Epigenetic inactivation of the RAS-effector gene *RASSF2* in lung cancers

KYOICHI KAIRA[1], NORIAKI SUNAGA[1], YOSHIO TOMIZAWA[3], NORIKO YANAGITANI[1], TAMOTSU ISHIZUKA[1], RYUSEI SAITO[3], TAKASHI NAKAJIMA[2] and MASATOMO MORI[1]

Departments of [1]Medicine and Molecular Science, [2]Tumor Pathology, Gunma University Graduate School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511; [3]Department of Respiratory Medicine, National Nishigunma Hospital, 2854 Kanai, Shibukawa, Gunma 377-8511, Japan

Received December 28, 2006; Accepted March 16, 2007

Abstract. *RASSF2*, a member of the RAS association domain family 1 (*RASSF1*), is a candidate tumor suppressor gene (TSG) that is silenced by promoter hypermethylation in several human cancers. In this study, we examined the expression of *RASSF2* mRNA and the promoter methylation status in lung cancer cell lines and in tumor samples of 106 primary non-small cell lung cancers (NSCLCs) by methylation-specific PCR. *RASSF2* expression was absent in 26% of small cell lung cancers (SCLCs; n=27 lines) and 50% of NSCLCs (n=42 lines). Promoter methylation of *RASSF2* was found in 18% of the SCLC cell lines (n=22) and 62% of the NSCLC cell lines (n=26), and the methylation status was tightly associated with the loss of *RASSF2* expression. *RASSF2* expression was restored by treatment with 5-aza-2-deoxycytidine and/or trichostatin-A in the NSCLC cell lines which were absent of the expression. *RASSF2* methylation was found in 31% of primary NSCLC tumors, and methylation was more frequent in the specimens from non-smokers (18 of 40, 45%) than in the specimens from smokers (15 of 66, 23%, P=0.014). We also examined the association of *RASSF2* methylation with mutations of KRAS and EGFR and with promoter hypermethylation of *RASSF1A*; however, we could not find a significant association between *RASSF2* methylation and these genetic and epigenetic changes. Our results indicate that aberrant methylation of the *RASSF2* gene with the subsequent loss of *RASSF2* expression plays an important role in the pathogenesis of lung cancers.

Correspondence to: Dr Noriaki Sunaga, Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511, Japan
E-mail: nsunaga@showa.gunma-u.ac.jp

Key words: *RASSF2*, tumor suppressor gene, lung cancer, promoter hypermethylation

Introduction

Recent advances in molecular genetics have revealed that multiple alterations accumulate in a multi-step manner via both genetic and epigenetic mechanisms during the process of lung carcinogenesis (1). Aberrant DNA hypermethylation of CpG sites in the promoter regions of a gene is a tumor-acquired epigenetic event that results in the inactivation of tumor suppressor genes (TSGs) (2). The aberrant promoter hypermethylation of several TSGs is frequently observed in lung cancers and seems to play an important role in lung cancer development (3).

RAS family genes encode small GTPase proteins involved in the signal transduction of extracellular signals (4). RAS proteins interact with a wide spectrum of regulators and downstream effectors to produce various cellular responses, including cell proliferation, differentiation, and apoptosis (4). RAS association domain family 1 (*RASSF1*) has been identified as a potential tumor suppressor that can serve as a RAS effector (5,6). *RASSF1A*, a major transcript of *RASSF1*, is frequently inactivated due to promoter hypermethylation in a variety of human cancers, including lung cancer (5,7,8). *RASSF2*, which is located at 20p13, was recently identified as a third member of the *RASSF1* family (9). The *RASSF2* protein binds to KRAS in a GPT-dependent manner via the RAS effector domain, but it displays only a weak interaction with HRAS (9). Some studies have reported that *RASSF2* expression was frequently down-regulated in lung cancer cell lines, and the forced expression of *RASSF2* inhibited the growth of tumor cells and promoted both cell cycle arrest and apoptosis (9). RNAi-mediated silencing of *RASSF2* expression enhanced the anchorage-independent growth of cells transformed by KRAS (10). Moreover, the *RASSF2* gene was silenced via promoter hypermethylation in cancers of the colon, stomach, and nasopharynx (10-12). These results indicate that the epigenetic inactivation of the *RASSF2* gene plays a role in the pathogenesis of various human cancers. However, it is not clarified whether *RASSF2* is inactivated by promoter hypermethylation in lung cancers. Therefore, we examined the expression and promoter methylation status of *RASSF2* in lung cancer cell lines and primary NSCLC. We also examined the correlation of *RASSF2* methylation with

clinicopathological features and *KRAS* and *EGFR* mutations and *RASSF1A* methylation in patients with primary NSCLC.

Materials and methods

Cell lines, tumor samples and DNA preparation. Lung cancer cell lines used in the present study included the following: 27 small cell lung cancers (SCLCs) (NCI-H82, -H187, -H209, -H289, -H345, -H378, -H524, -H526, -H740, -H865, -H889, -H1045, -H1092, -H1184, -H1238, -H1339, -H1607, -H1618, -H1672, -H1963, -H2107, -H2141, -H2171, -H2227, HCC33, HCC970 and N417); and 42 non-small cell lung cancers (NSCLCs) (NCI-H23, -H157, -H322, -H358, -H460, -H520, -H661, -H1264, -H1299, -H1395, -H1437, -H1648, -H1666, -H1792, -H1819, -H1993, -H2009, -H2077, -H2087, -H2106, -H2122, -H2126, -H2347, -H2882, -H2887, -H3255, HCC15, HCC44, HCC78, HCC95, HCC193, HCC366, HCC461, HCC515, HCC827, HCC1171, HCC1195, HCC1359, HCC1883, HCC2450, A427 and A549). Cells were cultured in RPMI medium 1640 (Sigma, St. Louis, MO) supplemented with 10% bovine serum. Tumors were obtained from 106 patients with primary NSCLC who underwent surgery between 2001 and 2003 at the Gunma University School of Medicine Hospital, Gunma, Japan. Tumor samples were immediately frozen at surgery and kept at -80˚C until DNA extraction. Genomic DNAs were prepared by the method described previously (13) or by a DNeasy kit (Qiagen, Chatworth, CA), according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed as described previously (14). Briefly, total RNA was extracted from cells, and cDNA was synthesized using 2 μg of total RNA with the Superscript II first-strand synthesis using oligo (dT) primer System (Invitrogen, Carlsbad, CA). Aliquots of the reaction mixture were used for the subsequent PCR amplification. Primer sequences used were 5'-AAG ACA TCC GTG TTC ACA CC-3' and 5'-TCG TTC TCA TGG CTC AGA TT-3' for RASSF2 mRNA (462 bp). PCR was performed for 35 cycles, each of which consisted of denaturation at 94˚C for 30 sec, annealing at 55˚C for 30 sec, and extension at 72˚C for 1 min, followed by a final 10-min extension at 72˚C. The products were separated by electrophoresis on 2% agarose-TBE gels, and the bands were visualized by ethidium bromide staining. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control to confirm the success of the reaction.

5-Aza-2'-deoxycytidine (5-aza-dC) and/or trichostatin A (TSA) treatment. Cells were treated with 5-aza-dC and/or TSA as described (15). Briefly, cells were incubated for the first 48 h in a medium containing 10 μM 5-aza-dC (Sigma) or 0.5 μM TSA (Wako, Tokyo). For the study of synergism, 10 μM 5-aza-dC was present in culture for 48 h and then for another 24 h with the addition of 0.5 μM TSA. Total RNA was then isolated with Rneasy minikit (Qiagen), and RT-PCR analysis was carried out with the *RASSF2* oligonucleotide primers.

Methylation-specific PCR (MSP). Bisulphite modification of DNA was performed by BisulFast DNA modification kit (Toyobo Co., Ltd., Life Science Department, Osaka, Japan), according to the manufacturer's instructions. The modified DNA was used as a template for MSP with primers specific for either the modified methylated or the modified unmethylated *RASSF2* promoter sequences. Treated DNAs were PCR-amplified with the following primer sets including CpG island of *RASSF2* as described (11): 5'-CGA AGG AGG GCG GGG AGATC-3' (sense) and 5'-TCC GCC GCC GTC TTC TAA ACG-3' (antisense) for the methylated *RASSF2* sequence (148 bp); and 5'-GTT TTG AAG GAG GGT GGG GAG ATT-3' (sense) and 5'-AAT CCA CCA CCA TCT TCT AAA CA-3' (antisense) for the unmethylated *RASSF2* sequence (154 bp). PCR amplification consisted of 15 min at 95˚C, followed by 45 cycles of 15-sec denaturation at 95˚C, 15 sec of annealing at 55˚C, 30 sec of extension at 72˚C, with final extension at 72˚C for 7 min for the methylated and unmethylated primers using HotStar Taq DNA polymerase (Qiagen). PCR products were separated in 3% agarose gels with ethidium bromide and visualized under UV illumination. As a positive control, SssI methylase (New England bioLabs,

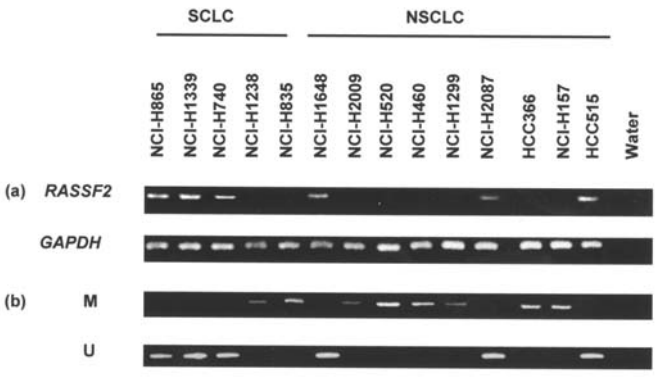

Figure 1. (a) Representative results of the RT-PCR-based analysis of the *RASSF2* gene in lung cancer cell lines. RT-PCR yielded the expected 475-bp DNA products. *GAPDH* was used as an internal control. (b) MSP-based analysis of *RASSF2* methylation in lung cancer cell lines. Lines labeled M and U denote products with methylated and unmethylated sequences, respectively.

Table I. Incidence of loss of *RASSF2* mRNA expression in lung cancer cell lines.

Cell line	No.	No. of *RASSF2* mRNA Expression negative samples (%)
Small cell lung cancer	27	7 (25.9
Non-small cell lung cancer	42	21 (50.0)
Adenocarcinoma	25	15 (60.0)
Squamous cell carcinoma	5	3 (60.0)
Large cell carcinoma	6	2 (33.3)
Adenosquamous cell carcinoma	1	1 (100)
Non-small cell carcinoma	5	1 (20.0)
Total	69	28 (40.5)

Table II. Correlation between expression and methylation of *RASSF2* in lung cancer cell lines.

	RASSF2		*RASSF2* promoter genotype			
	Total	Expression	Total	M[a]	U[a]	P-value[b]
Small cell lung cancer	22	+	16	1	15	0.04
		-	6	3	3	
Non-small cell lung cancer	26	+	9	1	8	<0.001
		-	17	15	2	

[a]M, methylated; U, unmethylated. [b]Fisher's exact test.

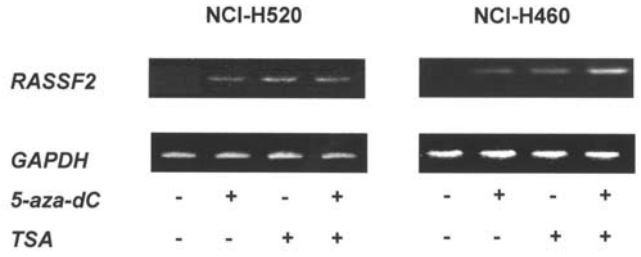

Figure 2. Restoration of *RASSF2* expression by 5-aza-dC and TSA in NCI-H520 and -H460. *RASSF2* expression was restored by 5-aza-dC and TSA in these cells.

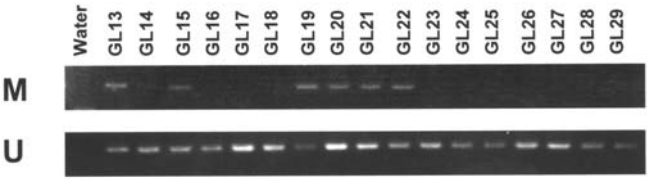

Figure 3. Representative results of the MSP-based analysis of *RASSF2* methylation in primary NSCLC tumors. Lines labeled M and U denote products with methylated and unmethylated sequences, respectively.

Inc., Beverly, MA, USA) was used to methylate the immortalized human bronchial epithelial cell line BEAS-2B, and was modified by sodium bisulphate as described above.

Methylation analysis of the RASSF1A and mutational analyses of the KRAS and EGFR gene. Mutations of *KRAS* and *EGFR*, and promoter methylation of *RASSF1A* were examined as reported previously (16,17). Briefly, *RASSF1A* methylation was analyzed by methylation-specific PCR method using genomic DNAs treated by sodium bisulfite (16). Four exons (exons 18-21), which code for tyrosine kinase domain of the *EGFR* gene, and codons 12 and 13 of the *KRAS* gene were amplified by PCR with EGFR- and KRAS-specific oligonucleotide primers as described (17). PCR products were sequenced with ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Statistical analysis. Fisher's exact test was used to examine the association of two categorical variables. A P-value of <0.05 was considered to be statistically significant. Statistical analysis was performed using StatView J-4.5 for Macintosh.

Results

Expression of the RASSF2 gene in lung cancer cell lines. We first examined the expression of *RASSF2* mRNA in lung cancer cell lines (Fig. 1a). *RASSF2* expression was absent in 26% of SCLC cell lines (n=27) and 50% of NSCLC cell lines (n=42; Table I). In the analysis of subtypes of NSCLCs, the expression was absent in 60% of adenocarcinomas (n=25), 60% of squamous cell carcinomas (n=5), 33% of large cell carcinomas (n=6), 20% of non-small cell carcinomas (n=5), and one adenosquamous cell carcinoma. The frequency of loss of *RASSF2* expression was significantly higher in NSCLCs than SCLCs (P=0.04).

Promoter hypermethylation of the RASSF2 gene. The methylation status of the *RASSF2* gene was determined by MSP in 22 SCLC cell lines and 26 NSCLC cell lines (Fig. 1b, Table II). The hypermethylation of RASSF2 was detected in 18% of SCLCs and 62% of NSCLCs. Three SCLCs and 15 NSCLCs that lacked *RASSF2* expression exhibited the hypermethylation, whereas the hypermethylation were not detected in 15 SCLCs and 8 NSCLCs that expressed *RASSF2*. The frequency of *RASSF2* methylation was significantly higher in NSCLCs than in SCLCs (P=0.002). There was a significant correlation between *RASSF2* methylation and loss of *RASSF2* expression in both SCLCs and NSCLCs (P=0.04 and P<0.001, respectively).

To assess whether the loss of *RASSF2* expression in lung cancer was a result of aberrant methylation and/or histone deacetylation, we examined the effect of the demethylating agent 5-aza-dC and/or histone deacetylase inhibitor TSA on the *RASSF2* expression in two cell lines (NCI-H520 and -H460) that lacked *RASSF2* expression (Fig. 2). The expression was restored by the treatment with 5-aza-dC and/or TSA in two cell lines (NCI-H520 and -H460) in which aberrant methylation was detected by MSP.

Correlation between RASSF2 methylation and clinicopathological features in primary NSCLC tumors. One hundred and six primary NSCLC tumor samples were examined for *RASSF2* methylation by MSP (Fig. 3). *RASSF2* methylation was detected in 31% of the NSCLC tumors. All tumor samples

Table III. Correlation of *RASSF2* methylation with clinicopathological characteristics of patients with non-small cell lung cancer.

Characteristics	No.	Methylation +	Methylation -	P-value[a]
Gender				
Male	68	20	48	NS
Female	38	13	25	
Age				
≤69	65	19	46	NS
>69	41	14	27	
Smoking history				
Smoker	66	15	51	0.014
Non-smoker	40	18	22	
Stage				
I + II	81	28	53	NS
III + IV	25	5	20	
Pathology				
Adenocarcinoma	57	19	38	NS
Squamous cell carcinoma	42	11	31	
Large cell carcinoma	2	0	2	
Adenosquamous cell carcinoma	5	3	2	
Vascular				
+	40	14	26	NS
-	59	17	42	
Unknown	7			
Lymphatic permeation				
+	53	16	37	NS
-	47	14	33	
Unknown	6			
Pleural involvement				
+	48	19	29	0.052
-	53	12	41	
Unknown	5			
EGFR				
WT[b]	81	26	55	NS
Mut[c]	25	7	18	
KRAS				
WT[b]	104	32	72	NS
Mut[d]	2	1	1	
RASSF1A				
M[e]	31	11	20	NS
U[e]	75	21	54	

[a]Fisher's exact test. [b]WT, wild-type. [c]Mut, mutation of the EGFR gene in the TK domain. [d]Mut, KRAS at codon 12 or 13. [e]M, methylation of the RASSF1A gene in the promoter region; U, unmethylation.

had unmethylated alleles. We then analyzed the relationship between the methylation of *RASSF2* and the clinicopathological characteristics of these patients (Table III). *RASSF2* methylation was significantly higher in non-smokers (18/22, 82%) than in smokers (15/51, 29%; P=0.014) than in smokers, and was marginally higher in tumors with pleural involvement (19/29, 66%) than in those without pleural involvement (12/41, 29%; P=0.052). There was no significant correlation of *RASSF2* methylation with age, gender, stage, pathology, lymphatic permeation, or vascular invasion. We also analyzed the association of *RASSF2* methylation with mutations of KRAS and EGFR, and *RASSF1A* methylation (Table III). However, we could not find significant association between *RASSF2* methylation and abnormalities of these genes.

Discussion

We demonstrated here that the expression of the *RASSF2* gene was absent in a substantial number of lung cancer cell lines, especially NSCLCs. A previous study showed that the lack of *RASSF2* expression was found in six of seven NSCLC cell lines (9). Therefore, we could confirm that the lack of RASSF2 expression was frequently observed in NSCLCs using a larger number of cell lines. In addition, we also demonstrated that the lack of *RASSF2* expression was observed in SCLC cell lines. These results indicate that *RASSF2* is down-regulated in both NSCLC and SCLC.

To analyze the mechanism of the negative expression of the *RASSF2* gene, we focused on examining the promoter hypermethylation of *RASSF2* in a panel of lung cancer cell lines and primary NSCLC tumors. Our MSP results showed that the *RASSF2* promoter region was subject to methylation in 42% of the lung cancer cell lines with a significant correlation between methylation status and *RASSF2* expression, and the frequency of methylation was higher in NSCLCs than SCLCs. Furthermore, the demethylating agent 5-aza-dC restored *RASSF2* expression in lung cancer cell lines. We also observed *RASSF2* methylation in 31% of primary NSCLC tumors. These results indicate that *RASSF2* is frequently hypermethylated in lung cancers (predominantly NSCLC), and promoter methylation functionally silences the expression of *RASSF2*. We also examined the effect of a histone deacetylase (HDAC) inhibitor, TSA, to assess whether the loss of *RASSF2* expression in lung cancers was the result of histone deacetylation. TSA also restored the *RASSF2* expression in these lung cancer cell lines, while the combined treatment of 5-aza-dC and TSA did not increase its expression. It is therefore possible that both histone deacetylation and promoter methylation play roles in silencing *RASSF2* expression; however, these two events occur independently.

We analyzed the correlation between the *RASSF2* methylation status and the clinicopathological features of NSCLC patients, and demonstrated that methylation was significantly more frequent in non-smokers. Although *KRAS* mutations and the aberrant methylations of genes such as $p16^{INK4a}$, *RASSF1A*, and *APC* in lung cancers have been closely related to smoking history (10-12,18), there are few reports of genetic or epigenetic alterations related to the absence of a history of smoking. Recently, we found that the

frequency of aberrant methylation of the *RUNX3* gene was higher in non-smokers than in smokers (19). The reason for this finding is unclear, but it is possible that some carcinogens other than those in tobacco smoke induce these molecular changes. *EGFR* mutations in the tyrosine kinase domain were the first genetic alterations that were observed to predominantly occur in NSCLC in non-smokers (20). In this study, we could not find a significant association between *EGFR* mutations and *RASSF2* methylation (Table III), suggesting that there are distinct roles for *RASSF2* and EGFR in the pathogenesis of NSCLC arising in non-smokers.

RASSF2, as well as *RASSF1A*, was identified as a negative regulator of KRAS (9). Because the activation of the RAS signaling pathway mediated by mutant KRAS is a well-known molecular abnormality leading to the malignant transformation of NSCLC (1,4), *RASSF2* could function as a tumor suppressor by inhibiting the activated RAS signaling. Akino *et al* showed that the siRNA-mediated silencing of *RASSF2* enhanced the anchorage-independent growth ability of cells transformed by KRAS, and KRAS mutations were more frequently observed in colorectal tumors with *RASSF2* methylation in comparison to those without methylation (10). This suggests that the inactivation of the *RASSF2* gene plays a role in tumorigenesis by enhancing the KRAS-mediated malignant transformation. However, Hessen *et al* reported an inverse association between KRAS mutations and RASSF2 methylation, suggesting that these alterations are mutually exclusive (18). The relationship between *RASSF2* and KRAS in the process of tumorigenesis is therefore still obscure. Because our series included only two tumors with KRAS mutations, further studies using a larger number of KRAS mutation-positive NSCLC samples will be necessary to clarify this issue.

In conclusion, aberrant methylation of the *RASSF2* gene and the subsequent loss of *RASSF2* expression was a common event in lung cancers, predominantly in NSCLCs. Although the mechanism by which *RASSF2* functions as a tumor suppressor remains unclear, the inactivation of the *RASSF2* gene via promoter hypermethylation may contribute to the pathogenesis of lung cancer. Our analysis of the correlation between *RASSF2* methylation and the clinicopathological features of 106 NSCLC tumor demonstrated that the frequency of methylation was significantly higher in tumors from non-smokers than in tumors from smokers. This finding suggests that *RASSF2* may play a role in the pathogenesis of NSCLC arising in non-smokers.

Acknowledgments

We thank Dr A.F. Gazdar and Dr J.D. Minna of the University of Texas Southwesten Medical Center, Dallas, TX, for providing cell lines. We also thank Dr H. Iijima and Dr H. Imai of the Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, Gunma, Japan for technical support. This work was supported in part by Grant 18790532 (to N.S.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and National Hospital Organization Policy Based Medical Services.

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