

Promoter Methylation of *CDKN2A*, *RAR β* , and *RASSF1A* in Non-Small Cell Lung Carcinoma: Quantitative Evaluation Using Pyrosequencing

Jung Uee Lee, M.D., Ph.D.¹, Hae Joung Sul, M.D., Ph.D.¹, Ji Woong Son, M.D., Ph.D.²

¹Department of Pathology, St. Mary's Hospital, The Catholic University of Korea School of Medicine, ²Department of Internal Medicine, Konyang University Hospital, Konyang University College of Medicine, Daejeon, Korea

Background: While qualitative analysis of methylation has been reviewed, the quantitative analysis of methylation has rarely been studied. We evaluated the methylation status of *CDKN2A*, *RAR β* , and *RASSF1A* promoter regions in non-small cell lung carcinomas (NSCLCs) by using pyrosequencing. Then, we evaluated the association between methylation at the promoter regions of these tumor suppressor genes and the clinicopathological parameters of the NSCLCs.

Methods: We collected tumor tissues from a total of 53 patients with NSCLCs and analyzed the methylation level of the *CDKN2A*, *RAR β* , and *RASSF1A* promoter regions by using pyrosequencing. In addition, we investigated the correlation between the hypermethylation of *CDKN2A* and the loss of p16^{INK4A} immunorepression.

Results: Hypermethylation of *CDKN2A*, *RAR β* , and *RASSF1A* promoter regions were 16 (30.2%), 22 (41.5%), and 21 tumors (39.6%), respectively. The incidence of hypermethylation at the *CDKN2A* promoter in the tumors was higher in undifferentiated large cell carcinomas than in other subtypes (p=0.002). Hypermethylation of *CDKN2A* was significantly associated with p16^{INK4A} immunorepression loss (p=0.045). With regard to the clinicopathological characteristics of NSCLC, certain histopathological subtypes were found to be strongly associated with the loss of p16^{INK4A} immunorepression (p=0.016). Squamous cell carcinoma and undifferentiated large cell carcinoma showed p16^{INK4A} immunorepression loss more frequently. The Kaplan-Meier survival curves analysis showed that methylation level and patient survival were barely related to one another.

Conclusion: We quantitatively analyzed the promoter methylation status by using pyrosequencing. We showed a significant correlation between *CDKN2A* hypermethylation and p16^{INK4A} immunorepression loss.

Key Words: DNA Methylation; Genes, p16; RASSF1 Protein, Human; Receptors, Retinoic Acid; Sequence Analysis, DNA; Carcinoma, Non-Small Cell Lung

Introduction

DNA methylation at the CpG islands within the promoter regions of tumor suppressor genes (TSGs) and cancer-associated genes plays a critical role in human cancers^{1,2}. Aberrant promoter methylation may affect the genes involved in cell cycle control (*p16INK4Ap15*, *Rb*, *p14*), DNA repair (*MGMT*, *hMLH1*), cell adhesion (*H-cadherin*, *CDH-1*), signal transduction (*RASSF1A*), apoptosis (*DAPK*, *TMS1*), and cell differentiation (*RAR β 2*)³. Cyclin-dependent kinase inhibitor 2A (*CDKN2A*), retinoic acid receptor beta (*RAR β*), and Ras association domain family member 1A (*RASSF1A*) are important

Address for correspondence: Hae Joung Sul, M.D., Ph.D.

Department of Pathology, St. Mary's Hospital, The Catholic University of Korea School of Medicine, 64, Daeheung-ro, Jung-gu, Daejeon 301-723, Korea
Phone: 82-42-220-9612, Fax: 82-42-252-9722
E-mail: sulpark@freechal.com

Address for co-correspondence: Ji Woong Son, M.D., Ph.D.

Department of Internal Medicine, Konyang University Hospital, 685, Gasuwon-dong, Seo-gu, Daejeon 302-718, Korea
Phone: 82-42-600-8817, Fax: 82-42-600-9090
E-mail: sk1609@hanmail.net

Received: Feb. 27, 2012

Revised: Mar. 22, 2012

Accepted: May 18, 2012

© It is identical to the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>).

TSGs that are frequently found in lung cancer patients. Hypermethylation at the promoter 5'-CpG island of these TSGs is frequently associated with gene silencing and occurs during carcinogenesis.

Among the methods used for assessing methylation, methylation-specific polymerase chain reaction (MSP), which is performed after bisulfite modification, is the most widely used method. The main advantages of MSP are that it is a very sensitive method, permitting the analysis of small and heterogeneous samples; it can be used on paraffin-embedded samples; it is specific for relevant CpG sites; and it eliminates the requirement for restriction enzymes, thus preventing problems resulting from and associated with incomplete enzymatic digestion. However, MSP tends to be more of a qualitative method than a quantitative one. Therefore, pyrosequencing is ideally suited for the simultaneous analysis and quantification of the degree of methylation at multiple CpG islands within each gene promoter region. Pyrosequencing is based on the sequencing-by-synthesis principle and is a bioluminometric real-time DNA sequencing technique that uses a cascade of 4 enzymatic reactions, producing light upon nucleotide incorporation⁴.

In this study, we analyzed methylation status at the *CDKN2A*, *RAR β* , and *RASSF1A* promoters in non-small cell lung carcinoma (NSCLC) tumor samples by using pyrosequencing. Then, we evaluated the association between methylation at the promoter regions of these TSGs and the clinicopathological parameters of the NSCLCs. In addition, we investigated the correlation between the hypermethylation of *CDKN2A* and the loss of p16^{INK4A} immunoexpression.

Materials and Methods

1. Patients and clinicopathological parameters

We analyzed 53 NSCLC patients who underwent surgery from 2000 to 2007 at Konyang University Hospital. This study had local ethics committee approval obtained from the Chungnam National University Hospital's Institutional Review Board. We retrieved these formal-

in-fixed, paraffin-embedded tissue blocks for analyzing of promoter methylation and immunohistochemical staining. We also reviewed the medical records of the NSCLC patients. The patients were characterized by clinicopathologic parameters (Table 1). Histopathological classification was assessed according to the World Health Organization (WHO) criteria. Tumor-node-metastasis (TNM) staging followed the American Joint Committee on Cancer (AJCC) staging system as revised in 2010 (7th edition). Squamous cell carcinoma (SqCC), adenocarcinoma, undifferentiated large cell carcinoma (LCC), which are major histological types of NSCLC, accounted for 32 (60.4%), 17 (32.1%), and 4 (7.5%), respectively. TNM stage following surgical resection was stage I in 24 (45.3%), stage II in 10 (18.9%), stage III in 14 (26.4%) and stage IV in 5 (9.4%). The median length of follow-up after the operation was 33 months (range, 1.5~80 months). Within follow-up periods, 23 (43.4%) patients died of cancer-related causes.

2. DNA extraction and bisulfite modification

All 53 samples were obtained by dissection of tumor areas and 13 samples were obtained by dissection of normal areas apart from tumors. Deparaffinization was carried out by adding 1 mL xylene to the microtube containing paraffin-embedded tissue sections and shaking incubation at normal temperature. This step repeated two or three times then spun down at 11,000 rpm for 3 minutes. The supernatant was removed, two identical washes with 100% ethanol for 15 minutes, spinning at 11,000 rpm for 3 minutes, was followed by drying at 37°C incubator for 15 minutes. DNA extraction was prepared using the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) as specified by the manufacturer's instructions.

Bisulfite treatment was performed to convert unmethylated cytosine to uracil and leaving methylated cytosine unchanged. Bisulfite treatment of 200 ng of each sample was subjected using the EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. The bisulfite converted DNA was eluted in 20 μ L of elution buffer

Table 1. Clinicopathological features and Immunoreactivity of p16^{INK4A}

Characteristics		p16 ^{INK4A} immunoreactivity		p-value
		Loss of p16	Expression of p16	
No. of patients	53	36 (67.9)	17 (32.1)	
Age, mean±SD, yr	62.49±9.01			
<60	18	11 (61.1)	7 (38.9)	0.446
≥60	35	25 (71.4)	10 (28.6)	
Gender				
Male	39 (73.6)	28 (71.8)	11 (28.2)	0.539
Female	14 (26.4)	8 (57.1)	6 (42.9)	
Smoking status				
Never	19 (35.8)	11 (57.9)	8 (42.1)	0.442
Former	9 (17.0)	6 (66.7)	3 (33.3)	
Current	25 (47.2)	19 (76.0)	6 (24.0)	
Survival				
Alive	30 (56.6)	20 (66.7)	10 (33.3)	0.247
Expire	23 (43.4)	16 (69.6)	7 (30.4)	
Pathological subtype				
SqCC	32 (60.4)	26 (81.2)	6 (18.8)	0.016
AdC	17 (32.1)	7 (41.1)	10 (58.9)	
LCC	4 (7.5)	3 (75.0)	1 (25.0)	
TNM Stage				
Stage I	24 (45.3)	15 (62.5)	9 (37.5)	0.702
Stage II	10 (18.9)	8 (80.0)	2 (20.0)	
Stage III	14 (26.4)	9 (64.3)	5 (35.7)	
Stage IV	5 (9.4)	4 (80.0)	1 (20.0)	
Methylation level of <i>CDKN2A</i> (mean±SD)		16.61±21.84	1.94±0.95	0.045
Hypermethylation		14 (87.5)	2 (12.5)	
Unmethylation		22 (59.5)	15 (40.5)	

Values are presented as mean±SD or number (%).

SqCC: squamous cell carcinoma; AdC: adenocarcinoma; LCC: undifferentiated large cell carcinoma; TNM: tumor-node-metastasis.

(Zymo Research). DNA samples were immediately stored at -20°C until to use.

3. Primer design and pyrosequencing methylation analysis

Polymerase chain reaction (PCR) assays were designed to amplify a part of the CpG islands in the *CDKN2A*, *RAR β* , and *RASSF1A* genes. Primers were designed by using the PSQ assay design program (Biotage, Charlotte, NC, USA). The primer sequence of *CDKN2A*, *RAR β* , and *RASSF1A* genes was listed in the Table 2.

PCR reaction was carried out in a volume of $50\ \mu\text{L}$ with 20 ng or less converted gDNA, $5\ \mu\text{L}$ of $10\times$ Taq buffer, 5 unit Hot/Start Taq polymerase (Enzynomics,

Daejeon, Korea), $4\ \mu\text{L}$ of each 2.5 mM dNTP mixture, $2\ \mu\text{L}$ of 10 pmole/ μL PCR primers. The amplification was carried out according to the general guidelines suggested by pyrosequencing: denaturing at 94°C for 15 minutes, followed by 40 cycles at 94°C for 30 seconds, at 58°C for 40 seconds, at 72°C for 300 seconds and a final extension at 72°C for 10 minutes. Confirmation of PCR product quality was established on 1.5% agarose gel stained with ethidium bromide. Pyrosequencing was performed using the PSQ96MA System (Biotage) according to manufacturer's instructions including single strand binding protein (PyroGold reagents; Biotage).

Table 2. PCR and sequencing primer for *CDKN2A*, *RAR β* , and *RASSF1A* pyrosequencing

Gene	Primer	Amplicon size (bp)	
<i>CDKN2A</i>	Forward	5'-AGGGGTTGGTTGGTTATTAG-3'	75
	5' biotinylated-reverse	5'-CTACCTACTCTCCCCCTCTC-3'	
	Sequencing primer	5'-GGTTGGTTATTAGAGGGT-3'	
<i>RARβ</i>	Forward	5'-GTTAAAGGGGGGATTAGAAT-3'	152
	5' biotinylated-reverse	5'-CTCCTTCCAAATAAATACTTACAA-3'	
	Sequencing primer	5'-GAGTTGTTTGAGGATTGG-3'	
<i>RASSF1A</i>	Forward	5'-AAGGAGGGAAGGAAGGGTAA-3'	146
	5' biotinylated-reverse	5'-CCTCCCCCAAATCCAAA-3'	
	Sequencing primer	5'-GGGAAGGAAGGGTAA-3'	

PCR: polymerase chain reaction.

4. Construction of tissue microarray and immunohistochemistry

All the hematoxylin and eosin stained slides for each case were reviewed, and the areas of interest were marked on each slide. The tissue microarrays (TMAs) were constructed using the 5 mm punch on the Bee-Cher arrayer (UNITMA, Seoul, Korea). The corresponding region was circled on the 'donor' paraffin block using a marker pen. The samples were then arrayed on a 'recipient' block. A total 53 samples were available for the array.

Four μ m-thick sections of the paraffin-embedded TMAs were deparaffinized with xylene and rehydrated in a series of graded alcohol solutions and microwave-treated for 25 minutes in a citrate buffer (pH 6.0). The antigen was retrieved with 0.01 M citrate buffer (pH 6.0) by heating the sample in autoclave (CHS-ACCE-860; JW Pharmaceutical, Seoul, Korea) at a controlled final temperature of 121°C for 15 minutes. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. Nonspecific binding sites were blocked by incubating in 10% normal goat serum diluted with phosphate-buffered saline (PBS). Tissue sections were then incubated with p16^{INK4A} mouse mAb (1:200, Abcam, Cambridge, MA, USA) for 60 minutes at room temperature. The sections were visualized with avidin-biotin-peroxidase complex and tissue arrays were counterstained with Harris' hematoxylin. Human brain tissue served as positive internal control for p16^{INK4A} staining.

Negative controls were incubated with PBS instead of a primary antibody.

5. Statistical analysis

All statistical analyses were performed using SPSS version 18 (SPSS Inc., Chicago, IL, USA) for windows. Group comparisons of the clinical variables were performed using chi-square tests or linear by linear association analysis. Comparisons of means were accomplished by independent sample t-tests or one-way ANOVA. The difference was considered to be statistically significant if the two-sided p-value was <0.05. Survival curves were plotted using the Kaplan and Meier method and statistical significance was determined by the Breslow test.

Results

1. Relationship between *CDKN2A* hypermethylation and p16^{INK4A} immunoeexpression and relationship between these factors and the clinicopathological parameters of NSCLC

We examined the relationship between the *CDKN2A* methylation level and p16^{INK4A} immunoeexpression in 53 NSCLC tumor tissue samples and also examined the clinicopathological parameters of the NSCLCs (Table 1). Loss of p16^{INK4A} immunoeexpression was found in 36 samples (67.9%). Hypermethylation of *CDKN2A* was significantly associated with loss of p16^{INK4A} immunoeexpression (p=0.045). In this study, we showed a sig-

nificant correlation between the *CDKN2A* gene hypermethylation status, which was identified by pyrosequencing, and the loss of p16^{INK4A} immunoexpression, which was detected by immunohistochemical analysis, in the NSCLC tumor samples.

With regard to the clinicopathological characteristics of NSCLC, certain histopathological subtypes were found to be strongly associated with the loss of p16^{INK4A} immunoexpression ($p=0.016$), i.e., SqCC and undifferentiated LCC showed more frequent loss of p16^{INK4A} immunoexpression. However, no other clinicopathological parameter was associated with the loss of p16^{INK4A} immunoexpression.

2. Methylation analysis of *CDKN2A*, *RAR β* , and *RASSF1A* promoters in tumor and normal lung tissues by using pyrosequencing

We evaluated the methylation status of the *CDKN2A*, *RAR β* , and *RASSF1A* gene promoters in 13 lung cancer tissue samples and 13 corresponding normal lung tissue samples by using pyrosequencing (Table 3, Figure 1). The mean methylation levels of normal lung tissues were 1.79, 5.07, and 3.15 and of tumor tissues were 12.16, 20.9, and 8.53 for *CDKN2A*, *RAR β* , and *RASSF1A*, respectively. On the basis of these results, we

defined promoter hypermethylation in a tumor tissue as a 2-fold increase in normal tissue⁵, i.e., 3.58, 10.14, and 6.30 in *CDKN2A*, *RAR β* , and *RASSF1A*, respectively. We further analyzed an additional 40 tumor tissues by using pyrosequencing (Table 4).

3. Methylation of *CDKN2A*, *RAR β* , and *RASSF1A* promoters and the correlation between their methylation status with the clinicopathological parameters of NSCLC

We evaluated the methylation status of the promoter regions of the *CDKN2A*, *RAR β* , and *RASSF1A* genes in 53 tumor tissue samples, as well as the correlation between methylation status of these promoter regions and the clinicopathological parameters of the samples (Tables 4, 5).

The incidence of hypermethylation at the *CDKN2A* promoter in the tumors was higher in the samples of undifferentiated LCC than in the samples of other subtypes ($p=0.002$) (Table 5). However, the number of LCC samples studied was too small to obtain meaningful results. The *RAR β* or *RASSF1A* gene promoters were not correlated with any clinicopathological parameters of NSCLC. We analyzed the effect of *CDKN2A*, *RAR β* or *RASSF1A* methylation on patient survival. Kaplan-

Table 3. Promoter methylation levels of normal tissue and tumor tissue

Case No.	<i>CDKN2A</i>		<i>RARβ</i>		<i>RASSF1A</i>	
	Normal	Tumor	Normal	Tumor	Normal	Tumor
1	1,79±0,85	1,99±1,03	10,44±3,51	40,00±5,39	4,10±2,63	3,25±2,37
2	1,56±1,37	0,82±1,00	4,88±3,21	3,20±3,03	1,93±1,51	3,73±5,54
3	1,78±1,17	33,99±4,13	6,20±2,12	0,00±0,00	2,55±1,30	2,95±0,81
4	1,74±0,98	4,45±2,23	5,58±4,61	0,54±0,00	5,50±2,06	3,40±6,52
5	1,49±1,33	41,36±2,82	5,11±0,78	37,51±7,84	3,28±1,45	4,15±5,25
6	1,84±1,69	1,70±1,45	2,70±0,53	24,60±1,57	2,74±2,16	2,56±1,18
7	2,15±0,63	2,46±0,93	4,05±1,33	0,00±0,00	5,06±3,97	26,40±5,73
8	1,83±1,23	25,64±2,73	4,06±0,75	29,60±3,35	3,47±1,57	3,31±2,62
9	1,75±1,33	1,74±1,57	3,18±0,76	3,16±0,52	2,07±1,40	16,93±1,46
10	2,31±1,67	1,86±0,63	7,90±2,93	50,10±4,49	1,67±1,43	3,02±1,56
11	1,43±1,31	1,55±0,55	3,16±0,76	10,06±3,31	3,88±2,75	34,20±4,63
12	1,59±1,88	1,10±1,24	4,60±1,05	27,27±4,81	1,56±1,88	2,53±4,60
13	1,98±1,02	39,48±2,81	4,01±0,71	45,68±1,80	3,19±1,73	4,52±3,09

Values are presented as mean±SD.



Figure 1. Detection of *CDKN2A*, *RAR β* , *RASSF1A* promoter hypermethylation in non-small cell lung cancer. Pyrograms illustrating the 6 CpGs in the *CDKN2A* promoter, the 5 CpGs in the *RAR β* promoter, and 6 CpGs in the *RASSF1A* promoter.

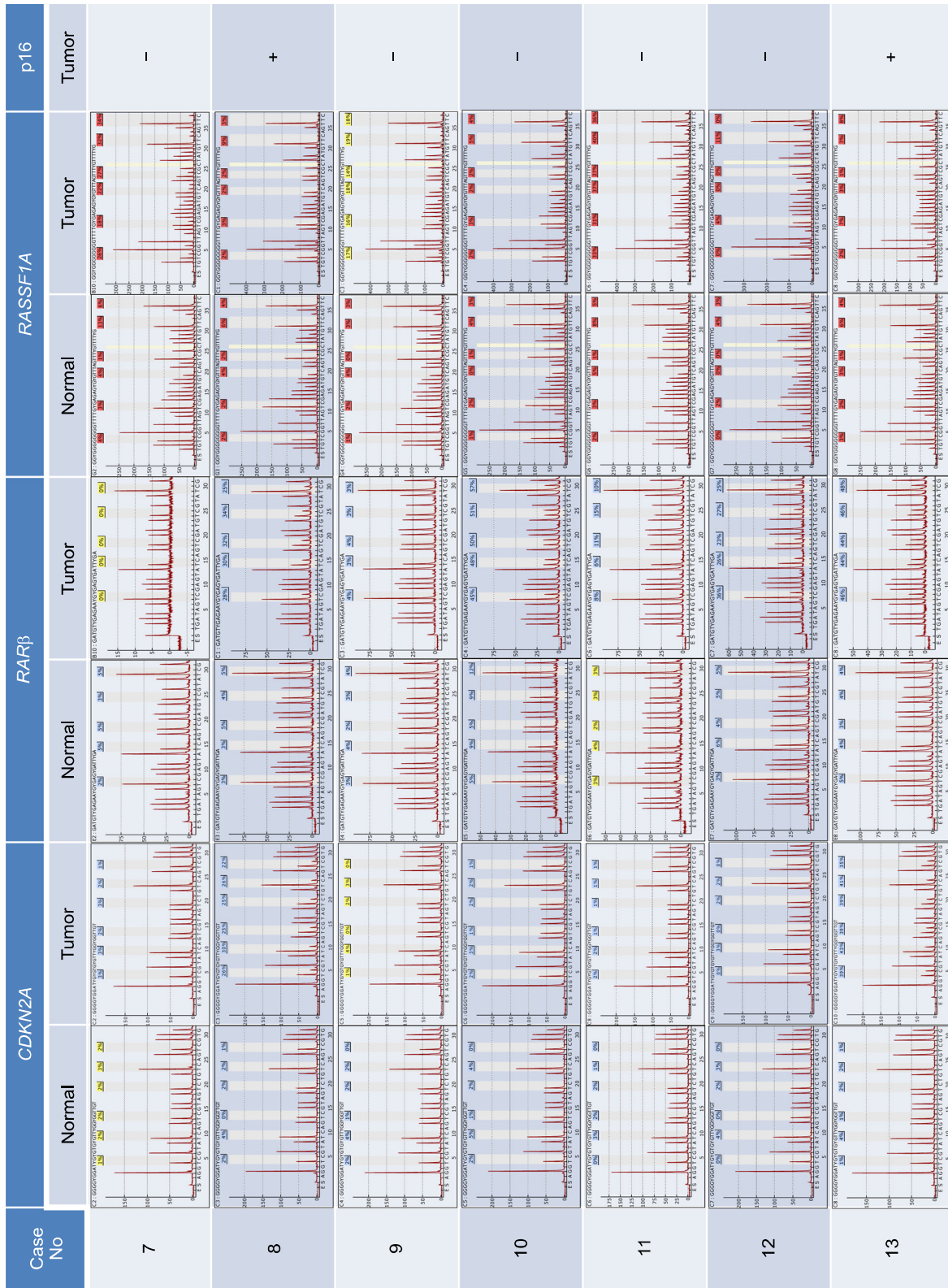


Figure 1. Continued.

Table 4. Clinicopathological features and the overall average promoter methylation levels

Characteristics	Methylation levels					
	<i>CDKN2A</i>	p-value	<i>RARβ</i>	p-value	<i>RASSF1A</i>	p-value
Age						
<60 yr	6.41±11.10	0,397	15.58±18.60	0,382	8.66±9.73	0,483
≥60 yr	14.73±21.88		15.55±15.86		12.02±14.20	
Gender						
Male	13.45±20.26	0,397	15.07±17.74	0,254	11.31±13.55	0,496
Female	7.61±15.79		16.94±13.68		9.67±11.09	
Smoking status						
Never	9.27±15.84	0,373	21.57±17.41	0,271	11.53±11.67	0,439
Former	5.59±8.09		9.86±12.90		8.70±11.49	
Current	16.19±23.45		13.05±16.47		11.16±14.49	
Survival						
Alive	12.90±21.62	0,397	16.00±16.60	0,477	13.68±14.65	0,475
Expire	10.62±15.90		15.00±17.10		7.22±9.12	
Pathological subtype						
AdC	3.72±7.99	0,373	18.36±17.09	0,493	9.66±10.61	0,373
SqCC	15.13±22.65		15.02±16.81		12.41±14.48	
LCC	20.96±12.46		7.97±14.44		3.79±1.16	
TNM Stage						
Stage I	11.27±19.83	0,417	15.87±17.61	0,364	12.38±14.28	0,494
Stage II	13.98±20.43		15.54±16.29		11.06±13.91	
Stage III	11.92±21.16		16.46±17.55		10.45±11.91	
Stage IV	10.79±11.16		11.58±14.28		4.48±3.85	

Values are presented as mean±SD or number.

AdC: adenocarcinoma; SqCC: squamous cell carcinoma; LCC: undifferentiated large cell carcinoma; TNM: tumor-node-metastasis.

Meier survival curves analysis showed that methylation level and patient survival were barely related (Figure 2).

Discussion

Lung cancer is the main cause of cancer-related deaths globally⁶. Lung cancer mainly comprises NSCLCs, which represent 85% of all lung cancer cases. Despite recent advances in early diagnosis and multimodality therapy, the prognosis for NSCLC is quite poor. Visbal et al,⁷ reported that the estimated 5-year survival rates were 15% in men and 19% in women. Therefore many studies have attempted to assess the specific molecular determinants that aid in detection of lung cancer at earlier stages and the mechanisms underlying tumor aggressiveness.

DNA hypermethylation refers to the addition of a methyl group to the cytosine ring of cytosines that pre-

cede a guanosine (CpG dinucleotides) resulting in the formation of methyl cytosine (5-methylcytosine). TSG contain CpG islands in their promoter regions, and many of them show evidence of methylation silencing^{8,9}. In this study, we evaluated the methylation status of tumor suppression genes associated with lung cancer, such as *CDKN2A*, *RAR β* , and *RASSF1A*, by using pyrosequencing. Resultantly, we showed that the methylation level of the promoter regions of the aforementioned genes was much higher in the tumor tissues than in the adjacent normal tissues. Some researchers have shown that hypermethylation of the CpG islands of the specific promoter regions of specific genes such as *RASSF1A*, *RUNX3*, *FHIT*, and *H-cadherin* was closely associated with poor prognostic factors of NSCLCs, such as advanced stage, lymph node metastasis, and adverse survival¹⁰⁻¹³. In this study, *CDKN2A* hypermethylation was associated with the histopathological subtype of

Table 5. Methylation status of promoters according to clinicopathological parameters

	<i>CDKN2A</i>			<i>RARβ</i>			<i>RASSF1A</i>		
	Hyper-methylation	Unmethylation	p-value	Hyper-methylation	Unmethylation	p-value	Hyper-methylation	Unmethylation	p-value
No. of case	16 (30.2)	37 (69.8)		22 (41.5)	31 (58.5)		21 (39.6)	32 (60.4)	
Age, yr									
<60	5 (27.8)	13 (72.2)	0.784	7 (38.9)	11 (61.1)	0.781	6 (33.3)	12 (66.7)	0.502
\geq 60	11 (31.4)	24 (68.6)		15 (42.9)	20 (57.1)		15 (42.9)	20 (57.1)	
Gender									
Male	14 (35.9)	25 (64.1)	0.131	15 (38.5)	24 (61.5)	0.452	16 (41.0)	23 (59.0)	0.727
Female	2 (14.3)	12 (85.7)		7 (50.0)	7 (50.0)		5 (35.7)	9 (64.3)	
Smoking status									
Never	5 (26.3)	14 (73.7)	0.668	11 (57.9)	8 (42.1)	0.150	8 (42.1)	11 (57.9)	0.905
Former	2 (22.2)	7 (77.8)		2 (22.2)	7 (77.8)		3 (33.3)	6 (66.7)	
Current	9 (36.0)	16 (64.0)		9 (36.0)	16 (64.0)		10 (40.0)	15 (60.0)	
Survival									
Alive	9 (30.0)	21 (70.0)	0.973	13 (43.3)	17 (56.7)	0.758	16 (53.3)	14 (46.7)	0.020
Expire	7 (30.4)	16 (69.6)		9 (39.1)	14 (60.9)		5 (21.7)	18 (78.3)	
Pathologic type									
AdC	10 (31.3)	22 (68.7)	0.002	13 (40.6)	19 (59.4)	0.714	15 (46.9)	17 (53.1)	0.177
SqCC	2 (11.8)	15 (88.2)		8 (47.1)	9 (52.9)		6 (35.3)	11 (64.7)	
LCC	4 (100.0)	0 (0.0)		1 (25.0)	3 (75.0)		0 (0.0)	4 (100.0)	
TNM stage									
Stage I	7 (29.2)	17 (70.8)	0.452	10 (41.7)	14 (58.3)	0.999	11 (45.8)	13 (54.2)	0.650
Stage II	3 (30.0)	7 (70.0)		4 (40.0)	6 (60.0)		3 (30.0)	7 (70.0)	
Stage III	3 (21.4)	11 (78.6)		6 (42.9)	8 (57.1)		6 (42.9)	8 (57.1)	
Stage IV	3 (60.0)	2 (40.0)		2 (40.0)	3 (60.0)		1 (20.0)	4 (80.0)	

Values presented as number (%).

AdC: adenocarcinoma; SqCC: squamous cell carcinoma; LCC: undifferentiated large cell carcinoma; TNM: tumor-node-metastasis.

NSCLCs, i.e., undifferentiated LCC, although other clinicopathological characteristics were not related with the methylation status of *CDKN2A*, *RAR β* , and *RASSF1A*.

We also evaluated the immunoeexpression of p16^{INK4A} in NSCLC samples. SqCC and undifferentiated LCC showed immunoeexpression loss of p16^{INK4A} more frequently than other NSCLC subtypes. Many studies showed that *CDKN2A* gene hypermethylation was associated with loss of p16^{INK4A} expression, whereas others showed that hypermethylation of the *CDKN2A* was related with overexpressed p16^{INK4A 1,14-16}. In this study, we demonstrated that hypermethylation of the *CDKN2A* was significantly associated with loss p16^{INK4A} immunoeexpression in NSCLCs.

Many efforts have been made to develop effective methods for detection of DNA methylation. MSP has

been found to provide useful results, mainly owing to its high sensitivity. However, MSP has several disadvantages: 1) This method yields qualitative rather than quantitative data; 2) It usually evaluates only a few CpG sites at the 3' end of the primers; 3) It lacks an internal control for evaluating the adequacy of bisulfite treatment, thus complicating the identification of false-positives results; 4) MSP is potentially susceptible to oversensitivity after numerous PCR cycles. In an attempt to overcome these disadvantages, we used pyrosequencing to determine the methylation status of the *CDKN2A*, *RAR β* , and *RASSF1A* promoters in NSCLC tumor samples. The advantages of pyrosequencing are that it is a highly reliable and quantitative sequencing-by-synthesis method with built-in internal controls for evaluating the adequacy of bisulfite treatment and

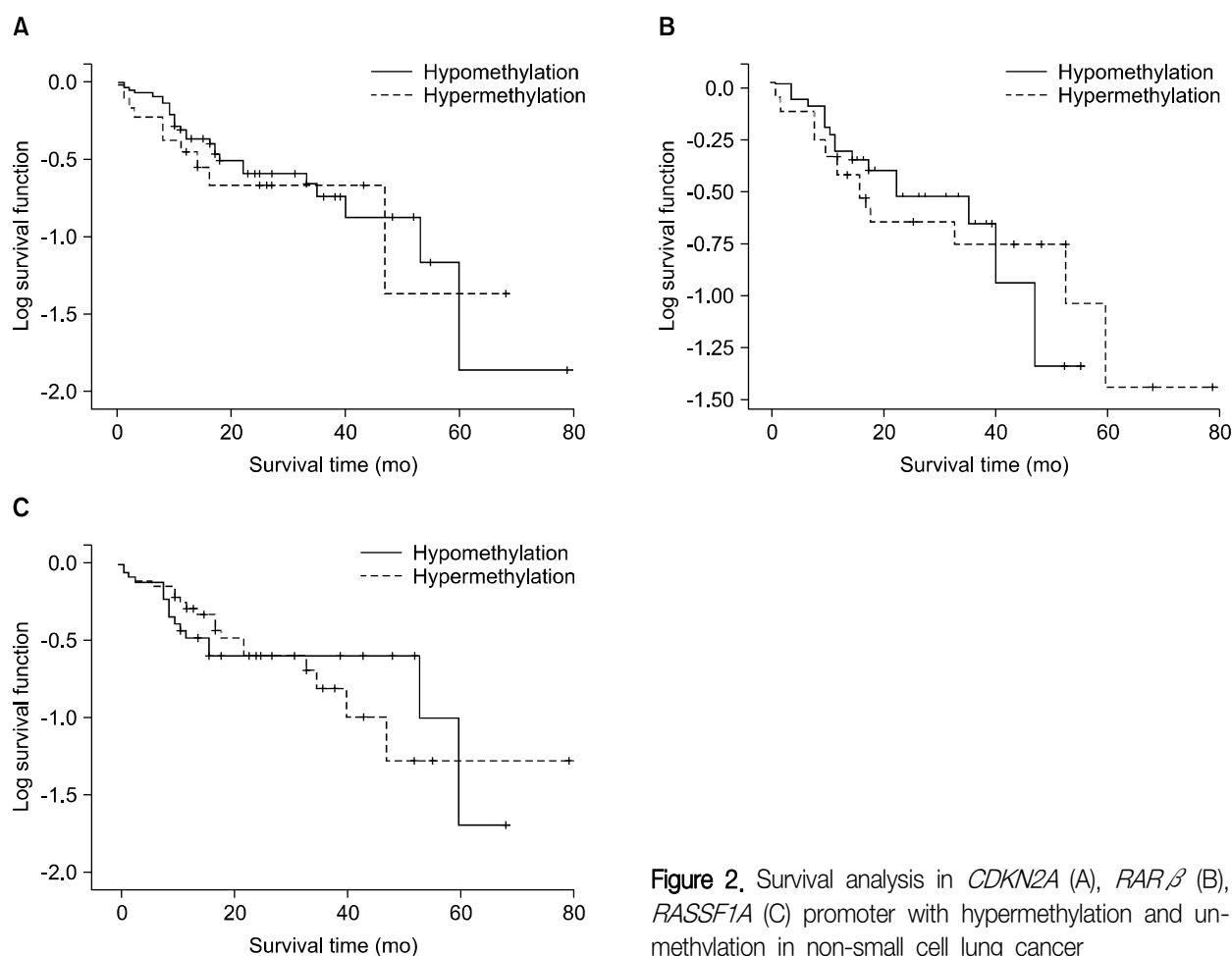


Figure 2. Survival analysis in *CDKN2A* (A), *RAR β* (B), *RASSF1A* (C) promoter with hypermethylation and unmethylation in non-small cell lung cancer.

can be used for analyzing DNA methylation at multiple CpG sites¹⁷⁻¹⁹. Pyrosequencing analysis can provide reproducible measurements of the average methylation levels in sequential CpG sites and can directly use PCR products, this method is rapid and accurate and can be used for simultaneous analysis of many samples. It is therefore well suited, for example, to clinical research applications where the methylation levels of several individuals need to be measured accurately over time to analyze the correlation with a disease status or response to treatment. However, owing to the limitation of pyrosequencing, i.e., sensitivity due to background noise²⁰, the application of this method to clinical samples such as plasma and saliva may have little value^{21,22}.

In conclusion, we quantitatively analyzed promoter methylation status by using pyrosequencing. We found that *CDKN2A* was hypermethylated in 30.2%, *RAR β* in

41.5%, and *RASSF1A* in 39.6% of the NSCLC samples. We showed a significant correlation between *CDKN2A* hypermethylation and p16^{INK4A} immunoexpression loss. Despite the theoretical advantages of pyrosequencing, no correlation was found between promoter methylation status and survival rates.

Acknowledgements

This work was supported by Konyang University Myunggok Research Fund of 2006.

References

1. 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.
2. Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E,

- Burger PC, et al. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat Med* 1995;1:686-92.
3. Buckingham L, Penfield Faber L, Kim A, Liptay M, Barger C, Basu S, et al. PTEN, RASSF1 and DAPK site-specific hypermethylation and outcome in surgically treated stage I and II nonsmall cell lung cancer patients. *Int J Cancer* 2010;126:1630-9.
 4. Huang TH, Perry MR, Laux DE. Methylation profiling of CpG islands in human breast cancer cells. *Hum Mol Genet* 1999;8:459-70.
 5. Ruike Y, Imanaka Y, Sato F, Shimizu K, Tsujimoto G. Genome-wide analysis of aberrant methylation in human breast cancer cells using methyl-DNA immunoprecipitation combined with high-throughput sequencing. *BMC Genomics* 2010;11:137.
 6. Herbst RS, Heymach JV, Lippman SM. Lung cancer. *N Engl J Med* 2008;359:1367-80.
 7. Visbal AL, Williams BA, Nichols FC 3rd, Marks RS, Jett JR, Aubry MC, et al. Gender differences in non-small-cell lung cancer survival: an analysis of 4,618 patients diagnosed between 1997 and 2002. *Ann Thorac Surg* 2004;78:209-15.
 8. Koh J, Enders GH, Dynlacht BD, Harlow E. Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. *Nature* 1995;375:506-10.
 9. Kim DH, Nelson HH, Wiencke JK, Zheng S, Christiani DC, Wain JC, et al. p16(INK4a) and histology-specific methylation of CpG islands by exposure to tobacco smoke in non-small cell lung cancer. *Cancer Res* 2001;61:3419-24.
 10. Danesi R, de Braud F, Fogli S, de Pas TM, Di Paolo A, Curigliano G, et al. Pharmacogenetics of anticancer drug sensitivity in non-small cell lung cancer. *Pharmacol Rev* 2003;55:57-103.
 11. Yanagawa N, Tamura G, Oizumi H, Takahashi N, Shimazaki Y, Motoyama T. Promoter hypermethylation of tumor suppressor and tumor-related genes in non-small cell lung cancers. *Cancer Sci* 2003;94:589-92.
 12. Brabender J, Usadel H, Metzger R, Schneider PM, Park J, Salonga D, et al. Quantitative O(6)-methylguanine DNA methyltransferase methylation analysis in curatively resected non-small cell lung cancer: associations with clinical outcome. *Clin Cancer Res* 2003;9:223-7.
 13. Field JK, Liloglou T, Warrak S, Burger M, Becker E, Berlin K, et al. Methylation discriminators in NSCLC identified by a microarray based approach. *Int J Oncol* 2005;27:105-11.
 14. Gonzalez-Zulueta M, Bender CM, Yang AS, Nguyen T, Beart RW, Van Tornout JM, et al. Methylation of the 5' CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. *Cancer Res* 1995;55:4531-5.
 15. Schmutte C, Jones PA. Involvement of DNA methylation in human carcinogenesis. *Biol Chem* 1998;379:377-88.
 16. El-Naggar AK, Lai S, Clayman G, Lee JK, Luna MA, Goepfert H, et al. Methylation, a major mechanism of p16/CDKN2 gene inactivation in head and neck squamous carcinoma. *Am J Pathol* 1997;151:1767-74.
 17. Herman JG, Graff JR, Myöhänen 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.
 18. Tost J, Gut IG. Analysis of gene-specific DNA methylation patterns by pyrosequencing technology. *Methods Mol Biol* 2007;373:89-102.
 19. González-Quevedo R, Iniesta P, Morán A, de Juan C, Sánchez-Pernaute A, Fernández C, et al. Cooperative role of telomerase activity and p16 expression in the prognosis of non-small-cell lung cancer. *J Clin Oncol* 2002;20:254-62.
 20. Shaw RJ, Liloglou T, Rogers SN, Brown JS, Vaughan ED, Lowe D, et al. Promoter methylation of p16, RARbeta, E-cadherin, cyclin A1 and cytoglobin in oral cancer: quantitative evaluation using pyrosequencing. *Br J Cancer* 2006;94:561-8.
 21. Shaw RJ, Hall GL, Lowe D, Liloglou T, Field JK, Sloan P, et al. The role of pyrosequencing in head and neck cancer epigenetics: correlation of quantitative methylation data with gene expression. *Arch Otolaryngol Head Neck Surg* 2008;134:251-6.
 22. Kontic M, Stojsic J, Jovanovic D, Bunjevacki V, Ognjanovic S, Kuriger J, et al. Aberrant promoter methylation of CDH13 and MGMT genes is associated with clinicopathologic characteristics of primary non-small-cell lung carcinoma. *Clin Lung Cancer* 2012;13:297-303.