Relationship of Ras Association Domain Family 1 Methylation and K-Ras Mutation in Primary Non-Small Cell Lung Cancer

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ABSTRACT

Recently, several groups have reported that Ras association domain family 1 (RASSF1A) interacts with Ras and mediates Ras-dependent apoptosis. However, the mechanism by which RASSF1A plays a role as a tumor suppressor in human cancer is unclear. In this study, we investigated the relationship between the RASSF1A methylation and K-ras mutation and their effects on patient’s survival in 242 primary non-small cell lung cancers (NSCLCs) to understand the role of RASSF1A in Ras-mediated oncogenic transformation. RASSF1A methylation was not found to be associated with the K-ras mutation in NSCLCs (P = 0.37). For patients with stage I adenocarcinoma, those with RASSF1A methylation and K-ras mutation had a poorer prognosis than those with either RASSF1A methylation or K-ras mutation (P = 0.001). In stage II–III adenocarcinoma patients, the median survival of those with RASSF1A methylation and K-ras mutation was 9 months, and this was poorer than that of those with either RASSF1A methylation or K-ras mutation (P = 0.001). The hazard of failure for those with RASSF1A methylation and K-ras mutation was 2.94 times higher compared with that of those with neither K-ras mutation nor RASSF1A methylation (95% confidence interval = 1.67–9.42; P = 0.01). Our results suggest that RASSF1A methylation and K-ras mutation are not mutually exclusive in NSCLC. In addition, RASSF1A methylation, in combination with K-ras mutation, may have an adverse synergistic effect on patient’s survival in NSCLCs.

INTRODUCTION

RASSF1A1 gene is a candidate tumor suppressor gene at 3p21.3, one of the most common regions showing loss of heterozygosity in lung cancer (1). The RASSF1A gene codes for two major transcripts, RASSF1A and RASSF1C, by alternative splicing and separate promoter use. RASSF1A is inactivated by the hypermethylation of CpG islands in many human cancers, including prostate cancer (2), nasopharyngeal cancer (3), ovarian cancer (4), medulloblastoma (5), breast cancer (4, 6, 7), and lung cancer (1, 4, 6, 8). Moreover, the exogenous expression of RASSF1A, but not RASSF1C, decreases colony formation in vitro and tumor formation in vivo (1, 6). However, the mechanism by which RASSF1A plays a role as a tumor suppressor in human cancer is not clear.

Recently, several groups have reported upon the existence of a relationship between RASSF1A and Ras. (1, 9). The small GTP-binding protein Ras plays an important role in mediating multiple intracellular signal transductions, including growth, apoptosis, and differentiation. Active GTP-bound Ras interacts with its downstream effectors and regulates the transduction of proliferative signals through the Raf/mitogen-activated protein kinase pathway and of the apoptosis through the Nore1/Mst1 and phosphatidylinositol 3’-kinase pathway (8). Nore1 is one of the potential Ras effectors or targets in mammalian cells (9). Nore1 was identified as a protein that interacts with active GTP-bound Ras in a yeast two-hybrid assay and is associated with Ras in vivo after epidermal growth factor or 12-O-tetradecanoylphorbol-13-acetate stimulation (9). RASSF1A was found to be the closest Ras effector homologue to Nore1 in mammalian cells (1). Moreover, the COOH-terminal 300 amino acid of RASSF1A, a segment encompassing a putative RA domain, shows high homology (50% identical) with Nore1 (1, 10). All three splice variants of RASSF1, i.e., A, B, and C, have an RA domain responsible for mediating interaction with Ras (1, 6, 11).

RASSF1A binds Ras in a GTP-dependent manner both in vitro and in vivo and mediates Ras-dependent apoptosis (11). The apoptosis induced by the ectopic expression of RASSF1A is enhanced by active Ras and inhibited by dominant negative Ras in 293-T cells (11). RASSF1A heterodimerizes with Nore1 through its nonhomologous NH2-terminal segments and thereby interacts with Ras (12). A new downstream target, Mst1 protein, of Ras was discovered recently. Mst1 forms a complex with Ras and Nore1 for proapoptotic signaling (8), suggesting that active Ras promotes apoptosis by forming a complex with Nore1-RASSF1-Mst1. These observations suggest that RASSF1A functions as a tumor suppressor through Ras-mediated apoptosis.

In addition, several observations have suggested that RASSF1A methylation and K-ras mutation are mutually exclusive. Most colorectal cancers with mutation of K-ras codon 12 or 13 do not have aberrant methylation of the RASSF1A promoter (13). Although the mutation of K-ras gene has been reported in ~30% of primary NSCLCs, especially in adenocarcinoma, K-ras mutations have never been reported in SCLC. In contrast, hypermethylation of RASSF1A occurs more frequently in small cell lung cancer than in NSCLC. The contrast in the prevalence of K-ras mutation and RASSF1A hypermethylation in SCLC and NSCLC also suggests that the two proteins act on the same pathway.

To understand the relationship between RASSF1A and Ras in NSCLC, we examined the methylation status of RASSF1A promoter and mutations of K-ras codons 12, 13, and 61 in 242 primary NSCLCs. We also investigated whether alterations of K-ras and RASSF1A have an adverse synergistic effect on patient survival in NSCLC.

MATERIALS AND METHODS

Study Population. Two hundred forty-two consecutive patients who underwent surgical resection for NSCLC during the period between January 1994 and September 2002 in the Department of Thoracic Surgery at the Samsung Medical Center (Seoul, Korea) participated in the study. Only those with records of accurate pathological staging and histology were included in the study. Patients with two or more forms of cancer and those who died of causes other than lung cancer were excluded. Written informed consent for the use of

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3 The abbreviations used are: RASSF1, Ras association domain family 1; RA, Ras association; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; MSP, methylation-specific PCR; HR, hazard ratio; CI, confidence interval.
the paraffin-embedded tissues and clinical information was obtained from all patients before surgery. Information on sociodemographic characteristics was obtained from an interviewer-administered questionnaire, and histopathological diagnoses were fully documented.

DNA Extraction from Paraffin Tissue Blocks. Formalin-fixed, paraffin-embedded tissue blocks containing at least 75% neoplastic tissue were used for this study. Serial 10-μm thick tissue sections were cut from each paraffin block and transferred to slides. Sections were stained with H&E to classify tumor and normal areas before DNA extraction. Tumor areas were carefully microdissected from normal surrounding stromal tissues, placed in an Eppendorf tube, deparaffinized overnight at 63°C in xylene, and then vortexed vigorously. After centrifuging at full speed for 5 min, the supernatant was removed, and ethanol was added to remove residual xylene and subsequently removed by centrifugation. After ethanol evaporation, the tissue pellet was resuspended in lysis buffer ATL (Qiagen), and the DNA was isolated according to the manufacturer’s instructions (Qiagen).

Methylation Analysis of RASSF1A Promoter. The methylation status of the RASSF1A promoter was determined by MSP, as described by Herman et al. (14). Two sets of primers were designed, one specific for DNA methylated at the promoter region of each gene and the other specific for unmethylated DNA. The primers used for MSP have been described previously (1). DNA from 242 NSCLCs was digested with 100 U of restriction endonuclease HpaII (Promega) at 37°C for 3 h. The mixtures were then incubated at 55°C for 3 min. The PCR product of each sample was sequenced with the primers described (HpaII methylated or Tsp458I unmethylated), using BigDye terminator Mix, 1 pmol each of the sense and antisense primers, 2.5 mM MgCl₂, and 0.1 units of TaqPolymerase (Nova-Taq; Genenmed). The primer sets for codons 12, 13, and 61 Mutations by Automatic Sequencing. Mutations of codons 12, 13, and 61 of the K-ras gene were examined by directly sequencing the PCR products. PCR was performed on a total volume of 50 μl, which contained 5 μl of DNA, 5 pmol each of the sense and antisense primers, 2.5 mM each of deoxynucleotide triphosphate, 10× PCR buffer (with 2.5 mM MgCl₂; Genenmed), 10× enhancer, and 2.5 units of TaqDNA Polymerase (Novag-Taq; Genenmed). The primers used for codons 12, 13, and 61 of the K-ras gene were 5′-TGAAGTACGTCATTGATA-CACGG-3′ (sense) and 5′-GGGAGTAAAGTTCCCATATTAATGGT-3′ (antisense) flanking codons 12 and 13 and 5′-GCGTTTAGTGCCATTGT-3’ (sense) and 5′-AAAACGGGATATTACCTACCTCA-3’ (antisense) for codon 61. The following PCR conditions were used: 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s, followed by extension at 72°C for 10 min. For DNA sequencing, PCR was performed in a total volume of 10 μl containing the purified PCR products (20–50 ng), 1.6 pmol primer, 1 μl of BigDye terminator Mix, 1× adding buffer, and 0.1 units of Taq Polymerase. The following cycling conditions were used: 25 cycles of 96°C for 10 s, 50°C for 5 s, and 72°C for 3 s, followed by 5 cycles of 96°C for 10 s, 50°C for 5 s, and 72°C for 3 min. The PCR product of each sample was sequenced with a 373 DNA sequencer (Applied Biosystems, Foster City, CA) using dye primer conditions as recommended by the manufacturer.

Statistical Analysis. Associations between RASSF1A methylation and the K-ras mutation and between these and the clinicopathological parameters were analyzed using the Wilcoxon’s rank-sum test for continuous variables or Fisher’s exact test (or Pearson χ² test) for categorical variables. The effects of RASSF1A methylation and/or K-ras mutation on patient survival were estimated using the Kaplan-Meier method, and the difference between the survival curves of the two groups was analyzed by the log-rank test. The Cox proportional hazard model was used to estimate the HR of independent factors influencing patient’s survival, after controlling for potential confounding factors such as age, sex, and disease stage. All Ps were based on two-sided statistical analysis.

RESULTS

Clinicopathological Characteristics of K-Ras Mutation and RASSF1A Methylation. In this study, mutations of K-ras codons 12, 13, and 61 and the methylation status of RASSF1A promoter were determined in 242 archival NSCLCs (Fig. 1). The clinicopathological characteristics of the 242 patients are listed in Table 1. Forty-four (18%) of the 242 NSCLCs samples examined showed a mutation at either codons 12, 13, or 61 of the K-ras gene. Of that 44, 38 (86%) were at codon 12, 5 (11%) at codon 13, and 1 (2%) at codon 61. The RASSF1A promoter was found to be methylated in 80 (33%) of the 242 NSCLCs analyzed. Neither K-ras mutation nor RASSF1A methylation was significantly associated with patient’s age (Table 1). K-ras mutation occurred in 26 of 147 men (18%) and in 18 of 95 women (19%), but the gender difference was not statistically significant (P = 0.80). RASSF1A methylation occurred at a similar frequency in men and women. The histological subtypes were adenocarcinoma (52%), squamous cell carcinoma (35%), and other types (13%). K-ras mutations were present in 35 of 125 adenocarcinomas (28%), 3 of 84 squamous cell carcinomas (4%), and 6 of the 33 other types of NSCLC (18%). The differences in the frequencies of these histologies were statistically significant (P < 0.001). Of 35 adenocarcinomas with a K-ras mutation, 30 were at codon 12 of K-ras, 4 at codon 13, and 1 at codon 61. The K-ras mutation in squamous cell carcinoma occurred only at codon 12. The RASSF1A methylation occurred more frequently in adenocarcinoma (49 of 125; 39%) than in squamous cell carcinoma (22 of 84; 26%). The K-ras gene was mutated in 20% of stage I tumors, 14% of stage II, 15% of stage III tumors, and 30% of stage IV tumors, which showed no significant relationship between K-ras mutation and disease stage. In addition, no association was found between RASSF1A methylation and disease stage (Table 1).

K-ras mutation occurred mainly in adenocarcinoma. Thus, the association between clinicopathological characteristics and K-ras mutation and RASSF1A methylation was examined in adenocarcinoma. Neither K-ras mutation nor RASSF1A methylation was associated with patient age, gender, and stage in adenocarcinoma (Table 1).

Relationship between K-Ras Mutation and RASSF1A Methylation. Of 242 patients examined, 130 (54%) had neither K-ras mutation nor RASSF1A methylation, whereas 12 (5%) had both K-ras mutation and RASSF1A methylation (Table 2). The prevalence of RASSF1A methylation in patients with K-ras mutation (12 of 44, 27%) was lower than in those with the wild-type K-ras (68 of 195, 35%), but this difference was not significantly different (P = 0.37; Fig. 2). The data were stratified to further analyze the relationship between K-ras mutation and RASSF1A methylation according to histology. For the 125 adenocarcinomas studied, 51 (41%) had neither K-ras mutation nor RASSF1A methylation, and 10 (8%) had both K-ras mutation and RASSF1A methylation (Table 2). The K-ras mutation without RASSF1A methylation was observed in 25 of 125 (20%), and 39 of 125 (31%) showed RASSF1A methylation without K-ras mutation (Fig. 2). The association between K-ras mutation and RASSF1A methylation was not statistically significant (P = 0.13).

For the 84 squamous cell carcinomas examined, 56 (67%) had abnormalities of neither K-ras nor RASSF1A. The K-ras mutation was detected in 5% (3 of 59) of tumors without the RASSF1A methylation and in 4% (1 of 25) of tumors with the RASSF1A methylation (Fig. 2). A single case had both K-ras mutation and RASSF1A methylation. However, K-ras mutation was not associated with RASSF1A methylation in squamous cell lung cancers (P = 1.00).

Survival Analysis. Kaplan-Meier estimator of the survivorship function was used to examine the effect of K-ras mutation and/or RASSF1A methylation on patient survival (Fig. 3). The survival of groups with mutated K-ras and/or methylated RASSF1A were compared by using the log-rank test. The data were stratified by disease stage because stage is an independent risk factor in NSCLC. The Kaplan-Meier survival curves demonstrated that the overall survival time in stage I–III adenocarcinoma patients was significantly shorter in patients having K-ras mutation and RASSF1A methylation (Fig. 3). The median survival time for stage I adenocarcinoma with K-ras mutation and RASSF1A methylation was 17 months, compared with either K-ras mutation only (28 months) or RASSF1A methylation only (46 months; Fig. 3A). Patients that lacked the K-ras mutation and the RASSF1A methylation had better survival (median, 76 months) than those that
lacked only *K-ras* mutation or *RASSF1A* methylation. The differences in the survivals for all four groups in stage I adenocarcinoma was statistically significant by the log-rank test (*P* < 0.001; Fig. 3A). The disease-free survival time in stage II–III adenocarcinoma (Fig. 3B) also was shorter for those having *K-ras* mutation and *RASSF1A* methylation than for those with either *K-ras* mutation or *RASSF1A* methylation, and this difference did reach a statistical significance (*P* < 0.001). The median survival time for those with the *K-ras* mutation and the *RASSF1A* methylation was 9 months and those without *K-ras* mutation and *RASSF1A* methylation 42

### Table 1 Clinicopathological characteristics in all cases (n = 242) and adenocarcinomas (n = 125)*

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<tr>
<td></td>
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<tr>
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<tr>
<td>Others</td>
<td>27</td>
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<td>24</td>
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*Values are mean ± SE.

*Adeno, adenocarcinoma; Squam, squamous cell carcinoma.
Table 2: Relationship between K-ras mutation and RASSF1A methylation

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<th>Total</th>
<th>Adenocarcinoma</th>
<th>Squamous cell carcinoma</th>
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<tr>
<td>K-ras Mutated</td>
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<tr>
<td>RASSF1A Methylation</td>
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<tr>
<td>Methylated</td>
<td>12</td>
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<tr>
<td>Unmethylated</td>
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<td>Wild-type</td>
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<td>39</td>
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<tr>
<td></td>
<td>130</td>
<td>51</td>
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<tr>
<td>P*</td>
<td>0.37</td>
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* Pearson χ² test.

Cox Proportional Hazard Analysis. The hazard of failure for patients with K-ras mutation and RASSF1A methylation was estimated by Cox proportional hazard analysis (Table 3). Age, sex, duration of smoking, and stage were controlled for as potential confounding factors. Patients without K-ras mutation and RASSF1A methylation were used as a reference group. K-ras mutation only significantly affected a patient’s prognosis (HR = 2.31, 95% CI = 1.62–5.97, P = 0.008), and hypermethylation of the RASSF1A promoter carried a 1.97 times higher risk of failure compared with those without K-ras mutation and RASSF1A methylation (95% CI = 1.42–4.35, P = 0.03). The hazard of failure for those with K-ras mutation and RASSF1A methylation was ~2.94 times higher than the reference group (95% CI = 1.67–9.42, P = 0.01).

DISCUSSION

In this study, we investigated the relationship between RASSF1A methylation and K-ras mutation in primary NSCLC in an attempt to understand the role of RASSF1A in Ras-mediated oncogenic transformation. We also studied their effects on patient prognosis in adenocarcinoma.

The prevalence of RASSF1A methylation and K-ras mutation in this study occurred at a frequency similar to that found by other studies (1, 4, 6, 15–18). In our data, no association was found between RASSF1A methylation and K-ras mutation in 242 primary NSCLCs. The prevalence of the RASSF1A methylation in patients with the K-ras wild-type was higher than in those with the K-ras mutant, but this difference was not statistically significant (P = 0.37). These results are not consistent with other recent observations, which have reported the interaction between RASSF1 and Ras.

On the basis of many observations suggesting that RASSF1A mediates Ras-dependent apoptosis (1, 6, 8, 11), it was hypothesized that RASSF1A inactivation is closely related to Ras activation in human cancers and thus contributes to malignant transformation by inhibiting Ras-mediated apoptosis. However, the absence of an association between RASSF1A methylation and K-ras mutation in our study does not support this hypothesis. Rather, our study suggests that RASSF1A inactivation contribute to malignant transformation by some distinct mechanism other than Ras-mediated antiapoptosis signaling. RASSF1A methylation in the Ras-mediated apoptosis pathway may not be functionally equivalent to Ras mutation or RASSF1A may have a minor role in Ras-mediated apoptosis. The fact that both proteins were found to be independent prognostic factors and showed an adverse synergistic effect on patient’s survival in this study supports the notion that RASSF1A methylation is not an alternative to Ras mutation in the pathogenesis of NSCLC.

How does RASSF1A function as a tumor suppressor irrespective of Ras-mediated apoptosis? Recently, there have been studies that report that RASSF1A suppresses malignant transformation by inhibiting the activation of cyclin D1 accumulation and that it is involved in DNA damage response. Shivakumar et al. (19) reported that reintroduction of RASSF1A in lung cancer cell H1299 that does not express RASSF1A results in growth arrest but not apoptosis and that RASSF1A blocks G1-S cell cycle progression by inhibiting the accumulation of cyclin D1 at the level of translational control. Both Ras and RASSF1A are involved in the cell cycle progression through the regulation of a common target, cyclin D1 (20–22). The inactivation of RASSF1A by CpG island hypermethylation and the activation of K-ras may cooperate to accumulate cyclin D1 and to direct cell cycle progression through a different pathway.

RASSF1A has a putative ATM kinase phosphorylation site (1, 4, 23), and ATM kinase plays a pivotal role in S-phase delay in response to DNA damage, suggesting that RASSF1 participates in a DNA damage-induced signaling pathway and thereby controls cell cycle progression and cell growth.

Our data are not consistent with the study of van Engeland et al. (13), which found that RASSF1A methylation occurs mainly in colorectal cancers without K-ras mutation, which implied that RASSF1A methylation is an alternative way of affecting Ras signaling. The different associations of the K-ras mutation and RASSF1A methylation in colorectal cancer and NSCLC may result from tissue specificity or from exposure to different environmental factors. RASSF1A, in Ras-mediated apoptosis, may play a minor role in NSCLC but could participate in a key event in the pathogenesis of colorectal cancer. Different types of cells in vivo may respond differently to Ras-mediated apoptotic signals. It is also possible that the Ras-mediated

months. The median survival for those with K-ras mutation but without RASSF1A methylation was 17 months.

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apoptosis pathway through RASSF1A may be not switched on in specific cell types (24).

If RASSF1A affects cell growth independently of Ras, it can also be an independent prognostic factor, and alterations in both genes could produce an adverse synergistic effect on patient prognosis. In this study, we compared survivals in a group of patients with K-ras mutation and RASSF1A methylation in stage I–III adenocarcinoma and found that K-ras mutation and RASSF1A methylation were significantly associated with a poor prognosis in stage I and II–III adenocarcinoma. This observation that RASSF1A methylation was an independent prognostic factor of survival was consistent with others (6, 18). The hazard of failure for patients with K-ras mutation and RASSF1A methylation was higher than that of those without K-ras mutation and RASSF1A methylation (HR = 2.94, 95% CI = 1.67–9.42, P = 0.01).

It is not clear how RASSF1A and Ras affect patients’ adverse prognosis synergistically in NSCLC. RASSF1A may affect patient prognosis by participating in cell growth arrest, rather than apoptosis. Although the timing of RASSF1A methylation and K-ras mutation in NSCLC is not clear, one explanation is that either genetic or epigenetic change may lead to an additional growth advantage for the transformed cell or promote malignant transformation. The loss of heterozygosity of chromosome 3p is known to be the earliest event in the pathogenesis of NSCLCs. Therefore, it is possible that RASSF1A methylation occurs first in some NSCLC and that the subsequent mutation of K-ras may be incidental to the methylation of RASSF1A.

In conclusion, our results suggest that RASSF1A is involved in the pathogenesis of NSCLC through a distinct mechanism other than Ras-mediated pathway. In addition, they indicate that RASSF1A methylation and K-ras mutation may be viewed as independent prognostic factors and that these factors act synergistically to the detriment of prognosis in NSCLC.

**ACKNOWLEDGMENTS**
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**REFERENCES**

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**Table 3** Estimation of the hazard of failure by Cox proportional hazard analysisa

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<td>K-ras mutated</td>
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<td>0.008</td>
</tr>
<tr>
<td>RASSF1A methylated</td>
<td>1.97</td>
<td>1.42–4.35</td>
<td>0.03</td>
</tr>
<tr>
<td>K-ras mutated/RASSF1A methylated</td>
<td>2.94</td>
<td>1.67–9.42</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*a Adjusted for age, sex, stage, and duration of smoking.*


Relationship of Ras Association Domain Family 1 Methylation and K-Ras Mutation in Primary Non-Small Cell Lung Cancer

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