

Aberrant methylation of *RASGRF2* and *RASSF1A* in human non-small cell lung cancer

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Abstract. Aberrant methylation of promoter CpG that causes silencing of tumor suppressor genes (TSGs) may play a key role in the carcinogenesis of many cancer types. *RASSF1A*, regarded as a TSG, has been extensively studied in lung cancer and other malignant tumors, whereas *RASGRF2* has only been reported to possibly play a role in the pathogenesis of pancreatic cancer cell lines. The aims of our study were to i) determine the methylation profile of *RASGRF2* and ii) compare the methylation profiles of *RASGRF2* with *RASSF1A* in lung cancer. We examined *RASGRF2* expression by reverse transcription PCR and aberrant methylation of *RASGRF2* by methylation-specific PCR in lung cancer cell lines. Loss of *RASGRF2* expression was presented in 36% lung cancer cell lines while aberrant methylation of *RASGRF2* was present in 30% (3/10) non-small cell lung cancer (NSCLC) cell lines and in 25% (1/4) small cell lung cancer (SCLC) cell lines. The concordance between loss of expression and aberrant methylation of *RASGRF2* was 86% (12/14). *RASGRF2* expression was restored after treatment with the demethylating agent, 5-aza-2'-deoxycytidine in all four cell lines tested that downregulated *RASGRF2* expression. Among primary NSCLC, *RASGRF2* and *RASSF1A* methylation was observed in 34% (39/114) and 39% (44/114) of cases respectively, while it was observed in only 7% (4/57) and none of the corresponding non-malignant lung tissue. There is no correlation between *RASGRF2* and *RASSF1A* methylation status. Both *RASGRF2* and *RASSF1A* methylation did not associate with clinical characteristics. Frequent methylation and silencing of *RASGRF2* in tumor

cells may play an important role, different from that of *RASSF1A*, in the carcinogenesis of NSCLC.

Introduction

Aberrant methylation of promoter CpG that causes silencing of tumor suppressor genes (TSGs) may play a key role in the carcinogenesis of many cancer types. Recent advances in molecular genetics have showed that multiple TSGs are involved in human lung cancer pathogenesis (1).

Ras association domain family 1A (*RASSF1A*) is located within a 120-kb region of chromosome 3p21, a region that is epigenetically inactivated at high frequency in non-small cell lung cancer (NSCLC) (2). Further studies reveal that, in addition to loss of heterozygosity (LOH), promoter hypermethylation of *RASSF1A* is another mechanism of this gene's loss of expression (3-5). It has been shown that *RASSF1A* bind to the Ras-GTP binding protein, Nore1, which is consistent with the role of a negative effector of Ras oncoprotein (6). *RASSF1A* could induce cell cycle arrest by engaging the RB cell cycle checkpoint. Inhibition of cyclin D1 by *RASSF1A* occurs post-transcriptionally and is likely at the level of translational control. These results strongly suggest that *RASSF1A* is an important human tumor suppressor protein acting at the level of G(1)/S-phase cell cycle progression (7).

Ras protein-specific guanine nucleotide-releasing factor 2 (*RASGRF2*) was originally found by Anborgh *et al* (8); who, using a yeast 2-hybrid screen of a brain cDNA library with the Dbl homology (DH) domain of *RASGRF1* as bait, followed by 5-prime and 3-prime RACE, isolated a full-length cDNA encoding *RASGRF2*. *RASGRF2* is involved in H-Ras signaling (9) and abundantly expressed in a variety of tissue, such as brain, heart and lung (8). However, its expression is decreased in rat mammary carcinomas (10). Recently, it was reported that *RASGRF2* was abundantly expressed in at least one of two ductal epithelial cell lines, but there was loss of expression due to promoter methylation in pancreatic cancer cell lines, using high-throughput microarray analysis (11). Among the 12 genes silenced by methylation of 5' regions, *RASGRF2* was methylated at about 17%, which is higher than a few other genes. This gene is located at a region of chromosome

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Table I. Clinical characteristics and *RASGRF2* and *RASSF1A* methylation of lung cancer patients.

Clinical factors	No. of cases	No. of <i>RASGRF2</i> methylation (%)	p-value ^a	No. of <i>RASSF1A</i> methylation (%)	p-value ^a
Gender					
Male	65	25 (38)	0.32	24 (37)	0.70
Female	49	14 (29)		20 (41)	
Age					
≤66 ^b	58	20 (34)	>0.99	19 (33)	0.25
>66	56	19 (34)		25 (45)	
Smoke					
Never	42	12 (29)	0.41	18 (43)	0.55
Smoker	72	27 (38)		26 (36)	
Histology					
Adenocarcinoma	85	28 (38)	0.30 ^c	36 (42)	0.10
Squamous cell carcinoma	27	11 (41)		7 (26)	
Others (ad-sq, large)	2	0		1 (50)	
PT					
T1	50	15 (30)	0.26	19 (38)	0.53
T2, 3, 4	64	24 (38)		25 (39)	
pN					
N0	68	25 (37)	0.31	24 (35)	0.86
N1, 2, 3	46	14 (30)		20 (43)	
pStage					
I	50	18 (36)	0.71	19 (38)	0.53
II, III, IV	64	21 (33)		25 (39)	

^aFisher's exact probability test; ^bDivided into two groups by median age; ^cAdenocarcinoma vs. squamous cell carcinoma; Ad-sq, adenosquamous cell carcinoma.

5q13, a locus which frequently shows an allelic imbalance in lung cancer, and was speculated to act as a TSG (12). This prompted us to examine the methylation status of *RASGRF2* in lung cancer.

Imbalance of the Ras signaling pathway is a major hallmark of human cancer. Activated Ras proteins interact with a broad range of effector proteins to induce a diverse series of biological consequences. *RASSF1A* and *RASGRF2*, correlating with Ras-effector, were investigated in this study. We examined the methylation, by methylation-specific PCR (MSP), of *RASSF1A* and *RASGRF2* in lung cancer cell lines, and analyzed the methylation status of primary lung cancer, and correlated them with the clinicopathological features. We examined the mRNA expression of *RASGRF2* by reverse transcription PCR (RT-PCR) and treated those cell lines which showed loss of *RASGRF2* mRNA expression with 5-Aza-CdR.

Materials and methods

Cell lines. Ten non-small cell lung cancer (NSCLC) and 4 small cell lung cancer (SCLC) cell lines were used in this study. These cell lines were established and provided by Dr Adi F. Gazdar. Cell lines were grown in RPMI-1640 medium supplemented with 5% fetal bovine serum and incubated in 5% CO₂ at 37°C. Normal tracheal RNA was obtained from Clontech (Palo Alto, CA), and non-malignant human bronchial

epithelial cells (NHBE) were cultured as reported previously (unpublished data).

Clinical samples. Surgically resected specimens of 114 patients with primary lung cancer and 57 adjacent non-malignant lung tissue samples were obtained from Chiba Cancer Center, Japan, after obtaining Institutional Review Board approval and informed consent. Samples were immediately frozen and stored at -80°C until used. The clinical characteristics of these patients are detailed in Table I.

Methylation assay. Genomic DNA was obtained from lung cancer cell lines, cultured non-malignant cells, primary tumors and adjacent non-malignant tissue, by digestion with 20 mg/ml proteinase K (Life Technologies, Inc.), followed by standard phenol/chloroform (1:1) extraction and ethanol precipitation (13). One µg of genomic DNA was further subjected to bisulfite treatment following the protocol of EZ DNA methylation kit (ZYMO Research, Orange, CA). The modified DNA was used as a template for MSP. DNA methylation patterns in the CpG island of *RASGRF2* were determined by the method of MSP as reported previously (11). Primer sequences of *RASGRF2* for the unmethylated reaction were: 5'-TGGAGAGTGTGTTTGGTTTGA-3' (sense), and 5'-CCAACCAACAAAAACACCCC-3' (anti-sense), which amplify a 117-bp product. Primer sequences of

RASGRF2 for the methylated reaction were: 5'-GGCGGAGAG CGTGTTC-3' (sense), and 5'-CCGACCGACGAAAAACG-3' (antisense), which amplify a 119-bp product. Primer sequences of *RASSF1A* for the unmethylated reaction were: 5'-GGTT TTGTGAGAGTGTGTTT-3' (sense), and 5'-CACTAACA AACACAAACCAAC-3' (antisense). Primer sequences of *RASSF1A* for the methylated reaction were: 5'-GGGTTTTGCG AGAGCGCG-3' (sense), and 5'-GCTAACAAACGCGAA CCG-3' (antisense). Universal methylated DNA (Chemicon, CA), which was subjected to bisulfite treatment, was used as a positive control for methylated alleles. Negative controls without DNA were included in each assay. Nine μ l of each PCR product was loaded on 2% agarose gels stained with ethidium bromide. Results were confirmed by repeating bisulfite treatment and MSP for all samples.

Reverse transcription-PCR for gene expression

RASGRF2 mRNA expression was analyzed by RT-PCR. Total RNA was obtained from cell lines (NHBEC, 10 NSCLC and 4 SCLC), primary tumors and adjacent non-malignant tissue by single-step method. Reverse transcription reaction was performed on 5 μ g of total RNA with the SuperScriptII First-Strand Synthesis using oligo (dT) primer system (Life Technologies Inc.), and aliquots of the reaction mixture were used for the subsequent PCR amplification. Expression of β -actin was used as an internal control to confirm the success of the reverse transcription reaction. The forward PCR amplification primer of *RASGRF2* was 5'-ACATTTTGATTGA GAGGAAGT-3', and the reverse primer 5'-CGCATTCGTT CTTTGGTTT-3'. The forward PCR amplification primer of β -actin was 5'-CAACTGGGACGACATGGAGA-3', and the reverse primer 5'-ACGTACATGGTGGGGTGTG-3'. These primer sequences were identical to the human target genes, as was confirmed by BLAST searches. PCR products were analyzed on 2% agarose gels stained with ethidium bromide. NHBEC and normal trachea were used as normal controls for RT-PCR.

5-aza-2'-deoxycytidine (5-Aza-CdR) treatment. Methylated cell lines that downregulated gene expression were incubated in culture medium with 1 μ M of the demethylating agent 5-Aza-CdR (Sigma-Aldrich, St. Louis, MO) for 6 days, with medium changes on days 1, 3 and 5. Cells were harvested and RNA was extracted on day 6.

Statistical analysis. The differences of methylation between two groups were analyzed by using Fisher's exact test. Survival was calculated from the date of initial diagnosis until death or the date of the last follow-up. Survival was analyzed according to the Kaplan-Meier method, and differences in their distribution were evaluated by means of the log-rank test. All p-values are two-sided. The probability value of $p < 0.05$ was regarded as statistically significant.

Results

Aberrant methylation of *RASSF1A* and *RASGRF2* in cell lines. Detailed results of aberrant methylation of *RASSF1A* and *RASGRF2* in cell lines are shown in Fig. 1. Aberrant methylation of *RASSF1A* was observed in 79% (11/14) of

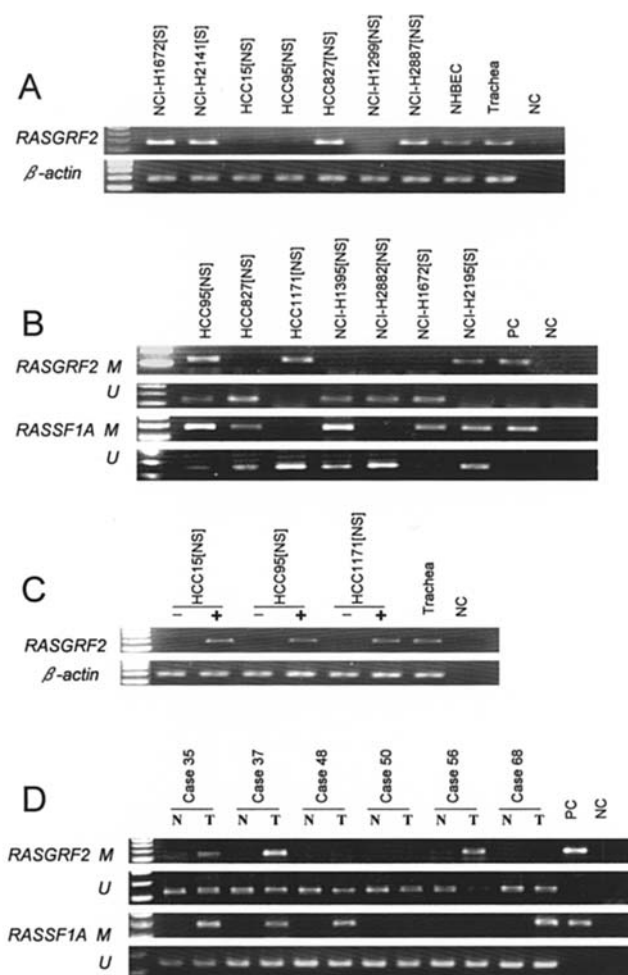


Figure 1. A, Representative examples of RT-PCR assay for *RASGRF2* RNA expression in NSCLC and SCLC cell lines. Expression of β -actin was used as a control for cDNA normalization. NHBEC and trachea were used as positive control. NC, negative control; NS, NSCLC; S, SCLC. Lanes that do not show a band represent samples with loss of expression. B, Methylation analysis of *RASGRF2* and *RASSF1A* in cell lines. Lane M, amplified product with primers recognizing methylated sequence. Lane U, amplified product with primers recognizing unmethylated sequence. PC, positive control; NC, negative control. C, Re-expression of *RASGRF2* after treatment with 5-Aza-2'-deoxycytidine (5-Aza-CdR). The expression of *RASGRF2* lost in those cell lines can be restored after treatment with 5-Aza-CdR. -, Cell line without 5-Aza-CdR; +, Cell line with 5-Aza-CdR; Trachea was used as positive control. D, Representative examples of methylation analysis of *RASGRF2* and *RASSF1A* in tumor specimens. N, non-malignant lung tissue; T, tumor.

lung cancer cell lines; in 100% (4/4) of SCLC cell lines and in 70% (7/10) of NSCLC cell lines. Aberrant methylation of both genes was absent in NHBEC. However, aberrant methylation of *RASGRF2* was observed in 29% (4/14) of lung cancer cell lines; in 30% (3/10) of NSCLC cell lines and 25% (1/4) of SCLC cell lines. Only two cell lines (NCI-H524 and NCI-H1299) demonstrated loss of expression and lack of methylation of *RASGRF2*. The concordance between gene expression and methylation of *RASGRF2* was 90% (9/10) in NSCLC cell lines, and 75% (3/4) in SCLC cell lines (overall concordance was 86%). There was no correlation between *RASGRF2* and *RASSF1A* methylation status.

Expression of *RASGRF2* in cell lines. *RASGRF2* expression was examined by RT-PCR, representative examples are shown

in Fig. 1. Expression of *RASGRF2* was present in NHBE and normal trachea. However, loss of *RASGRF2* expression was observed in 36% (5/14) of lung cancer cell lines; in 40% (4/10) of NSCLC cell lines, and 25% (1/4) of SCLC cell lines.

5-Aza-CdR treatment. To confirm that the promoter methylation was responsible for silencing the *RASGRF2* expression, we treated methylated NSCLC cell lines (HCC15, HCC95, HCC1171, NCI-H2195) that showed downregulated *RASGRF2* expression with the demethylating agent 5-Aza-CdR. *RASGRF2* expression was restored after the treatment in all four cell lines tested (Fig. 1).

Aberrant methylation of *RASSF1A* and *RASGRF2* in clinical samples. *RASSF1A* and *RASGRF2* methylation of primary tumors and corresponding non-malignant tissue is detailed in Table I and representative samples are illustrated in Fig. 1. *RASSF1A* methylation was observed in 39% (44/114) of tumors compared to none in corresponding non-malignant tissue. *RASGRF2* methylation was observed in 34% (39/114) of tumors while only 7% (4/57) was observed in corresponding non-malignant tissue. Methylation of both genes was tumor specific when compared with that of corresponding non-malignant lung tissue ($p < 0.0001$). There was no relationship between *RASSF1A* methylation and *RASGRF2* methylation. *RASSF1A* and *RASGRF2* methylation with clinicopathological features was also examined. There were no significant correlations with gender, age, smoking history (ever vs. never smoker), or histology (adenocarcinoma vs. squamous cell carcinoma), in either gene. *RASSF1A* and *RASGRF2* methylation statuses did not correlate with survival.

Expression of *RASGRF2* in clinical samples. We checked 17 clinical samples of *RASGRF2* expression by RT-PCR to confirm the relationship between methylation and expression, representative examples are shown in Fig. 1. Expression of *RASGRF2* was present in non-malignant tissue. However, loss of *RASGRF2* expression was observed in 53% (9/17) of NSCLC tumors. The loss of expression was highly correlated with methylation. The concordance between gene expression and methylation of *RASGRF2* was 65% (11/17).

Discussion

Recently, Hagihara *et al* reported that *RASGRF2* is frequently methylated in pancreatic cancer and thought to be a candidate for a TSG (11). However, there is no report about the role of *RASGRF2* in lung cancer. To understand the role of the *RASGRF2* gene in lung cancer, we examined the expression of *RASGRF2*. *RASGRF2* was expressed in tracheal cells, and cultured airway epithelial cells, whereas lung cancer cell lines had 36% loss of expression. 5-Aza-CdR treatment restored the expression of the gene in RT-PCR-negative cell lines, indicating methylation as a mechanism of transcriptional silencing of the gene. Moreover, methylation of *RASGRF2* was present in 34% of NSCLC with a tumor-specific manner. There are several reports about the correlation between aberrant methylation of TSGs and carcinogenesis of lung cancer (14). *RASGRF2* may be added to the list of TSGs. To

our knowledge, this is the first report that demonstrates the methylation of *RASGRF2* promoter in lung cancer.

We found that *RASGRF2* expression was decreased both in lung cancer cell lines and clinical samples, which was the same as in rat mammary carcinomas (10) and in pancreatic cancer cell lines (11). *RASGRF2* has been reported to be involved in H-Ras signaling (9). RAS GTPases cycle between an inactive GDP-bound state and an active GTP-bound state. As guanine-nucleotide exchange factors, *RASGRF2* stimulate the conversion of the GDP-bound form into the active form. However, the methylation changes in our experiment of *RASGRF2* were contrary to the changes expected if the H-Ras pathway were activated. *RASGRF2* expression has been shown to be nuclear in lung cancer, but little is known about the role of *RASGRF2* expression during carcinogenesis. Ras GAP protein generates potent anti-apoptotic signals after cleavage by caspase and may play an anti-apoptotic role in tumor cell lines (15,16). Whether *RASGRF2* acts as a TSG by activating apoptotic signals, as well as activating the *K-ras* gene, is unknown and requires further study.

Inactivation of TSGs may occur via multiple mechanisms, including allelic loss, gene mutation, or by methylation of CpG sites in promoter regions. *RASSF1A* is frequently silenced in lung cancer due to promoter hypermethylation in a few studies, while the mutations of *RASSF1A* are rare. The frequency of *RASSF1A* promoter hypermethylation is about 70-100% in SCLC tumors whereas it is 30-60% in NSCLC samples (14). We found hypermethylation of *RASSF1A* in 100% (4/4) of SCLC and 70% (7/10) of NSCLC cell lines, in 39% (44/114) of primary tumors and none in corresponding non-malignant tissue. The results were highly consistent with the previous reports (14). We did not examine the expression of *RASSF1A* because it was confirmed by the previous study (17). However, there was no correlation between *RASSF1A* and *RASGRF2* methylation status. The reason might be that *RASSF1A* is one of the Ras-effectors, whereas *RASGRF2* regulate the RAS GTPases cycle.

Neither *RASGRF2* nor *RASSF1A* showed a significant association with methylation and patients' clinical characteristics. The prognostic association of *RASSF1A* methylation in lung cancer is a controversial issue (18-20). The lack of significance in the association of methylation with the outcome may result from the limited number of patients in our study or other factors. However, the frequent methylation of *RASGRF2* and *RASSF1A* genes in lung cancer, without any correlation with clinical factors, would suggest that inactivation of these genes might be a ubiquitous mechanism stimulating tumor cell growth, rather than a means of further subdividing tumors into constituent biological or prognostic groups.

In conclusion, we demonstrated frequent inactivation of the *RASGRF2* gene through aberrant methylation of the promoter in NSCLC cell lines. We also frequently found methylation of *RASGRF2* in primary NSCLC. Aberrant methylation of the *RASGRF2* gene appears to be an important factor in the pathogenesis of NSCLC, a different role compared to *RASSF1A*. Our findings of a frequent acquired tumor-related epigenetic alteration favor the candidacy of *RASGRF2* as a TSG.

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