according to differences in Duke's stage was significantly different (P=0.016). This study found that the five patients at Duke's D stage included in the methylated group and Duke's B and C stages were in the unmethylated group. This is consistent with earlier studies that found that the more advanced type is commonly observed in methylated groups[11]. These results suggested that DNA methylation of the *p16INK4a* gene can be utilized as an indicator of poor prognosis. However, the DNA methylation status of the *p16INK4a* gene in tumor tissue was not found to be correlated with differences in Duke's stage according to a multivariate logistic regression test conducted as part of this study. In the Kaplan-Meier survival analysis, the methylated group showed a low survival rate compared to be the unmethylated group, but the difference was not significant. Therefore, additional studies of *p16INK4a* gene methylation with more samples are expected to identify the prognostic value of *p16INK4a* gene methylation as a diagnostic method.

Methods

Study Population

The study populations consisted of 52 patients (male: 25, female: 27) who gave informed consent from patients who had undergone surgical resection and had been diagnosed as having colorectal cancer in Dong-A University Hospital from January of 2002 to January of 2005. The study design was approved by the Committee on Human Research of Dong-A University Hospital.

Table 4. Primer sequences and annealing temperatures for PCR reactions for promoter regions of the *p16INK4a* gene.

Site	Primer sequences (5′→3′)	Annealing temperature (°C)
p16INK4a-1	Forward: 5′-ACGCCTTTGCTGGCAGGCGGG-3′ Reverse: 5′-CGCCAGAGCCAGCGTTGGCAAG-3′	68
p16INK4a-2	Forward: 5′-CTTGCCAACGCTGGCTCTGGCG-3′ Reverse: 5′-CCTCTGGAGGGACCGCGGTATC-3′	69
p16INK4a-3	Forward: 5′-GATACCGCGGTCCCTCCAGAGG-3′ Reverse: 5′-CTCCGCAGCCGCCGAGCGCACT-3′	67
p16INK4a-4	Forward: 5′-AGTGCGCTCGGCGGCTGCGGAG-3′ Reverse: 5′-GCCAGTCAGCCGAAGGCTCCATG-3′	67

DNA Extraction

Immediately after surgical resection, tumor specimens and adjacent normal specimens were prepared as samples by a pathologist and stored at −80°C. DNA samples (10-20 mg) were obtained from tumor and non-tumorous tissue samples using Wizard genomic DNA purification kits (Promega, Madison, USA), according to the instructions of the manufacturer.

p16INK4a Hypermethylation Analysis

Real-time PCR (ABI PRISM 7000 Sequence Detection System, Applied Biosystems, Faster City, USA) was used to quantify genomic target sequences using the SYBR Green 2X PCR Master Mix (Applied Biosystems, USA) for detection. *p16INK4a* gene methyla-

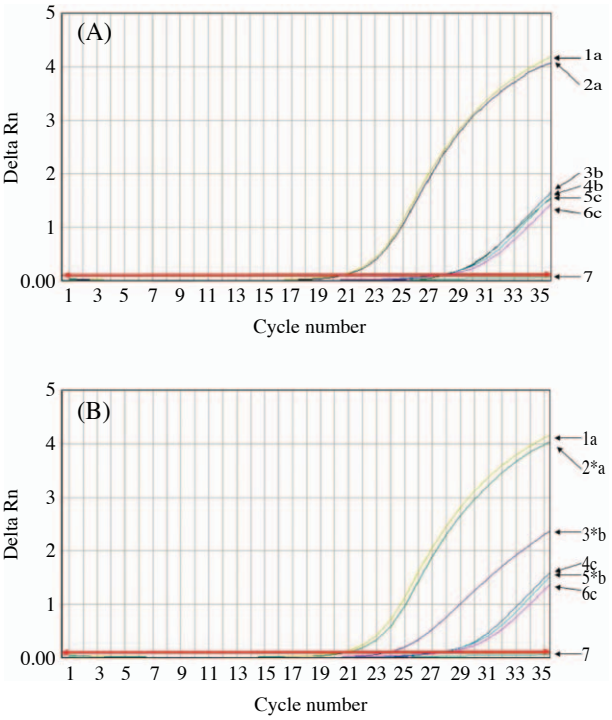

Figure 4. Results of *p16*^{INK4a} gene promoter methylation of normal (A) and tumor (B) in tissues by real-time PCR. 2, 3, 5: normal tissues (A); 2*, 3*, 5*: tumor tissue (B); 1, 4, 6: positive control (Wi-38); 7: negative control (water); a: no-cutting DNA amplification; b: *Hpa* II-cutting DNA amplification; c: *Msp* I-cutting DNA amplification. Delta Rn: The magnitude of the fluorescence signal generated during the PCR test at each time point.

tion statuses were determined by Real-time Methylation Specific PCR followed by restriction digestion.

One μg of genomic DNA was incubated for seven days at 37˚C with *Msp* I and *Hpa* II (New England BioLab, Beverly, MA USA). When the external C in the sequence CCGG of *p16*^{INK4a} is methylated, *Msp* I and *Hpa* II cannot cleave *p16*^{INK4a}. However, unlike *Hpa* II, *Msp* I can cleave this sequence when the internal C residue is methylated.

The PCR reaction mixtures each contained each genomic DNA, 5 pmol of primers, and the SYBR green 2X PCR master mix (Applied Biosystems, USA) in a final volume of 20 μL. The primer sequence and target sites are shown in the Figure 3 and Table 4.

The standard amplification protocol consisted of an initial denaturation step lasting 10 min at 95˚C, which was followed successively 35 amplification cycles at 94˚C for 15 s each at different annealing temperature (67, 68 and 69˚C) for 30 s and 72˚C for 30 s (Figure 4, Table 4). A standard curve was established with a 10-fold dilution series of DNA ranging from 1×10^0 to 1×10^3 ng. The diluting test DNA sample used for the standard curve was a known DNA concentration (814 ng/μL) of Wi-38 (Figure 5). After PCR, each amplification reaction was checked using a dissociation curve.

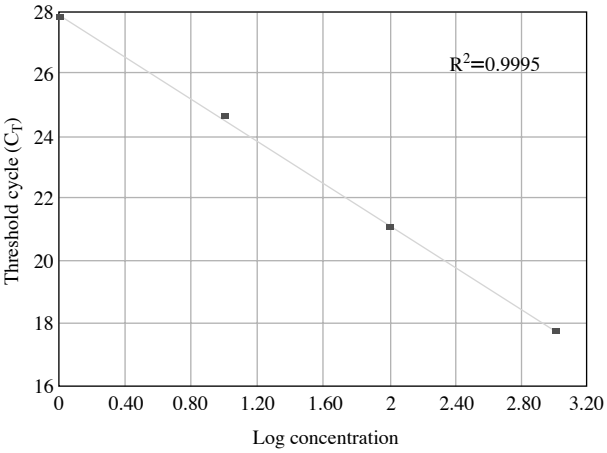

Figure 5. Result of serial dilutions to determine the detection limits of the real-time PCR protocol showing the initial DNA amounts used in the amplification step.

Calculation of Methylation

Raw data were evaluated using ABI 7000 System software. The status of methylation in each sample was expressed as a threshold cycle (C_T) ratio. The C_T denotes is the fractional cycle number at which the fluorescence signal reaches an arbitrary but defined threshold value within the early exponential phase of the reaction. C_T values are proportional to the logarithm of the initial copy numbers of the target; they were used to determine the initial copy numbers of samples. The C_T ratio reflects methylation. It was calculated as follows: C_T ratio=(C_T of untreated target genes−C_T of treated *Hpa* II)/(C_T of untreated target genes−C_T of treated *Msp* I); the values of the C_T ratio are between 0 and 1. In a completely non-methylated state, the C_T ratio of the *p16*^{INK4a} gene is 1; thus, a lower C_T ratio reflects a higher level of methylation.

Human cell line Wi-38 (KCLB No. 10075.1, epithelium, lung) was used as a positive control for methylated alleles. Water blanks were used as negative controls.

Statistical Analyses

The concordance of DNA methylation of the *p16*^{INK4a} gene between normal tissue and tumor tissue was analyzed using Kendall's tau B. The association between the DNA methylation status of the *p16*^{INK4a} gene and the clinicopathological features was analyz-

ed using a chi-square test or a t-test. The correlation between the clinicopathological features and DNA methylation status of the $p16^{INK4a}$ gene were evaluated in a multivariate logistic regression test. The survival analysis was performed by the Kaplan-Meier method via a log rank test. A probability level of 0.05 was used as the criterion of statistical significance and all the statistical analyses in this study were performed using the SPSS program version 12.0 (Chicago, IL, USA).

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