Quantitative promoter hypermethylation analysis of *RASSF1A* in lung cancer: Comparison with methylation-specific PCR technique and clinical significance

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Abstract. Lung cancer is the major health problem and leading cause of cancer-related deaths worldwide owing to late diagnosis and poor prognosis. Aberrant promoter methylation is an important mechanism for silencing tumor-suppressor genes in cancer and a promising tool for the development of molecular biomarkers. *Ras association domain family 1A* (*RASSF1A*), a pivotal gatekeeper of cell cycle progression, is highly methylated in a wide range of human sporadic tumors, including lung cancer. However, no significant prognostic implications have been observed in most studies qualitatively analyzed by methylation-specific PCR (MSP). We found that the *RASSF1A* promoter was aberrantly methylated in 44.7 and 37.4% of the tumors by pyrosequencing (PS) and MSP methods, respectively. *RASSF1A* methylation evaluated by the two methods was more frequent in ever-smokers and tumors with *TP53* mutation than in never-smokers and tumors without *TP53* mutation, respectively. Univariate and multivariate analyses revealed that strong methylation was an unfavorable prognostic factor with stage I (adjusted HR, 2.25; 95% CI 1.03-4.90; P=0.003) and squamous cell carcinoma patients (adjusted HR=2.25, 95% CI 1.03-4.90, P=0.042). Taken together, these results suggested that quantitative PS could gain wider applications in clinical samples as a promising method for early detection screening and prognosis compared with MSP.

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Introduction

Lung cancer, the leading cause of cancer-related mortality worldwide, is unique among common malignancies in that it does not have a proven screening or early detection strategy (1). Of note, lung cancer is a heterogeneous group of diseases made up of entities characterized by distinct clinical, pathological, morphologic and genetic features (2). However, the molecular basis of these variations in behavior and epidemiology is not yet well known.

Aberrant methylation of CpG islands within the gene promoter associated with transcriptional inactivation is as common as gene mutations are in human cancer (3). This change may also drive cells toward a certain oncogenic pathway, predisposing cells to the accumulation of genetic mutations (4). A wealth of evidence has indicated that multiple tumor-related and tumor suppressor genes (TSGs) are frequently methylated in lung cancer (5-7). In addition, tobacco smokers are predisposed to acquire multiple epigenetic alterations in key cellular regulatory genes within the respiratory tract (8). Accordingly, identification of aberrantly methylated genes may improve the clinical management of lung cancer by facilitating earlier disease diagnosis and providing more accurate prognostic information. There is now a wide range of methods to analyze the methylation status (9). Methylation-specific PCR (MSP) and pyrosequencing (PS) are highly rapid, sensitive and robust methods, thus amenable to broad usage (10,11). The former information is considered only qualitative, while the latter offers quantitative analysis of multiple CpG sites.

Ras association domain family 1A (*RASSF1A*), a negative effector of RAS, functions as a tumor suppressor in cancer through several distinct pathways, including apoptosis, genomic and microtubule stability, and cell cycle regulation (12). *RASSF1A* methylation is frequently found in a variety of human cancers, including lung cancer (13,14). To date, the prevalence of *RASSF1A* methylation is exhibited in 30-40% of non-small cell lung cancers (NSCLCs), which have mainly been determined by MSP analysis (12). However, only one study of methylation status by PS analysis has been performed in NSCLCs (15). Furthermore, there is no report to compare *RASSF1A* methylation status in NSCLCs by qualitative MSP and quantitative PS methods, and correlate

their results with survival outcomes of patients with NSCLC. Herein, to determine which method may have greater clinical utility, we evaluated the methylation status of the *RASSF1A* gene promoter by means of MSP and PS in a large number of NSCLC tissue specimens, and then compared the results.

Materials and methods

Patients and tissue samples. Tumor and corresponding non-malignant lung tissue specimens were provided by the National Biobank of Korea, Kyungpook National University Hospital (KNUH; Daegu, Korea), which is supported by the Ministry of Health, Welfare and Family Affairs. All materials derived from the National Biobank were obtained under Institutional Review Board approved protocols. The clinicopathological characteristics of the patients are summarized in Table I. A total of 140 males and 66 females with a mean age of 62.5±8.6 years were included in this study. There were 60 never-smokers and 146 ever-smokers (current- or former-smokers) with a mean pack-years of 27.5±24.6. The histological types of NSCLSs were 84 cases of squamous cell carcinomas (SCCs) and 122 adenocarcinomas (ACs). Regarding the pathological stages, there were 130 cases at stage I and 76 cases at stage II-IV. All of the tumor and macroscopically normal lung tissue samples were obtained at the time of surgery, rapidly frozen in liquid nitrogen and stored at -80˚C until genomic DNA preparation. Only tumors with >80% of the tumor components were sent for DNA extraction and methylation analysis. The macroscopically normal lung tissues were confirmed to be normal by H&E staining. In addition, mutations of the *TP53* (entire coding exons) and *EGFR* (exons 18-21) genes were detected by PCR-based direct sequencing.

Genomic DNA isolation and bisulfite treatment. Genomic DNA was extracted from primary tumors and corresponding non-malignant lung tissues using a QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. Extracted DNA samples were treated with sodium bisulfite (Sigma, St. Louis, MO, USA) and purified by using a Wizard DNA clean-up system (Promega Corporation, Madison, WI, USA), as described previously (16). CpGenome™ Universal methylated and unmethylated DNA (Chemicon, Temecula, CA, USA) were treated the same way and were used as a positive control for the methylated and unmethylated genes in MSP and PS, respectively.

Pyrosequencing. Methylation of the *RASSF1A* gene promoter was quantitatively analyzed by PS method. Briefly, bisulfite-modified DNA was amplified using forward (5'-GTTAGGGTTCGGATGTGGGGATTT-3') and reverse primers (biotin-5'-TACCCTTCCTTCCCTCCTTC-3') through PCR, enabling the conversion of the PCR product to a single-stranded DNA template suitable for PS. All samples were heated to 95˚C for 5 min and then amplified for 45 cycles at 95˚C for 45 sec, 58˚C for 45 sec and 72˚C for 45 sec, followed by a final extension at 72˚C for 5 min. Confirmation of PCR product quality and freedom from contamination was established on 2% agarose gels with ethidium bromide staining. Following purification of the PCR product using Sepharose beads on PyroMark Vacuum Prep Workstation (Qiagen), PS was performed according to the manufacturer's specifications with sequencing primer (5'-AAAGTTGGTTTTTAGAAATA-3') using the PyroMark Q96MD System (Qiagen). A mean methylation index (MI) was calculated from the mean of the methylation percentage for all observed CpG sites. To set the controls for PS, we used CpGenome™ Universal methylated and unmethylated DNA that were consistently positive or negative with stable levels of methylation. We tested each DNA in triplicate and used their average in the statistical analyses.

Methylation-specific PCR. The methylation of the *RASSF1A* gene targeting the same region validated by PS was qualitatively performed on the bisulfite-treated DNA by using a MSP as previously described (17). All PCR amplifications were performed using reagents supplied in a GeneAmp DNA Amplification kit with AmpliTaq Gold as the polymerase (PE Applied Biosystems, Foster City, CA, USA) on PTC-100 (MJ Research, Watertown, MA, USA). Negative control samples without DNA were included for each set of PCR. PCR products were analyzed on 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet (UV) light. The reproducibility of the results was confirmed by repeating the MSP analysis for each DNA sample. A portion of the PCR products was purified using PCR purification kit (Qiagen) and directly sequenced to confirm their methylation status.

Statistical analysis. The relationship between the methylation and the clinicopathological characteristics was analyzed using a Chi-square test or Fisher's exact test for categorical variables. A logistic regression analysis was conducted to estimate the relationship between methylation and the covariates of age, gender, exposure to tobacco smoke and histology. Overall survival (OS) was measured from the day of surgery until the date of death or to the date of the last follow-up. The survival estimates were calculated using the Kaplan-Meier method. The differences in OS across different groups were compared using the log-rank test. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using multivariate Cox proportional hazards model.

Results

Methylation status of RASSF1A gene in NSCLCs. PS analysis was undertaken on 206 malignant and 40 non-malignant lung tissues resected from NSCLC patients. Accurate and reproducible estimates of methylated cytosine content were obtained in 100% of the tested samples and the representative programs are shown in Fig. 1A. Considering a maximal methylation index (MI) (4.69%) in all non-malignant samples, we used an MI ≥5 as a cut-off point for methylation-positive classification. The *RASSF1A* promoter was methylated in 92 (44.7%) of the 206 NSCLCs and 1 (2.5%) of 40 non-malignant samples (Table I), suggesting that promoter methylation of the *RASSF1A* gene is not an intrinsic, developmentally programmed event, but a *de novo*, tumor-specific event. Furthermore, using a median MI (17.36%) for 92 methylated samples as a cutoff point, the methylation-positive tumors were divided into two groups, weak-methylation (5≤ MI <17) and strong-methylation (MI ≥17). Methylation prevalence of the two groups was 28.8% (Table I). In addition, we also determined the methylation status

Table I. Correlation between *RASSF1A* methylation and clinicopathological features in the NSCLC patients.

Variables	Pyrosequencing						Methylation-specific PCR	
	Overall n (%)	P-value	Weak[a] n (%)	P-value	Strong[b] n (%)	P-value	Methylation n (%)	P-value
All subjects (n=206)	92 (44.7)		46 (28.8)		46 (28.8)		77 (37.4)	
Age (years)		0.025		0.291		0.011		0.195
≤63 (n=103)	38 (36.9)		22 (25.3)		16 (19.8)		34 (33.0)	
>63 (n=103)	54 (52.4)		24 (32.9)		30 (38.0)		43 (41.7)	
Gender		0.100		0.442		0.065		0.080
Female (n=66)	24 (36.4)		14 (25.0)		10 (19.2)		19 (28.8)	
Male (n=140)	68 (48.6)		32 (30.8)		36 (33.3)		58 (41.4)	
Smoking status		0.016		0.081		0.043		0.042
Never (n=60)	19 (31.7)		10 (19.6)		9 (18.0)		16 (26.7)	
Ever (n=146)	73 (50.0)		36 (33.0)		37 (33.6)		61 (41.8)	
Histological type		0.890		0.912		0.912		0.860
SCC (n=84)	38 (45.2)		19 (29.2)		19 (29.2)		32 (38.1)	
AC (n=122)	54 (44.3)		27 (28.4)		27 (28.4)		45 (36.9)	
Pathological stage		0.040		0.199		0.041		0.171
Stage I (n=130)	51 (39.2)		27 (25.5)		24 (23.3)		44 (33.8)	
Stage II-IIIA (n=76)	41 (53.9)		19 (35.2)		22 (38.6)		33 (43.4)	
P53 mutation[c]		0.001		0.099		<0.001		0.019
Absent (n=106)	33 (31.1)		21 (22.3)		12 (14.1)		31 (29.2)	
Present (n=66)	37 (56.1)		16 (35.6)		21 (42.0)		31 (47.0)	
EGFR mutation[d]		0.039		0.627		0.005		0.300
Absent (n=137)	52 (38.0)		30 (26.1)		22 (20.6)		48 (35.0)	
Present (n=41)	23 (56.1)		8 (30.8)		15 (45.5)		18 (43.9)	

[a]Weak methylation, 5≤ MI <17; [b]strong-methylation, MI ≥17. [c,d]The mutations of *P53* and *EGFR* genes were studied in 172 and 178 of the 206 NSCLCs. NSCLC, non-small cell lung cancer; MI, methylation index; SCC, squamous cell carcinoma; AC, adenocarcinoma.

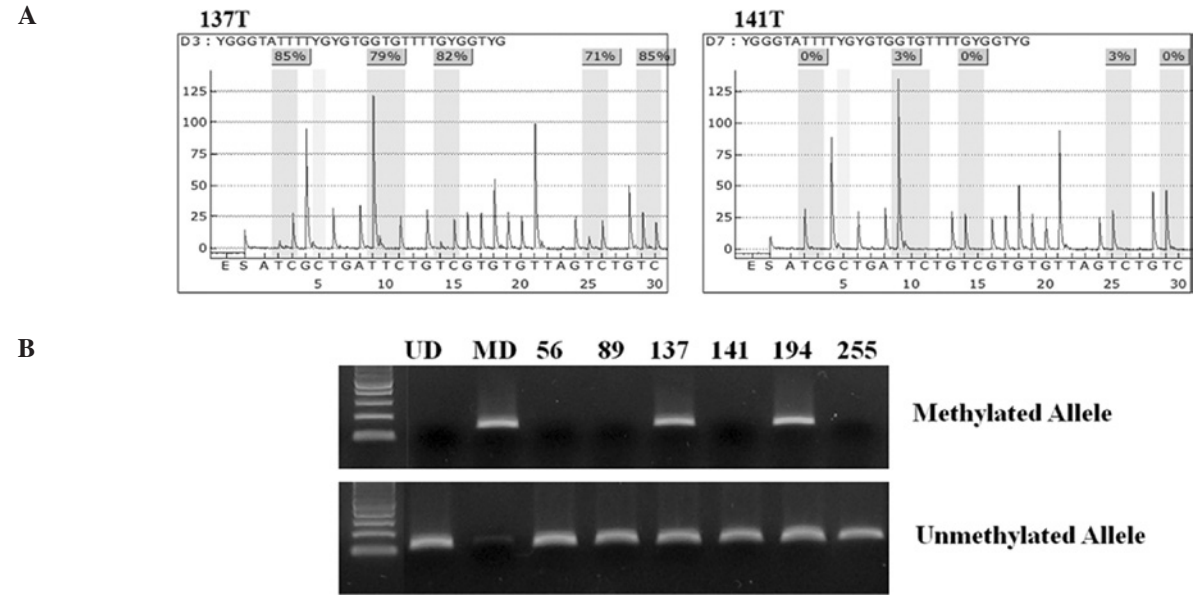

Figure 1. (A) Representative pyrograms and (B) methylation-specific PCR for the *RASSF1A* gene. (A) The letters on the axis represent the dispensation order; E, enzyme mix; S, substrate; A, G, C and T, nucleotides. Shaded bars encompassing T/C pairs, indicate five interrogated CpG's. The methylation of each CpG site was calculated as a percentage of C incorporation. (B) CpGenome™ universal methylated or unmethylated DNA was used as a positive control for the methylated or unmethylated products, respectively.

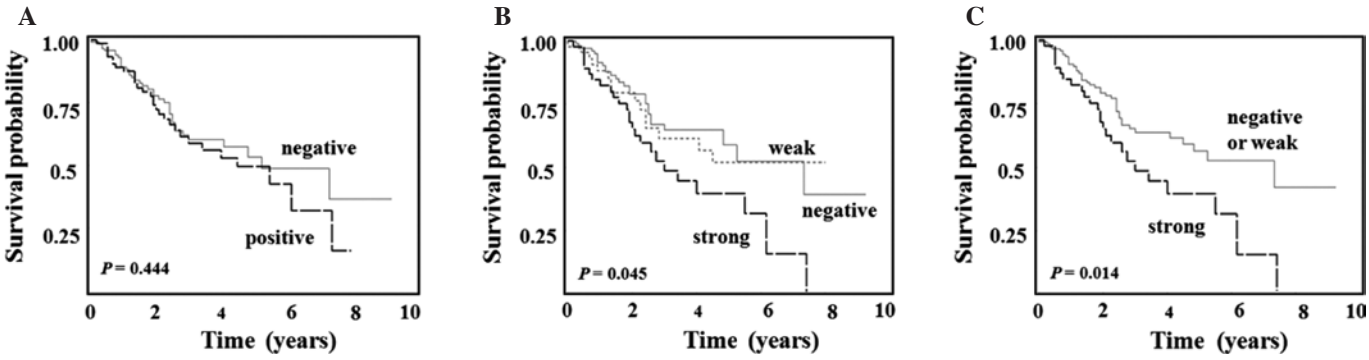

Figure 2. Overall survival curves of patients according to *RASSF1A* methylation status evaluated by (A) methylation-specific PCR and (B and C) by pyrosequencing. Negative, methylation index (MI) <5; weak, 5≤ MI <17; and strong, MI ≥17. P-values from log-rank test.

Table II. Association of the methylation level of the *RASSF1A* gene with overall survival in NSCLC patients.

Methylation status	No. of cases (%)[a]	No. of deaths (%)[b]	5 years (%)[c]	Overall survival			
				HR (95% CI)[d]	P-value	Adjusted HR (95% CI)[d]	P-value
Negative	114 (55.3)	27 (23.7)	58.9	1		1	
Weak	46 (22.3)	15 (32.6)	56.7	1.16 (0.62-2.19)	0.639	1.02 (0.54-1.93)	0.945
Strong	46 (22.3)	25 (54.4)	39.7	1.93 (1.12-3.33)	0.018	1.27 (0.72-2.25)	0.413
P-value[c]			0.045				
Negative or weak	160 (77.6)	42 (26.3)	56.5	1		1	
Strong	46 (22.3)	25 (54.4)	39.7	1.84 (1.12-3.01)	0.016	1.26 (0.75-2.13)	0.389
P-value[c]			0.014				

Negative methylation, MI <5; weak methylation, 5 ≤MI <17; strong methylation, MI ≥17. [a]Column percentage; [b]row percentage. [c]5-year survival rate: proportion of survival derived from Kaplan-Meier estimator. P-value was calculated by the log-rank test. [d]Hazard ratio (HR), 95% confidence interval (CI) and their corresponding P-values were calculated using Cox proportional hazard models and adjusted for age, gender, smoking status, tumor histology and stage. NSCLC, non-small cell lung cancer.

in 206 resected NSCLC samples using MSP. Representative examples of the MSP analysis are illustrated in Fig. 1B; methylation of the *RASSF1A* gene was found in 77 (37.4%) of the 206 NSCLCs (Table I). In comparison between PS- and MSP-based methylation assessment, when the strong-methylation in PS assay was considered as methylation-positive, the concordance rate of methylation-positive samples was 97.8%, but when the weak-methylation in PS assay was also included in the methylation-positive category, this led to a decrease in the concordance rate to 75% (data not shown).

Correlation of RASSF1A methylation status and clinicopathological characteristics. When methylation status was analyzed by PS assay, *RASSF1A* methylation was significantly associated with age, smoking status, pathological stage, *EGFR* and *TP53* mutation. In addition, these associations were more pronounced in the strong-methylation group compared to the weak-methylation group (Table I). MSP data revealed that *RASSF1A* methylation was significantly associated only with smoking status and *TP53* mutation (Table I).

Effect of RASSF1A methylation on survival outcome. Kaplan-Meier analysis revealed that *RASSF1A* methylation status determined by MSP did not significantly correlate with overall survival (OS) of the patients (Fig. 2A). However, according to the PS results the strong-methylation group was associated with a significantly worse OS compared to the negative-methylation group (P=0.045; Fig. 2B). In addition, the strong-methylation group exhibited a significantly worse OS compared to the combined negative- and weak-methylation group (P=0.014; Fig. 2C). Unfortunately, a multivariate survival analysis using Cox proportional hazards model revealed that strong-methylation of the *RASSF1A* gene did not effect the survival outcome (adjusted HR, 1.26; 95% CI 0.75-2.13; P=0.389; Table II). Notably, when the patients were stratified according to pathological stage and histological types, *RASSF1A* strong-methylation was significantly associated with a worse survival in patients with stage I (adjusted HR, 2.25; 95% CI 1.03-4.90; P=0.003) and SCC patients (adjusted HR, 2.25; 95% CI 1.03-4.90; P=0.042; Fig. 3 and Table III).

Discussion

In the present study, *RASSF1A* methylation was discovered in 44.7 and 37.4% of the tumors by PS and MSP assays, respectively, being consistent with the previous data that

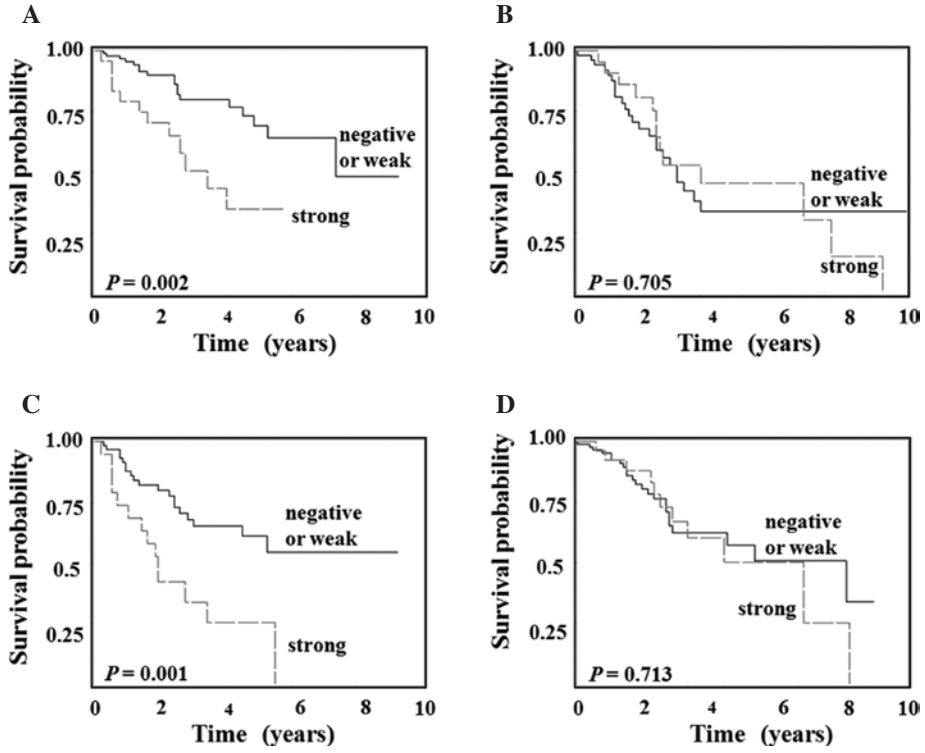

Figure 3. Kaplan-Meier survival curve of *RASSF1A* methylation levels according to pathological stage and tumor histology. P-values from log-rank test. (A) Stage I; (B) stage II-IIIA; (C) squamous cell carcinomas; (D) adenocarcinomas.

Table III. Multivariate analysis of overall survival with methylation level of *RASSF1A* according to pathological stage and histologic type.

Variables	Methylation status	No. of cases (%)[a]	No. of deaths (%)[b]	5 years (%)[c]	Overall survival			
					HR (95% CI)[d]	P-value	Adjusted HR (95% CI)[d]	P-value
Stage I	Negative or weak	106 (81.5)	18 (17.0)	69.1	1		1	
	Strong	24 (18.5)	12 (50.0)	34.6	1.84 (1.12-3.01)	0.016	3.10 (1.48-6.51)	0.003
	P-value[c]			0.002				
Stage II-IIIA	Negative or weak	54 (71.1)	24 (44.4)	33.8	1		1	
	Strong	22 (28.9)	13 (59.1)	45.3	0.88 (0.44-1.74)	0.708	0.85 (0.42-1.73)	0.652
	P-value[c]			0.705				
SCC	Negative or weak	65 (77.4)	19 (29.2)	61.1	1		1	
	Strong	19 (22.6)	14 (73.7)	25.3	3.01 (1.50-6.04)	0.002	2.25 (1.03-4.90)	0.042
	P-value[c]			0.001				
AC	Negative or weak	95 (77.9)	23 (24.2)	50.9	1		1	
	Strong	27 (22.1)	11 (40.7)	50.2	1.14 (0.56-2.35)	0.715	0.67 (0.31-1.47)	0.317
	P-value[c]			0.713				

Negative methylation, MI <5; weak methylation, 5 ≤MI <17; strong methylation, MI ≥17. [a]Column percentage; [b]row percentage. [c]5-year survival rate: proportion of survival derived from Kaplan-Meier estimator. P-value was calculated by log-rank test. [d]Hazard ratio (HR), 95% confidence interval (CI) and their corresponding P-values were calculated using Cox proportional hazard models and adjusted for age, gender, smoking status, tumor histology and stage. SCC, squamous cell carcinoma; AC, adenocarcinoma; MI, methylation index.

RASSF1A methylation is typically observed in 30-40% of NSCLCs (12-15). Moreover, regardless of the detection methods, *RASSF1A* methylation was more frequent in ever-smokers and *TP53* mutation-positive tumors than in never-smokers and *TP53* mutation-negative tumors, respectively.

Although MSP and PS data demonstrated no significant correlation between OS and methylation levels of the

RASSF1A promoter in NSCLC patients, the strong-methylation of *RASSF1A* measured by PS was an unfavorable prognostic factor for stage I and SCC patients. Although a significant association between methylation status of the *RASSF1A* promoter and poor prognosis has been reported in lung cancer patients (17-19), our finding is the first report regarding the association of *RASSF1A* methylation levels with survival outcome. In this regard, it is reasonable to speculate that PS assessment may be more informative of prognosis and have greater potential clinical utility than MSP, being compatible with a recent finding (20). Although MSP and PS methods are sensitive enough to detect the low concentration of methylated alleles in target cells, the present study emphasizes the need to be more cautious in clinical interpretation of low-levels of methylation. Moreover, faint bands may be frequently observed on MSP analysis, potentially leading to interpretive difficulties. Therefore, considering the tissue heterogeneity that primary tumor tissues usually contain different fractions of target sequences (partial or complete methylation) and tumor cells, quantitative PS is gaining wider applications in clinical samples as a promising method for early detection screening and prognosis than MSP. However, additional studies are required to clarify the prognostic value of the methylation level of the *RASSF1A* gene in NSCLC.

Notably, considering the statistical power for the small number of methylated samples above background threshold, we used the median level of methylation observed by PS to classify tumors as weak vs. strong, and an association with survival was observed only in the 'strong tumors'. In actuality, when samples were repeated commencing with bisulfate medication, this cutoff would exclude or include some different tumors that, in turn, would probably affect the observed association to prognosis, indicating that this finding may simply be due to chance. However, we tested each tumor DNA in three replicates and used their average in the statistical analyses, ruling out this possibility. In addition, log-rank test of patients OS through a series of methylation levels seen by PS revealed that the lowest P-value was observed around the median level (data not shown). Similarly, Shaw *et al* have divided the methylated samples into a weak- and strong-methylated group using a median split, which is fortunately necessary to silence mRNA expression for the *RASSF1A* gene (21). Although there was an acute shortage of reasoning rationale for using median MI as a cutoff, our observation may offer new insight for the clinical management of methylation levels observed by PS.

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