Prognostic Significance of *p16^{INK4a}* Hypermethylation in Non-small Cell Lung Cancer is Evident by Quantitative DNA Methylation Analysis

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Abstract. *Background: p16^{INK4a} is a tumor suppressor gene frequently inactivated by aberrant promoter hypermethylation. In the present study, p16^{INK4a} methylation was evaluated in non-small cell lung cancer (NSCLC) using a quantitative assay and the clinical significance of the methylation was explored. Materials and Methods: A total of 244 tumor samples from formalin-fixed paraffin-embedded archives were examined in this study. p16^{INK4a} methylation was analyzed by the fluorescence-based, real-time methylation-specific PCR assay, MethyLight. The quantitative methylation value was expressed as the percentage of methylated reference (PMR). Results: The median level of p16^{INK4} methylation was 0.55 PMR (range 0.00-503.4). The p16^{INK4} methylation value was significantly higher in males (p=0.005) and in squamous cell carcinoma (p=0.018). Prognostic analysis using the Cox proportional hazard model showed that the p16^{INK4a} methylation value was a significant prognostic factor (odds ratio, 1.005; 95%CI, 1.003 to 1.008; p<0.0001). The p16^{INK4a} methylation value remained a significant prognostic factor (p=0.0004) in multivariate analysis including age, gender, histological type and clinical stage. Specimens were then classified into hypermethylated or non-hypermethylated groups based on the p16^{INK4a} methylation value using various cut-offs from 1 to 100 PMR. There was no significant difference in prognosis between the two groups using a cut-off value of 1 PMR. On the other hand, there was a significant difference using 6 PMR or more as the cut-off value (p<0.01). Conclusion: These results provide clear evidence for the prognostic significance of p16^{INK4a} methylation in NSCLC using quantitative DNA methylation analysis. Careful assessment of DNA methylation is needed because qualitative methylation analysis may overestimate low levels of methylation, which have less clinical significance.*

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Despite recent advances in cancer therapy, lung cancer remains one of the major causes of cancer death worldwide. The cumulative 5-year survival rate of non-small cell lung cancer (NSCLC) is about 60% even in patients in clinical stage I. Therefore, precise methods for prediction of prognosis are required for the optimal selection from among the available modalities of lung cancer therapy.

Aberrant promoter methylation is a common epigenetic modification in human cancer (1). Methylation leads to silencing of tumor suppressor genes and plays an important role in tumorigenesis. It has been suggested that aberrant promoter methylation may be useful as a prognostic marker in many type of cancers. Indeed, APC (2), DAP-kinase (3), FHIT (4) and MGMT (5) methylation were reported to be linked with clinical prognosis in patients with NSCLC.

p16^{INK4a} is a tumor suppressor gene frequently inactivated by aberrant promoter hypermethylation (6, 7). Although there have been a number of studies of the prognostic significance of *p16^{INK4a}* methylation in NSCLC, the results were not consistent (8-13). This inconsistency may be attributable to qualitative methods of the DNA methylation analysis used in previous studies. Ogino *et al.* showed that the CpG island methylator phenotype of colorectal cancer was best characterized by quantitative DNA methylation analysis (14). They suggested that qualitative methods may give positive results for tumors with low levels of methylation, which are of little or no biological significance. Previous studies on *p16^{INK4a}* methylation in NSCLC may have evaluated such low levels of methylation as hypermethylated, making the prognostic significance of *p16^{INK4a}* methylation unclear. In the present study, *p16^{INK4a}* methylation was evaluated in NSCLC using the quantitative MethyLight method and the clinical significance of methylation was explored. The results indicate that the prognostic significance of *p16^{INK4a}* methylation is evident with quantitative DNA methylation analysis.

Materials and Methods

Sample collection and DNA isolation. A total of 244 primary tumors collected from NSCLC patients diagnosed by pathological examination were included in this study. All tissue samples were obtained from surgically resected tissues, which were fixed in formalin and embedded in paraffin followed by histological diagnosis with H&E staining. Tumor tissue was dissected manually from formalin-fixed paraffin-embedded tissue sections 10 μm thick. After deparaffinization using xylene and ethanol, genomic DNA was isolated using a QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Approval of this project was obtained from the Kanazawa University School of Medicine Ethics Committee.

DNA methylation analysis. The DNA was subjected to bisulfite treatment using a CpGenome DNA Modification Kit (Chemicon, Temecula, CA, USA) according to the manufacturer's protocol. Quantitative DNA methylation analysis was performed by the fluorescence-based, real-time PCR assay, MethyLight, as described previously (15). Two sets of primers and probes used to specifically amplify the bisulfite-converted *p16^{INK4a}* and ACTB sequence as a DNA loading control, were designed previously (16). The specificities of the reactions for methylated DNA were confirmed separately using human sperm DNA (unmethylated) and SssI methylase (New England Biolabs, Ipswich, MA, USA)-treated sperm DNA (methylated). The percentage of molecules fully methylated at *p16^{INK4a}* was calculated by dividing the *p16^{INK4a}*: ACTB ratio of the sample by the *p16^{INK4a}*: ACTB ratio of SssI-treated sperm DNA and multiplying by 100. The abbreviation, percentage of methylated reference (PMR) was used, to indicate this measurement as described previously (16).

Statistical analysis. The associations of the PMR as a continuous value with clinicopathological variables were analyzed by the Mann-Whitney *U*-test or the Kruskal-Wallis test. The statistical significance of the *p16^{INK4a}* methylation value as a prognostic factor was evaluated using the Cox proportional hazard regression model. The cumulative survival rate was calculated by the Kaplan-Meier method and statistical significance was analyzed by Log-rank test.

Results

p16^{INK4a} promoter methylation and clinicopathological features in NSCLC. The methylation level of the *p16^{INK4a}* gene was successfully measured in all 244 tumor samples. The associations between *p16^{INK4a}* methylation and clinicopathological features are shown in Table I. Significant associations of the *p16^{INK4a}* methylation level with gender ($p=0.005$) and histopathological type ($p=0.018$) were observed. There were no correlations between methylation and other clinicopathological features, including age, T factor, N factor, distant metastasis or clinical stage.

p16^{INK4a} promoter methylation and prognosis. Prognostic information was available in 238 patients. The median follow-up time was 45.0 months (range 2-149 months). Cox proportional hazard regression analysis indicated that the *p16^{INK4a}* methylation value in cancer tissue was a significant prognostic factor (odds ratio, 1.005; 95%CI, 1.003 to 1.008; $p<0.0001$). Multivariate analysis including age, gender, histological type, clinical stage, and *p16^{INK4a}* methylation value indicated that *p16^{INK4a}* methylation remained significant as a prognostic factor ($p=0.0004$) together with age ($p=0.0009$), gender ($p<0.0001$) and clinical stage ($p<0.0001$).

To explore what level of PMR in *p16^{INK4a}* methylation was clinically significant as a prognostic factor, a variety of cut-off values of PMR were set, followed by grouping of the samples into hypermethylated or non-hypermethylated groups. The groups were compared with regard to prognosis by the Kaplan-Meier method and the Log-rank test. Cut-off values from 1 to 100 PMR were tested. Representative results are shown in Table II, in which the number of hypermethylated cases and *p*-value by the Log-rank test are listed together with the cut-off values used. There were no significant differences in prognosis between the two groups with a cut-off value of 1 PMR, whereas borderline significance, from $p=0.03$ to 0.05, was observed when using a cut-off value of 2 to 5 PMR. On the other hand, a significant difference was observed with a cut-off value of 6 PMR or more ($p<0.01$). These results suggest that methylation showing more than 6 PMR has clinical significance as a prognostic factor. The frequency of the samples judged as hypermethylated was less than 20% with this level of cut-off value. The prognostic difference between the hypermethylated and non-hypermethylated cases remained significant in either clinical stage I or stage III sub-groups using a cut-off value of more than 10 PMR. Statistical

Table I. *Relationship between clinicopathological features and p16^{INK4a} methylation.*

	n	*p16^{INK4a}* methylation		*P*-value
Total	244	0.55	(0.00-503.4)	
Age				
≤65	123	0.50	(0.00-132.5)	0.222
65<	121	0.62	(0.00-503.4)	
Gender				
male	153	0.71	(0.00-503.4)	0.005
female	91	0.41	(0.00-350.6)	
Stage				
I	141	0.61	(0.00-503.4)	0.146
II	20	0.77	(0.04-64.70)	
III	78	0.40	(0.00-350.6)	
IV	5	0.26	(0.00-107.7)	
Histology				
adeno	148	0.44	(0.00-350.6)	0.018
squamous	87	1.04	(0.00-503.4)	
large	3	0.36	(0.20-24.72)	
others	6	0.93	(0.03-107.7)	

p16^{INK4a} methylation was expressed as median (range).

Table II. *Frequency of $p16^{INK4a}$ hypermethylation and the p-values by the Log-rank test when analyzed with the indicated cut-off value.*

Cut-off value	$p16^{INK4a}$ hypermethylated samples	*P*-value
1	87 (36.6)	0.1729
2	62 (26.1)	0.0435
4	49 (20.6)	0.0492
6	43 (18.1)	0.0067
8	41 (17.2)	0.0053
10	36 (15.1)	0.0016
15	31 (13.0)	0.0003
20	29 (12.2)	0.0001
30	22 (9.2)	<0.0001
50	17 (7.1)	<0.0001
100	8 (3.4)	0.0021

$p16^{INK4a}$ hypermethylated samples are expressed as the number (%).

analyses in clinical stage II and IV were not available because of the small number of patients in these sub-groups. As representative results, the Kaplan-Meier survival curves using a cut-off value of 10 PMR are shown in Figure 1.

Discussion

In the present study, we explored the prognostic significance of $p16^{INK4a}$ methylation in NSCLC using the MethyLight method of quantitative DNA methylation analysis. The results clearly demonstrate the prognostic significance of $p16^{INK4a}$ methylation. In particular, methylation of more than 6 PMR was a significant indicator of poor prognosis. On the other hand, there was no significant difference in prognosis between the hypermethylated and non-hypermethylated groups when using a cut-off value of 1 PMR. The results suggest that a low level of methylation of approximately 1 PMR is of limited clinical significance. The frequency of the samples judged as hypermethylated was 36.6% with a cut-off value of 1 PMR. Previous studies of the prognostic significance of $p16^{INK4a}$ methylation indicated hypermethylation frequencies ranging from 25 to 83% (8-13). A negative prognostic correlation was observed in studies showing $p16^{INK4a}$ hypermethylation frequencies of 39, 67 and 83%, whereas a positive correlation was demonstrated in those of 25, 40.4 and 49%. The negative studies may have overvalued the low-level methylation and underestimated the prognostic significance of $p16^{INK4a}$ methylation. All previous studies on $p16^{INK4a}$ methylation used qualitative methylation analysis, methylation-specific PCR (MSP). It has been reported that MSP can constantly detect 0.1% methylated DNA (17). This high sensitivity of MSP is accompanied by a risk of overvaluing low-level methylation, which has no clinical significance. Therefore, quantitative evaluation of DNA methylation may be more

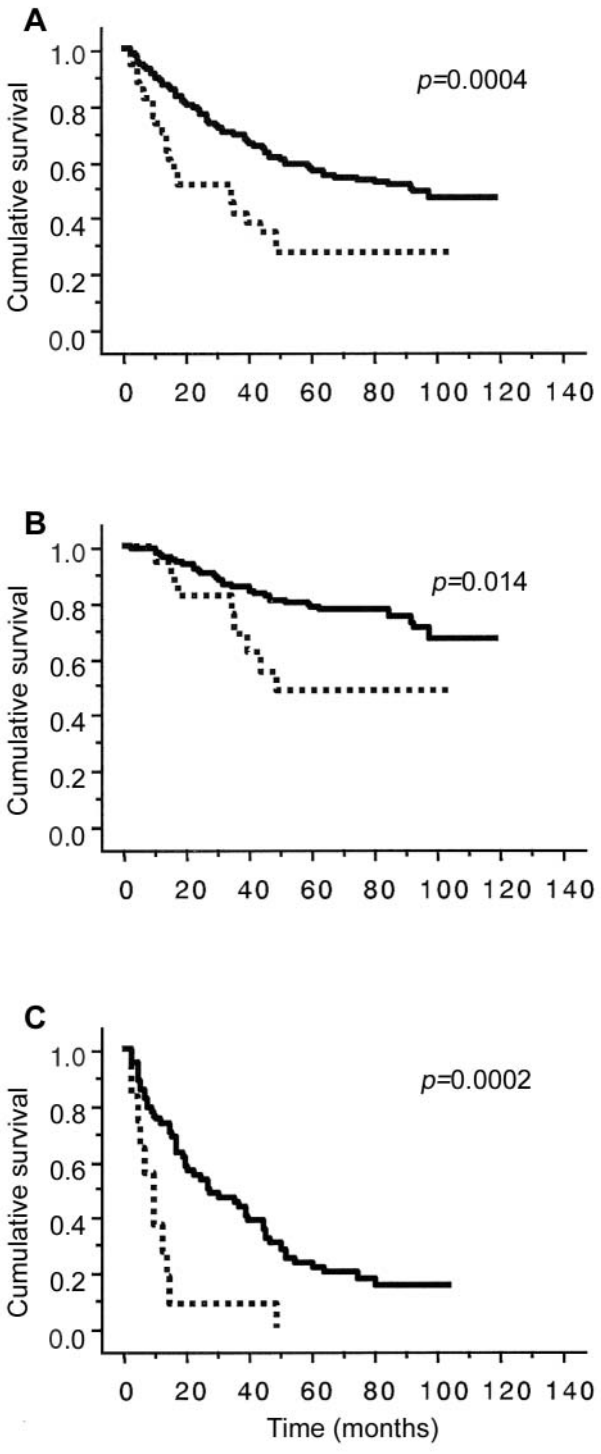

Figure 1. *Cumulative survival curves were constructed for all patients (A), those with clinical stage I disease (B) and those with clinical stage III disease (C) by the Kaplan-Meier method, using a cut-off value of 10 PMR. The dotted line indicates patients with $p16^{INK4a}$ hypermethylation (≥10 PMR) and the solid line indicates those without hypermethylation (<10 PMR). There was a significant difference in prognosis between the patients with and without hypermethylation in all groups.*

optimal for exploring the clinical significance of a given aberrant promoter methylation.

Another advantage of the present study was the relatively large number of samples analyzed. DNA obtained from formalin-fixed paraffin-embedded archive tissues was sufficient for our methylation analysis. The use of these archive specimens enabled us to analyze a large number of samples retrospectively. The archives also have the great advantage in that molecular events can be explored in association with a variety of clinical information, including long-term survival rates. Availability of formalin-fixed paraffin-embedded archives for DNA methylation analysis may advance our knowledge of methylation markers. The exploration of prognostic markers has been performed by analyzing DNA, mRNA and protein. However, targeting mRNA or protein for molecular analysis is intrinsically vulnerable to assay error because of the target's instability. Specific handling of clinical samples is often required to analyze mRNA or protein. On the other hand, alterations in DNA were suggested to be more stable than the changes in mRNA or protein during the clinical handling from tumor resection to the final assay. The results of the present study demonstrate that formalin-fixed paraffin-embedded tissue, which is generally used in clinical routine work, is sufficient for DNA methylation analysis. The convenience for sample handling may facilitate the use of DNA methylation markers in the clinical setting.

In conclusion, we provided clear evidence of the prognostic significance of $p16^{INK4a}$ methylation in NSCLC using quantitative DNA methylation analysis. The implications of aberrant promoter methylation for clinical significance should be analyzed with quantitative methods, because qualitative evaluation may have overvalued low-level methylation, which has less clinical significance.

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References

1 Jones PA and Baylin SB: The fundamental role of epigenetic events in cancer. Nat Rev Genet *3*: 415-428, 2002.

2 Brabender J, Usadel H, Danenberg KD, Metzger R, Schneider PM, Lord RV, Wickramasinghe K, Lum CE, Park J, Salonga D, Singer J, Sidransky D, Holscher AH, Meltzer SJ and Danenberg PV: Adenomatous polyposis coli gene promoter hypermethylation in non-small cell lung cancer is associated with survival. Oncogene *20*: 3528-3532, 2001.

3 Tang X, Khuri FR, Lee JJ, Kemp BL, Liu D, Hong WK and Mao L: Hypermethylation of the death-associated protein (DAP) kinase promoter and aggressiveness in stage I non-small-cell lung cancer. J Natl Cancer Inst *92*: 1511-1516, 2000.

4 Maruyama R, Sugio K, Yoshino I, Maehara Y and Gazdar AF: Hypermethylation of FHIT as a prognostic marker in nonsmall cell lung carcinoma. Cancer *100*: 1472-1477, 2004.

5 Brabender J, Usadel H, Metzger R, Schneider PM, Park J, Salonga D, Tsao-Wei DD, Groshen S, Lord RV, Takebe N, Schneider S, Holscher AH, Danenberg KD and Danenberg PV: Quantitative o(6)-methylguanine DNA methyltransferase methylation analysis in curatively resected non-small cell lung cancer: associations with clinical outcome. Clin Cancer Res *9*: 223-227, 2003.

6 Merlo A, Herman JG, Mao L, Lee DJ, Gabrielson E, Burger PC, Baylin SB and Sidransky D: 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat Med *1*: 686-692, 1995.

7 Herman JG, Merlo A, Mao L, Lapidus RG, Issa JP, Davidson NE, Sidransky D and Baylin SB: Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res *55*: 4525-4530, 1995.

8 Toyooka S, Suzuki M, Maruyama R, Toyooka KO, Tsukuda K, Fukuyama Y, Iizasa T, Aoe M, Date H, Fujisawa T, Shimizu N and Gazdar AF: The relationship between aberrant methylation and survival in non-small-cell lung cancers. Br J Cancer *91*: 771-774, 2004.

9 Wang J, Lee JJ, Wang L, Liu DD, Lu C, Fan YH, Hong WK and Mao L: Value of $p16^{INK4a}$ and RASSF1A promoter hypermethylation in prognosis of patients with resectable non-small cell lung cancer. Clin Cancer Res *10*: 6119-6125, 2004.

10 Kim YT, Park SJ, Lee SH, Kang HJ, Hahn S, Kang CH, Sung SW and Kim JH: Prognostic implication of aberrant promoter hypermethylation of CpG islands in adenocarcinoma of the lung. J Thorac Cardiovasc Surg *130*: 1378. Epub Oct 13, 2005.

11 Kim YT, Lee SH, Sung SW and Kim JH: Can aberrant promoter hypermethylation of CpG islands predict the clinical outcome of non-small cell lung cancer after curative resection? Ann Thorac Surg *79*: 1180-1188, 2005.

12 Safar AM, Spencer H, 3rd, Su X, Coffey M, Cooney CA, Ratnasinghe LD, Hutchins LF and Fan CY: Methylation profiling of archived non-small cell lung cancer: a promising prognostic system. Clin Cancer Res *11*: 4400-4405, 2005.

13 Tanaka R, Wang D, Morishita Y, Inadome Y, Minami Y, Iijima T, Fukai S, Goya T and Noguchi M: Loss of function of p16 gene and prognosis of pulmonary adenocarcinoma. Cancer *103*: 608-615, 2005.

14 Ogino S, Cantor M, Kawasaki T, Brahmandam M, Kirkner G, Weisenberger DJ, Campan M, Laird PW, Loda M and Fuchs CS: CpG island methylator phenotype (CIMP) of colorectal cancer is best characterized by quantitative DNA methylation analysis and prospective cohort studies. Gut *11*: in press, 2006.

15 Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, Danenberg PV and Laird PW: MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res *28*: E32, 2000.

16 Eads CA, Lord RV, Wickramasinghe K, Long TI, Kurumboor SK, Bernstein L, Peters JH, DeMeester SR, DeMeester TR, Skinner KA and Laird PW: Epigenetic patterns in the progression of esophageal adenocarcinoma. Cancer Res *61*: 3410-3418, 2001.

17 Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA *93*: 9821-9826, 1996.

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