Clinicopathological Significance of Epigenetic Inactivation of *RASSF1A* at 3p21.3 in Stage I Lung Adenocarcinoma[1]

Yoshio Tomizawa, Takashi Kohno, Haruhiko Kondo, Ayaka Otsuka, Michiho Nishioka, Toshiro Niki, Tesshi Yamada, Arafumi Maeshima, Kimio Yoshimura, Ryusei Saito, John D. Minna, and Jun Yokota[2]

Biology Division [Y. T., T. K., A. O., M. N., J. Y.], Pathology Division [T. N., T. Y., A. M.], and Cancer Information and Epidemiology Division [K. Y.], National Cancer Center Research Institute and Thoracic Surgery Division [H. K.], National Cancer Center Hospital, Tokyo 104-0045, Japan; First Department of Internal Medicine, School of Medicine, University of Gunma, Gunma, 371-8511, Japan [Y. T., R. S.]; and Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, Texas 75390 [J. D. M.]

ABSTRACT

Purpose: **Chromosome 3p is deleted frequently in various types of human cancers, including lung cancer. Recently, the *RASSF1A* gene was isolated from the 3p21.3 region homozygously deleted in lung and breast cancer cell lines, and it was shown to be inactivated by hypermethylation of the promoter region in lung cancers. In this study, we investigated the pathogenetic and clinicopathological significances of *RASSF1A* methylation in the development and/or progression of lung adenocarcinoma.**

Experimental Design: **Association of *RASSF1A* methylation with clinicopathological features, allelic imbalance at 3p21.3, *p53* mutations, and K-*ras* mutations was examined in 110 stage I lung adenocarcinomas.**

Results: **Thirty-five of 110 (32%) tumors showed *RASSF1A* methylation. *RASSF1A* methylation was dominantly detected in tumors with vascular invasion ($P = 0.0242$) or pleural involvement ($P = 0.0305$), and was observed more frequently in poorly differentiated tumors than in well ($P = 0.0005$) or moderately ($P = 0.0835$) differentiated tumors. Furthermore, *RASSF1A* methylation correlated with adverse survival by univariate analysis ($P = 0.0368$; log-rank test) as well as multivariate analysis ($P = 0.032$,; risk ratio 2.357; 95% confidence interval, 1.075–5.169). The correlation between *RASSF1A* methylation and allelic imbalance at 3p21.3 was significant ($P = 0.0005$), whereas the correlation between *RASSF1A* methylation and *p53* mutation was borderline ($P = 0.0842$). However, there was no correlation or inverse correlation between *RASSF1A* methylation and K-*ras* mutation ($P = 0.2193$).**

Conclusions: **These results indicated that epigenetic inactivation of *RASSF1A* plays an important role in the progression of lung adenocarcinoma, and that *RASSF1A* hypermethylation appears to be a useful molecular marker for the prognosis of patients with stage I lung adenocarcinoma.**

INTRODUCTION

Recent advances in the molecular genetics of human cancers have revealed that multiple TSGs[3] are involved in human lung carcinogenesis (1). Chromosome 3p allele loss is a frequent event in the development of lung cancer (2–5). Multiple 3p regions have been identified as showing frequent allelic losses in lung and other cancers by detailed allelotyping studies. This suggests that there are several TSGs on 3p (5–7). Homozygous deletions have been found at 3p21.3 in lung cancer and breast cancer cell lines, and the 630-kb 3p21.3 homozygous deletion region has been studied extensively (8). The *RASSF1* gene was isolated from this region as a candidate TSG (9–11). The *RASSF1* locus encodes several major transcripts by alternative promoter selection and alternative mRNA splicing. *RASSF1A*, one of the several transcripts, encodes a M_r 39,000 predicted peptide with a Ras association domain and a predicted NH_2-terminal diacylglycerol binding domain. Transfection and expression of *RASSF1A* in lung cancer cells resulted in suppression of colony formation, anchorage-independent soft agar growth, and nude mouse tumorigenicity (9, 10). Mutations of *RASSF1A* are uncommon, whereas the lack of expression is common in lung cancer (9, 10). *RASSF1A* was expressed in normal bronchial epithelial cells but was not expressed in 80–100% of small cell lung cancer and 30–60% of NSCLC (9, 10, 12, 13). A putative promoter region of the *RASSF1A* gene was highly methylated in lung tumors but not in normal lung tissues, and methylation of this region was associated with reduction of *RASSF1A* transcription (9, 10). It was reported recently that *RASSF1A* methylation in 107 resected NSCLCs was associated with impaired patient survival (10). However, because the stage

Received 11/29/01; revised 4/2/02; accepted 4/3/02.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported in part by a Grant-in-Aid from the Ministry of Health, Labor, and Welfare for the 2nd-term Comprehensive 10-Year Strategy for Cancer Control; Grants-in-Aid from the Ministry of Health, Labor, and Welfare and from the Ministry of Education, Culture, Sports, Science and Technology, Japan; National Cancer Institute USA Grants Specialized Programs of Research Excellence P50 CA70907 and R01 CA71618; and the G. Harold and Leila Y. Mathers Charitable Foundation.
[2] To whom requests for reprints should be addressed, at Biology Division, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan. Phone: 81-3-3547-5272; Fax: 81-3-3542-0807.

[3] The abbreviations used are: TSG, tumor suppressor gene; NSCLC, non-small cell lung cancer; CI, confidence interval; LOH, loss of heterozygosity; AI, allelic imbalance; MSP, methylation-specific PCR; LBAC, localized bronchioloalveolar carcinoma.

Table 1 Correlation of *RASSF1A* methylation with clinicopathological characteristics in patients with stage I lung adenocarcinomas

Clinicopathological feature	Subset	No. of *RASSF1A* methylation (%)		P^a
		+	−	
Gender	Male	24 (38)	40 (62)	0.1506
	Female	11 (24)	35 (76)	
Age	<60	14 (34)	27 (66)	0.8326
	60–69	12 (31)	27 (69)	
	>69	9 (30)	21 (70)	
Smoking history	Smoker	22 (35)	40 (65)	0.4117
	Nonsmoker	13 (27)	35 (73)	
T stage	T1	23 (31)	52 (69)	0.8264
	T2	12 (34)	23 (66)	
Differentiation	Poorly	11 (65)	6 (35)	0.0005 (poorly *vs.* well)
	Moderately	14 (38)	23 (62)	0.0835 (poorly *vs.* moderately)
	Well	10 (18)	46 (82)	
Vascular invasion	+	15 (48)	16 (52)	0.0242
	−	20 (25)	59 (75)	
Lymphatic permeation	+	7 (32)	15 (68)	>0.9999
	−	28 (32)	60 (68)	
Pleural involvement	+	13 (50)	13 (50)	0.0305
	−	22 (26)	62 (74)	
Histologic typing of small adenocarcinoma of the lung[b]	A	0 (0)	3 (100)	0.2585 (A, B *vs.* C, D)
	B	2 (25)	6 (75)	
	C	5 (28)	13 (72)	
	D	4 (100)	0 (0)	
	E	0	0	
	F	1 (50)	1 (50)	

[a] Fisher's exact test.
[b] Ref. 25, the greatest dimension of the tumor is ≤2 cm.

of the disease was not adjusted, it is still unclear whether the *RASSF1A* methylation is an independent prognostic factor and how *RASSF1A* methylation correlates with clinicopathological features. Therefore, it is important to examine *RASSF1A* methylation in a large number and defined subset of primary lung cancers to assess the role of *RASSF1A* expression in multistage lung carcinogenesis.

Lung cancer is a major cause of cancer-related deaths in the world. In recent years, adenocarcinoma has replaced squamous cell carcinoma as the most frequent histological subtype in lung cancers (14, 15). Prognosis of patients is largely dependent on the stage of the disease. However, even in patients with stage I NSCLC, the 5-year survival rate is ~65% (16, 17). Because there are only a few prognostic parameters that can estimate survival, it is important to identify a marker for high-risk early stage patients who should benefit from more aggressive treatment approaches.

Here, we investigated the association of *RASSF1A* methylation with clinicopathological features in 110 cases of stage I lung adenocarcinoma by the MSP method. *RASSF1A* methylation was detected dominantly in tumors with vascular invasion or pleural involvement, and was observed more frequently in poorly differentiated tumors than in well-differentiated tumors. Furthermore, *RASSF1A* methylation correlated with adverse survival in patients with stage I lung adenocarcinoma. The results indicated that epigenetic inactivation of *RASSF1A* plays an important role in the acquisition of aggressive phenotypes in lung adenocarcinoma and that *RASSF1A* methylation is a marker for prognosis of patients with stage I lung adenocarcinoma.

MATERIALS AND METHODS

Patients and Tissues. Primary lung tumors and corresponding normal lung tissues were obtained from 110 patients with lung adenocarcinoma. All of the patients underwent curative resection with systematic mediastinal lymph node dissection at the National Cancer Center Hospital, Tokyo, Japan, from December 1986 to December 1996. None of the patients had been treated before operation. All of the cases were pathologically diagnosed as being stage I lung adenocarcinoma according to the Tumor-Node-Metastasis classification of malignant tumors (18). Detailed information on these patients is summarized in Table 1. The median follow-up period in all of the patients was 101 months. Tumors and normal tissues were frozen and stored at −80°C until DNA extraction. Genomic DNAs were prepared by the method described previously (4) or by a QIAamp DNA Mini kit (Qiagen, Tokyo, Japan).

Methylation Analysis of *RASSF1A*. The methylation status of the *RASSF1A* was analyzed by MSP, as described previously (10), using bisulfite modified genomic DNA (19, 20). Briefly, 1 μg of genomic DNA was denatured with 0.2 M NaOH, and 10 mM hydroquinone (Sigma) and 3 M sodium bisulfite (Sigma) were added and incubated at 55°C for 16 h. Afterward, modified DNA was purified using Wizard DNA purification resin (Promega) followed by ethanol precipitation. Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines (but not methylated cytosines) to uracil, which are then converted to thymidine during the subsequent PCR step, giving sequence differences between methylated and unmethylated DNA. PCR primers that distinguish between these

methylated and unmethylated DNA sequences were then used. The primers to detect the methylated form were 5′-GGGTTTTGCGAGAGCGCG-3′ (forward) and 5′-GCTAACAAACGCGAACCG-3′ (reverse), and the primers to detect the unmethylated form were 5′-GGTTTTGTGAGAGTGTGTTTAG-3′ (forward) and 5′-CACTAACAAACACAAACCAAA-3′ (reverse). MSP cycling conditions consisted of one incubation of 15 min at 95°C, followed by 35 cycles of a 30 s denaturation at 94°C, 50 s at an annealing temperature (64°C for methylation-specific and 59°C for unmethylated-specific primers), a 30 s extension at 72°C, and a final extension at 72°C for 10 min. PCR products were separated in 2% agarose gels and visualized under UV illumination.

AI Analysis of the *RASSF1A* Locus. The following microsatellite loci were examined for AI at 3p21.3: *D3S4622/LUCA4.1*, *D3S4604/LUCA19.1*, and *D3S4597/P1.5*, because these markers were mapped to the regions flanking and close to the *RASSF1* gene locus (5). Primers were end labeled with fluorochromes FAM, HEX, or NED. PCR amplifications were carried out in a 20 μl reaction mixture containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 pmol of each primer, 250 μM of each deoxynucleotide triphosphate, and 0.5 unit of Taq DNA polymerase (Takara, Osaka, Japan). Thirty-five cycles of 94°C (30 s) for denaturation, 55°C (30 s) for annealing, and 72°C (30 s) for extension were performed to amplify DNA fragments. The loading mix was prepared with 12 μl of deionized formamide and 0.5 μl of ABI size standard 400HD (Perkin-Elmer). Each loading sample contained this loading mix plus 0.33 μl each of FAM-, HEX-, and NED-labeled PCR products. The fluorescent signals were laser scanned, and the data were stored electronically by the ABI310 sequencer/genotyper. The intensity of the peak heights was calculated by the Genotyper2.1 software to ascertain the ratios of allele intensities in tumor DNA as compared with the corresponding normal tissue DNA. Normal tissue samples showed small differences in the percentage of allele ratio because of artificial signal variation (SD 5.4%, $n = 40$). Thus, a signal ratio in the range of 0.76–1.0 could occur even without AI in a tumor because the mean −2 SD per mean +2 SD was 0.76. Therefore, a sample was scored as having AI if the signal ratio was <0.76.

Mutational Analysis of the K-*ras* Gene. Codons 12, 13, and 61 of the K-*ras* gene were examined for mutation by PCR amplification and direct sequencing of PCR products. The primer sets for codons 12 and 13 of the K-*ras* gene were 5′-GACTGAATATAAACTTGTGG-3′ (forward) and 5′-CTATTGTTGGATCATATTCG-3′ (reverse), and the primer sets for codon 61 were 5′-TTCCTACAGGAAGCAAGTAG (forward) and 5′-CACAAAGAAAGCCCTCCCCA-3′ (reverse), as reported previously (21). Thirty-five cycles of 94°C (30 s) for denaturation, 55°C (60 s) for annealing, and 72°C (30 s) for extension were performed to amplify DNA fragments for codons 12 and 13 of the K-*ras* gene. Touchdown PCR was used for the amplification of DNA fragment for codon 61 of the K-*ras* gene. Touchdown PCR had an initial denaturation step at 94°C for 5 min, which was followed by 10 touchdown cycles of 30 s at 94°C, 1 min at 60°C with a decrease in the annealing temperature by 1°C each cycle, and 30 s at 72°C. This was followed by 25 cycles of 30 s at 94°C, 1 min at 50°C, and 30 s at 72°C, with a final extension at 72°C for 10 min. PCR products were purified for sequencing by a QIAquick PCR Purification kit (Qiagen). Cycle sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Norwalk, CT) and the ABI PRISM 310 DNA Sequencer (Perkin-Elmer).

Mutational Analysis of the *p53* Gene. Ten portions of genomic DNA fragments covering the entire coding region of the *p53* gene, between exon 2 and exon 11, were amplified by PCR with *p53*-specific oligonucleotide primers: 5′-TTGGAAGTGTCTCATGCTGG-3′ (forward) and 5′-GTGGTGGCCTGCCCTTCC-3′ (reverse) for exon 2, 5′-CCCCTAGCAGAGACCTGTG-3′ (forward) and 5′-CCCTCCAGGTCCCCAGC-3′ (reverse) for exon 3, 5′-GAGGACCTGGTCCTCTGAC-3′ (forward) and 5′-CCAGGCATTGAAGTCTCATG-3′ (reverse) for exon 4, 5′-ACTTGTGCCCTGACTTTCAAC-3′ (forward), and 5′-CAACCAGCCCTGTCGTCTC-3′ (reverse) for exon 5, 5′-AGGGTCCCCAGGCCTCTG-3′ (forward) and 5′-AACCCCTCCTCCCAGAGAC-3′ (reverse) for exon 6, 5′-GCGCACTGGCCTCATCTTG-3′ (forward) and 5′-GCCAGTGTGCAGGGTGGC-3′ (reverse) for exon 7, 5′-GGTAGGACCTGATTTCCTTAC-3′ (forward) and 5′-TCTCCTCCACCGCTTCTTG-3′ (reverse) for exon 8, 5′-GTGCAGTTATGCCTCAGATTC-3′ (forward) and 5′-AAACTTTCCACTTGATAAGAGG-3′ (reverse) for exon 9, 5′-ATCTTTTAACTCAGGTACTGTG-3′ (forward) and 5′-GAGTAGGGCCAGGAAGGG-3′ (reverse) for exon 10, and 5′-ACCCTCTCACTCATGTGATG-3′ (forward) and 5′-GATGGGGGTGGGAGGCTG-3′ (reverse) for exon 11. PCR amplification was carried out in a 25-μl reaction mixture containing 0.6 unit of TaKaRa Ex Taq (Takara) for exons 4 and 5, and AmpliTaq Gold (Perkin-Elmer) for exons 2, 3, and 6–11. Thirty-five cycles of 94°C (60 s) for denaturation, 60°C (60 s) for annealing, and 72°C (90 s) for extension were performed to amplify DNA fragments for exons 4 and 5. Thirty-five cycles (for exons 7 and 10) or 40 cycles (exons 2, 3, 6, 8, 9, and 11) of 94°C (20 s) for denaturation, 58°C (30 s) for annealing, and 72°C (30 s) for extension were performed to amplify DNA fragments. The sizes of PCR products were from 99 to 367 bp. Denatured and reannealed PCR products were analyzed by the WAVE DNA Fragment Analysis System and WAVEMAKER Software 4.0 (TRANSGENOMIC Inc., Omaha, NE). PCR products with detected variant peaks by the WAVE DNA Fragment Analysis System were purified by a QIAquick PCR Purification kit (Qiagen) for sequencing. Cycle sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) and the ABI PRISM 310 DNA Sequencer (Perkin-Elmer).

Statistical Analysis. Fisher's exact test was used to examine the association of two categorical variables. The survival curves were estimated by the Kaplan-Meier method, and the resulting curves were compared using the log-rank test. A P of <0.05 was considered to be statistically significant. The joint effect of covariables was examined using the stepwise Cox proportional hazards regression model. The stepwise procedure was used to select significant independent variables. The probability of the statistic used to test whether a variable should be included or excluded was 0.2. Statistical analysis was performed

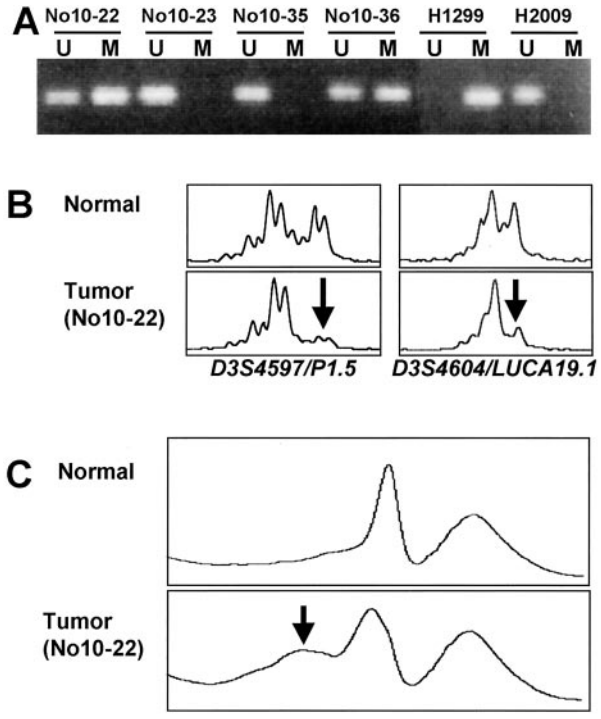

Fig. 1 *A*, methylation analysis of *RASSF1A* in primary stage I lung adenocarcinoma by MSP. *U*, amplified product with primers recognizing unmethylated sequences; *M*, amplified product with primers recognizing methylated sequences. NCI-H1299 is used as a positive control for the methylated form. NCI-H2009 is used as a positive control for the unmethylated form. *B*, AI analysis at 3p21.3 in primary lung adenocarcinoma. *Arrows* indicate AI at *D3S4597/P1.5* (*left*) and at *D3S4604/LUCA19.1* (*right*) in tumor (*No10–22*). *C*, mutational analysis of the *p53* gene by the WAVE DNA Fragment Analysis System. Variant peak is indicated by an *arrow*. The mutation of the *p53* gene in this sample (*No10–22*) was confirmed by sequencing [Arg (AGG) to Met (ATG) at codon 249].

using SPSS for Windows statistical package (SPSS Inc., Chicago, IL).

RESULTS

Correlation between *RASSF1A* Methylation and Clinicopathological Characteristics of Stage I Lung Adenocarcinoma. One hundred and ten stage I lung adenocarcinomas were examined for *RASSF1A* methylation by MSP (10). The site examined by MSP was the one of which the methylation was associated with reduced *RASSF1A* expression (10). Representative results are shown in Fig. 1*A*. *RASSF1A* methylation was detected in 35 of 110 tumors (32%). We then analyzed the relationship between *RASSF1A* methylation and clinicopathological characteristics of these patients. The results are summarized in Table 1. *RASSF1A* methylation was observed preferentially in poorly differentiated subtypes. The difference in frequency between poorly and well differentiated was statistically significant ($P = 0.0005$), and the difference between poorly and moderately differentiated was near significance ($P = 0.0835$). *RASSF1A* methylation was detected dominantly in tumors with vascular invasion ($P = 0.0242$) or pleural involvement ($P = 0.0305$). There was no significant correlation of *RASSF1A* methylation with gender, age, smoking history, T stage, and lymphatic permeation within the tumor.

Table 2 Correlation of *RASSF1A* methylation with genetic alterations in stage I lung adenocarcinoma

Genetic alteration		No. of *RASSF1A* methylation (%) +	−	P^a
AI at 3p21.3	+	22 (52)	20 (48)	0.0005
	−	11 (18)	50 (82)	
	NI[b]	2 (29)	5 (71)	
p53 mutation	+	16 (43)	21 (57)	0.0842
	−	19 (26)	54 (74)	
K-*ras* mutation	+	2 (15)	11 (85)	0.2193
	−	33 (34)	64 (66)	

[a] Fisher's exact test.
[b] NI, not informative.

Correlation of *RASSF1A* Methylation with AI at 3p21.3, *p53* Mutations, and K-*ras* Mutations in Stage I Lung Adenocarcinoma. AI at 3p21.3 was examined in 110 stage I adenocarcinomas by PCR amplification of three polymorphic loci. Representative results are shown in Fig. 1*B*. The numbers of patients informative for each polymorphic locus were 78 cases (71%) for *D3S4622/LUCA4.1*, 84 cases (76%) for *D3S4604/LUCA19.1*, and 71 cases (65%) for *D3S4597/P1.5*, respectively. The frequencies of AI at these loci were 34 of 78 (44%) at *D3S4622/LUCA4.1*, 34 of 84 (40%) at *D3S4604/LUCA19.1*, and 24 of 71 (34%) at *D3S4597/P1.5*, respectively. In total, 103 of 110 (94%) cases were informative for the allelic status of 3p21.3, and the frequency of AI was 42 of 103 (41%). These tumors were also examined for mutations in exons 2–11 of the *p53* gene by the WAVE DNA Fragment Analysis System. Representative results are shown in Fig. 1*C*. *p53* mutations were detected in 39 of 110 cases (35%). Because 2 of 39 cases showed a silent *p53* mutation, we counted 37 of 110 as cases with *p53* mutation in this analysis. We also examined these 110 tumors for mutations at codons 12, 13, and 61 of the K-*ras* gene. Mutations in the K-*ras* gene were detected in 13 tumors (12%): 11 in codon 12, 1 in codon 13, and 1 in codon 61. The frequencies of AI at 3p21.3, *p53* mutations, and K-*ras* mutations in primary lung adenocarcinomas in this study were comparable with those in previous studies (5, 21–24).

We then analyzed the correlation of *RASSF1A* methylation with AI at 3p21.3, *p53* mutations, and K-*ras* mutations (Table 2). Among 103 informative cases, 22 of 42 (52%) tumors with AI at 3p21.3 showed *RASSF1A* methylation, whereas 11 of 61 (18%) tumors without AI at 3p21.3 showed *RASSF1A* methylation. The correlation between *RASSF1A* methylation and AI at 3p21.3 was statistically significant ($P = 0.0005$). *RASSF1A* methylation was detected in 16 of 37 (43%) tumors with *p53* mutations and detected in 19 of 73 (26%) tumors without *p53* mutations. The statistical significance of the correlation between *RASSF1A* methylation and *p53* mutation was borderline ($P = 0.0842$). We found K-*ras* mutations in 13 tumors. Among the 13 tumors, 11 (85%) tumors did not show *RASSF1A* methylation, whereas 2 (15%) tumors showed *RASSF1A* methylation. However, there was no statistically significant correlation or inverse correlation between *RASSF1A* methylation and K-*ras* mutation ($P = 0.2193$).

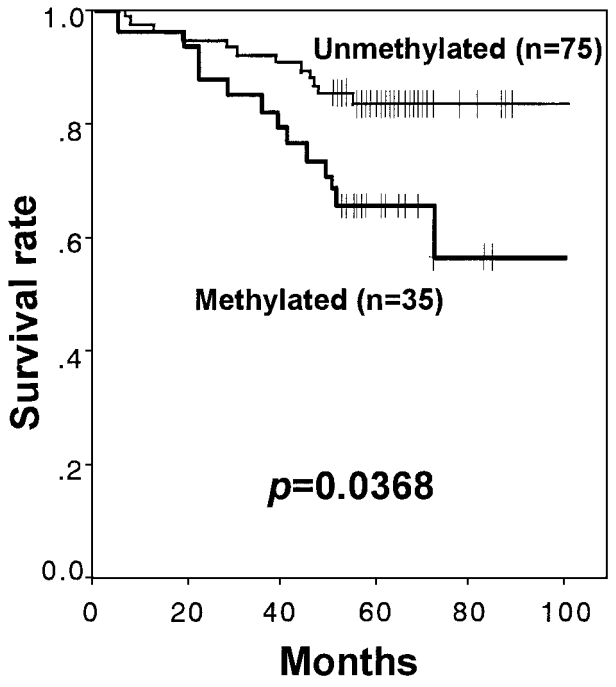

Fig. 2 Kaplan-Meier survival curves for patients with stage I lung adenocarcinoma classified according to *RASSF1A* methylation and the resulting curves were compared using the log-rank test ($P = 0.0368$). The *tick mark* indicates the follow-up period of each patient.

Correlation between *RASSF1A* Methylation and Prognosis of Patients with Stage I Lung Adenocarcinoma. We analyzed the effect of *RASSF1A* methylation on patient survival. Kaplan-Meier survival estimates showed that the patients with *RASSF1A* methylation tended to have a poorer survival than the patients without *RASSF1A* methylation (Fig. 2). The difference in the prognosis of these two groups was statistically significant ($P = 0.0368$; log-rank test). Univariate survival analysis of clinicopathological factors showed that gender was significantly associated with survival, and age and T stage were associated with survival although they did not reach statistical significance (Table 3). Smoking history, differentiation, vascular invasion, lymphatic permeation, pleural involvement, *p53* mutation, K-*ras* mutation, and AI at 3p21.3 did not account for the adverse survival (Table 3). Subsequently, a multivariate stepwise Cox proportional hazard regression analysis was performed using all of these clinicopathological and molecular factors. *RASSF1A* methylation was a significantly unfavorable prognosis factor independent of other clinicopathological and molecular factors ($P = 0.032$; risk ratio 2.357; 95% CI, 1.075–5.169; Table 3). Age was also an unfavorable prognosis factor; however, no other factor was considered as being an independent prognostic marker ($P > 0.05$; Table 3).

DISCUSSION

We demonstrate here that *RASSF1A* methylation is significantly associated with a poorer survival of patients with stage I lung adenocarcinoma. It was reported recently that *RASSF1A* promoter hypermethylation in resected NSCLCs was associated with impaired patient survival (10); thus, the present study agrees with this report. In the previous study, patients with NSCLCs of various stages and histological types were analyzed (10), whereas in this study, patients with stage I lung adenocarcinoma were exclusively analyzed. These results suggest that tumors with *RASSF1A* methylation are more aggressive than tumors without *RASSF1A* methylation in lung adenocarcinoma. In fact, *RASSF1A* methylation was more commonly detected in the poorly differentiated type than in the well- or moderately differentiated types, suggesting that *RASSF1A* methylation is related to the degree of differentiation or the stage of progression in the adenocarcinoma. Furthermore, *RASSF1A* methylation was detected dominantly in tumors with vascular invasion or pleural involvement. These results also indicate the tumors with *RASSF1A* methylation are more aggressive than tumors without *RASSF1A* methylation.

Noguchi *et al.* (25) have reported histological subtypes for the small size lung adenocarcinoma measuring ≤2 cm in the greatest dimension. In this report, type C (LBAC with foci of active fibroblastosis) is considered to be an advanced carcinoma, which is progressed from type A (LBAC) or type B (LBAC with foci of collapsed alveolar structure), and patients with type D (poorly differentiated adenocarcinoma) have a worse prognosis than patients with types A, B, and C. We classified small adenocarcinomas, up to 2 cm in dimension, used in the present study according to Noguchi's classification (Table 1). Interestingly, *RASSF1A* methylation was detected in all 4 of the type D tumors, but not in any of 3 type A tumors, and *RASSF1A* methylation was more frequently detected in types C and D tumors (9 of 22; 41%) than in types A and B tumors (2 of 11; 18%), although the difference did not reach statistical significance ($P = 0.2585$; Table 1). Therefore, it is possible that inactivation of *RASSF1A* could be involved in a series of morphologically distinct changes in carcinogenesis and progression of lung adenocarcinoma.

The *RASSF1A* gene is located at chromosome 3p21.3 that frequently undergoes LOH in lung cancers. Recently, it was reported that two of three breast cancers with LOH at 3p21.3 had hypermethylation of the *RASSF1A* promoter region (26). We demonstrated here that *RASSF1A* methylation significantly correlated with AI at 3p21.3 in lung adenocarcinoma ($P = 0.0005$). These results suggested that the *RASSF1A* gene is inactivated in accordance with the Knudson two-hit model, including epigenetic mechanisms of gene inactivation (27). However, there were a number of tumors with *RASSF1A* methylation without AI at 3p21.3 and with AI at 3p21.3 without *RASSF1A* methylation. It was also reported that only 6 of 22 NSCLC with 3p21.3 allele loss had *RASSF1A* methylation (12). In the cases with retention of 3p21.3 alleles with *RASSF1A* methylation, both alleles may be inactivated by hypermethylation of the promoter region of the *RASSF1A* gene. In the cases with AI at 3p21.3 alleles without *RASSF1A* methylation, other TSGs may be a target of AI. In fact, there are several candidate TSGs at 3p21.3 (8, 28–31).

Recent studies have shown that several genes on chromosome 3p other than *RASSF1* are also methylated in lung cancers. The *SEMA3B* gene located ~60 kb distal to *RASSF1* was shown to be inactivated by methylation in lung cancer cell lines (31). In addition, the *FHIT* gene at 3p14.2 and the *RAR*β gene at 3p24 have been shown to be inactivated by methylation in primary lung

Table 3 Overall survival of patients with stage I lung adenocarcinoma in relation to molecular and clinicopathological characteristics

Variable	Subset	Incidence[a] (%)	*P*	
			Univariate[b]	Multivariate[c]
Gender	Male	21/64 (33)	0.0150	0.077
	Female	5/46 (11)		(Risk ratio 2.440; 95% CI, 0.907–6.565)
Age	<60	5/41 (12)	0.0670	0.125 (<60 *vs.* 60–69)
	60–69	10/39 (26)		(Risk ratio 2.375; 95% CI, 0.786–7.175)
	>69	11/30 (37)		0.032 (<60 *vs.* ≥69)
				(Risk ratio 3.190; 95% CI, 1.084–9.388)
Smoking history	Smoker	18/62 (29)	0.1617	NS[d]
	Nonsmoker	8/48 (17)		
T stage	T1	13/75 (17)	0.0600	0.104
	T2	13/35 (37)		(Risk ratio 1.934; 95% CI, 0.873–4.292)
Differentiation	Poorly	4/17 (24)	0.9664	NS
	Moderately	9/37 (24)		
	Well	13/56 (23)		
Vascular invasion	+	11/31 (35)	0.1190	NS
	−	15/79 (19)		
Lymphatic permeation	+	7/22 (32)	0.1922	NS
	−	19/88 (22)		
Pleural involvement	+	8/26 (31)	0.4143	NS
	−	18/84 (21)		
RASSF1A methylation	+	13/35 (37)	0.0368	0.032
	−	13/75 (17)		(Risk ratio 2.357; 95% CI, 1.075–5.169)
p53 mutation	+	12/37 (32)	0.1630	NS
	−	14/73 (19)		
K-*ras* mutation	+	1/13 (8)	0.1818	NS
	−	25/97 (26)		
AI at 3p21.3	+	11/42 (26)	0.7243	NS
	−	12/61 (20)		

[a] Incidence is expressed by the number of patients who died of lung cancer per the number of patients in total.
[b] Log-rank test.
[c] Stepwise Cox proportional hazards regression model.
[d] NS, not selected in stepwise Cox proportional regression model.

cancers (32, 33). Therefore, it is likely that multiple genes on 3p are methylated in lung cancers. It is possible that the methylation of a gene among them correlates more strongly with AI on 3p as well as prognosis than that of *RASSF1A*. Additional genetic and functional studies should be done on those genes, including *RASSF1A*, to elucidate the pathogenetic significance of methylation of those genes for the development of lung cancers.

The presence of a Ras association domain in RASSF1A suggests that these proteins may function as effectors of Ras signaling (or signaling of a Ras-like molecule) in cells. Therefore, it was important to analyze the relationship between *RASSF1A* methylation and K-*ras* mutation in the current series of tumors. The present study showed that there was no significant correlation or inverse correlation between *RASSF1A* methylation and K-*ras* mutation. Therefore, it is possible that inactivation of *RASSF1A* does not activate K-ras signaling in tumorigenic mechanisms. However, because K-*ras* mutations were detected in only 13 cases, this negative correlation could be because of the few cases for statistical analysis. In fact, only 2 of 35 (6%) tumors with *RASSF1A* methylation showed K-*ras* mutations, implying that there may be an inverse correlation between *RASSF1A* methylation and K-*ras* mutation. It was reported that RASSF1C binds Ras in a GTP-dependent manner (11). Because RASSF1A has the identical Ras associate domain, it is possible that RASSF1A binds to Ras in the same manner as RASSF1C. Therefore, additional investigations in a larger population and functional studies to assess the role of *RASSF1A* in Ras-dependent growth control will be required.

Multiple TSGs are involved in lung carcinogenesis (1), and *p53* plays a central role in tumor progression (34). In the present study, we showed that *RASSF1A* methylation was weakly correlated with *p53* mutations. These results suggest that epigenetic inactivation of *RASSF1A* and *p53* mutations play a cooperative role in progression of lung adenocarcinoma. In fact, *p53* mutations were also detected more frequently in types C and D tumors (8 of 22; 36%) than in types A and B tumors (0 of 11; 0%; data not shown). However, because *RASSF1A* methylation but not *p53* mutation showed significant correlation with poor prognosis of patients with stage I adenocarcinoma, *RASSF1A* could play a more dominant role than *p53* in the progression of lung adenocarcinoma.

In conclusion, *RASSF1A* methylation was significantly correlated with vascular invasion, pleural involvement, poor differentiation, and poor survival of patients with stage I lung adenocarcinoma. As expected, there was also a correlation between *RASSF1A* methylation and AI at 3p21.3. These results indicate that epigenetic inactivation of *RASSF1A*, which appears to be one of the target TSGs at 3p21.3, plays an important role in the progression of lung adenocarcinoma. Additional investigations will be required to elucidate the importance of *RASSF1A* methylation as a diagnostic and prognostic marker in the management of early stage lung adenocarcinoma and to elucidate the role of *RASSF1A* in cell signaling.

ACKNOWLEDGMENTS

We thank Dr. Masatomo Mori, First Department of Internal Medicine, Gunma University, School of Medicine, Gunma, Japan, for his encouragement of this study.

REFERENCES

1. Kohno, T., and Yokota, J. How many tumor suppressor genes are involved in human lung carcinogenesis? Carcinogenesis (Lond.), *20:* 1403–1410, 1999.

2. Kok, K., Osinga, J., Carritt, B., Davis, M. B., van der Hout, A. H., van der Veen, A. Y., Landsvater, R. M., de Leij, L. F., Berendsen, H. H., Postmus, P. E., and *et al.* Deletion of a DNA sequence at the chromosomal region 3p21 in all major types of lung cancer. Nature (Lond.), *330:* 578–581, 1987.

3. Naylor, S. L., Johnson, B. E., Minna, J. D., and Sakaguchi, A. Y. Loss of heterozygosity of chromosome 3p markers in small-cell lung cancer. Nature (Lond.), *329:* 451–454, 1987.

4. Yokota, J., Wada, M., Shimosato, Y., Terada, M., and Sugimura, T. Loss of heterozygosity on chromosomes 3, 13, and 17 in small-cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. Proc. Natl. Acad. Sci. USA, *84:* 9252–9256, 1987.

5. Wistuba, I. I., Behrens, C., Virmani, A. K., Mele, G., Milchgrub, S., Girard, L., Fondon, J. W., Garner, H. R., McKay, B., Latif, F., Lerman, M. I., Lam, S., Gazdar, A. F., and Minna, J. D. High resolution chromosome 3p allelotyping of human lung cancer and preneoplastic/preinvasive bronchial epithelium reveals multiple, discontinuous sites of 3p allele loss and three regions of frequent breakpoints. Cancer Res. *60:* 1949–1960, 2000.

6. Hibi, K., Takahashi, T., Yamakawa, K., Ueda, R., Sekido, Y., Ariyoshi, Y., Suyama, M., Takagi, H., and Nakamura, Y. Three distinct regions involved in 3p deletion in human lung cancer. Oncogene, *7:* 445–449, 1992.

7. Killary, A. M., Wolf, M. E., Giambernardi, T. A., and Naylor, S. L. Definition of a tumor suppressor locus within human chromosome 3p21–p22. Proc. Natl. Acad. Sci. USA, *89:* 10877–10881, 1992.

8. Lerman, M. I., and Minna, J. D. The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. Cancer Res., *60:* 6116–6133, 2000.

9. Dammann, R., Li, C., Yoon, J. H., Chin, P. L., Bates, S., and Pfeifer, G. P. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat. Genet. *25:* 315–319, 2000.

10. Burbee, D. G., Forgacs, E., Zochbauer-Muller, S., Shivakumar, L., Fong, K., Gao, B., Randle, D., Kondo, M., Virmani, A., Bader, S., Sekido, Y., Latif, F., Milchgrub, S., Toyooka, S., Gazdar, A. F., Lerman, M. I., Zabarovsky, E., White, M., and Minna, J. D. Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J. Natl. Cancer Inst., *93:* 691–699, 2001.

11. Vos, M. D., Ellis, C. A., Bell, A., Birrer, M. J., and Clark, G. J. Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. J. Biol. Chem., *275:* 35669–35672, 2000.

12. Agathanggelou, A., Honorio, S., Macartney, D. P., Martinez, A., Dallol, A., Rader, J., Fullwood, P., Chauhan, A., Walker, R., Shaw, J. A., Hosoe, S., Lerman, M. I., Minna, J. D., Maher, E. R., and Latif, F. Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumors. Oncogene, *20:* 1509–1518, 2001.

13. Dammann, R., Takahashi, T., and Pfeifer, G. P. The CpG island of the novel tumor suppressor gene RASSF1A is intensely methylated in primary small cell lung carcinomas. Oncogene, *20:* 3563–3567, 2001.

14. Travis, W. D., Travis, L. B., and Devesa, S. S. Lung cancer. Cancer (Phila.), *75:* 191–202, 1995.

15. Thun, M. J., Lally, C. A., Flannery, J. T., Calle, E. E., Flanders, W. D., and Heath, C. W. Cigarette smoking and changes in the histopathology of lung cancer. J. Natl. Cancer Inst., *89:* 1580–1586, 1997.

16. Nesbitt, J. C., Putnam, J. B., Walsh, G. L., Roth, J. A., and Mountain, C. F. Survival in early-stage non-small cell lung cancer. Ann. Thorac. Surg., *60:* 466–472, 1995.

17. Strauss, G. M., Kwiatkowski, D. J., Harpole, D. H., Lynch, T. J., Skarin, A. T., and Sugarbaker, D. J. Molecular and pathologic markers in stage I non-small-cell carcinoma of the lung. J. Clin. Oncol., *13:* 1265–1279, 1995.

18. International Union Against Cancer (UICC): Lung (ICD-0 C-34). *In:* L. H. Sobin and C. H. Wittekind (eds.), TNM Classification of Malignant Tumors. 5th Ed., pp. 91–97. New York: Wiley-Liss, 1997.

19. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc. Natl. Acad. Sci. USA, *93:* 9821–9826, 1996.

20. Zochbauer-Muller, S., Fong, K. M., Virmani, A. K., Geradts, J., Gazdar, A. F., and Minna, J. D. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. Cancer Res., *61:* 249–255, 2001.

21. Sugio, K., Ishida, T., Yokoyama, H., Inoue, T., Sugimachi, K., and Sasazuki, T. ras gene mutations as a prognostic marker in adenocarcinoma of the human lung without lymph node metastasis. Cancer Res., *52:* 2903–2906, 1992.

22. Fukuyama, Y., Mitsudomi, T., Sugio, K., Ishida, T., Akazawa, K., and Sugimachi, K. K-ras and p53 mutations are an independent unfavourable prognostic indicator in patients with non-small-cell lung cancer. Br. J. Cancer, *75:* 1125–1130, 1997.

23. Sekido, Y., Fong, K. M., and Minna, J. D. Progress in understanding the molecular pathogenesis of human lung cancer. Biochim. Biophys. Acta *1378:* F21–59, 1998.

24. Mitsudomi, T., Hamajima, N., Ogawa, M., and Takahashi, T. Prognostic significance of p53 alterations in patients with non-small cell lung cancer: a meta-analysis, Clin Cancer Res., *6:* 4055–4063, 2000.

25. Noguchi, M., Morikawa, A., Kawasaki, M., Matsuno, Y., Yamada, T., Hirohashi, S., Kondo, H., and Shimosato, Y. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. Cancer (Phila.), *75:* 2844–2852, 1995.

26. Dammann, R., Yang, G., and Pfeifer, G. P. Hypermethylation of the cpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers. Cancer Res., *61:* 3105–3109, 2001.

27. Jones, P. A., and Laird, P. W. Cancer epigenetics comes of age. Nat. Genet., *21:* 163–167, 1999.

28. Sekido, Y., Bader, S., Latif, F., Chen, J. Y., Duh, F. M., Wei, M. H., Albanesi, J. P., Lee, C. C., Lerman, M. I., and Minna, J. D. Human semaphorins A(V) and IV reside in the 3p21.3 small cell lung cancer deletion region and demonstrate distinct expression patterns. Proc. Natl. Acad. Sci. USA, *93:* 4120–4125, 1996.

29. Gao, B., Sekido, Y., Maximov, A., Saad, M., Forgacs, E., Latif, F., Wei, M. H., Lerman, M., Lee, J. H., Perez-Reyes, E., Bezprozvanny, I., and Minna, J. D. Functional properties of a new voltage-dependent calcium channel $\alpha(2)\delta$ auxiliary subunit gene (CACNA2D2). J. Biol. Chem., *275:* 12237–12242, 2000.

30. Daigo, Y., Nishiwaki, T., Kawasoe, T., Tamari, M., Tsuchiya, E., and Nakamura, Y. Molecular cloning of a candidate tumor suppressor gene, DLC1, from chromosome 3p21.3. Cancer Res., *59:* 1966–1972, 1999.

31. Tomizawa, Y., Sekido, Y., Kondo, M., Gao, B., Yokota, J., Roche, J., Drabkin, H., Lerman, M. I., Gazdar, A. F., and Minna, J. D. Inhibition of lung cancer cell growth and induction of apoptosis after reexpression of 3p21.3 candidate tumor suppressor gene SEMA3B. Proc. Natl. Acad. Sci. USA, *98:* 13954–13959, 2001.

32. Virmani, A. K., Rathi, A., Zochbauer-Muller, S., Sacchi, N., Fukuyama, Y., Bryant, D., Maitra, A., Heda, S., Fong, K. M., Thunnissen, F., Minna, J. D., and Gazdar, A. F. Promoter methylation and silencing of the retinoic acid receptor-β gene in lung carcinomas. J. Natl. Cancer Inst., *92:* 1303–1307, 2000.

33. Zochbauer-Muller, S., Fong, K. M., Maitra, A., Lam, S., Geradts, J., Ashfaq, R., Virmani, A. K., Milchgrub, S., Gazdar, A. F., and Minna, J. D. 5′ CpG island methylation of the FHIT gene is correlated with loss of gene expression in lung and breast cancer. Cancer Res., *61:* 3581–3585, 2001.

34. Morgan, S. E., and Kastan, M. B. p53 and ATM: cell cycle, cell death, and cancer. Adv. Cancer Res., *71:* 1–25, 1997.

Clinical Cancer Research

Clinicopathological Significance of Epigenetic Inactivation of *RASSF1A* at 3p21.3 in Stage I Lung Adenocarcinoma

Yoshio Tomizawa, Takashi Kohno, Haruhiko Kondo, et al.

Clin Cancer Res 2002;8:2362-2368.