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# *RASSF1A* Promoter Methylation and *Kras2* Mutations in Non Small Cell Lung Cancer[1]

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**Abstract**

**In the present studies, we investigated the correlation between *RASSF1A* promoter methylation status and *Kras2* mutations in 65 primary non small cell lung cancer (NSCLC) including 33 adenocarcinomas, 12 large cell carcinomas, and 20 squamous cell carcinomas. Mutational analysis of *Kras2* showed: 30% (10 of 33) of adenocarcinomas, 25% (3 of 12) of large cell carcinomas, and only 5% (1 of 20) of squamous cell carcinomas contained activated *Kras2* mutation at codon 12 or 13. *RASSF1A* promoter region CpG island methylation was detected in adenocarcinomas (55%), large cell carcinomas (25%), and squamous cell carcinomas (25%). Interestingly, combined *RASSF1A* methylation and *Kras2* mutation data show that only ~7% adenocarcinomas/large cell carcinomas exhibited both *KRASSF1A* promoter methylation and *Kras2* mutation, whereas 24% adenocarcinomas, 50% large cell carcinomas, and 70% squamous cell carcinomas showed neither *Kras2* mutation nor *RASSF1A* promoter methylation. These results showed that the majority of the primary NSCLCs with *Kras2* mutations lack *RASSF1A* inactivation, and both *RASSF1A* inactivation and *Kras2* mutation events occur frequently in adenocarcinomas and large cell carcinomas. Our results indicate a trend of inverse relationship between *Kras2* activation and *RASSF1A* promoter methylation in the majority of human lung adenocarcinomas and large cell carcinomas.**

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**Keywords:** *Kras2*; *RASSF1A*; mutations; methylation; lung cancer.

## Introduction

Lung cancer is the leading cause of cancer death in men and women in the US [1]. Molecular changes in proto-oncogenes and tumor suppressor genes have been detected in all stages of lung tumorigenesis. Activating point mutations in the *ras* genes have been implicated in the pathogenesis of several human cancers including those of the lung, colon, and pancreas [2]. In particular, non small cell lung cancer (NSCLC), which accounts for 75% of lung cancers, has been shown to harbor *Kras2* mutations in 30% to 50% of lung adenocarcinomas. However, *Kras2* mutations were rarely detected in lung squamous cell carcinomas [3–7].

*RASSF1A*, a newly discovered Ras effector, is inactivated in a variety of human cancers including lung, colon, breast, prostate, thyroid, and renal cell carcinomas [8–15]. Allelic loss at 3p21.3 is one of the common events in the pathogenesis of lung cancer and many other cancers [14]. *RASSF1* gene at 3p21.3 encodes two major transcripts, RASSF1A and RASSF1C. Both RASSF1A and RASSF1C proteins possess the RAS-binding domain, which binds RAS in a GTP-dependent manner *in vivo* and *in vitro* [15]. The binding of *RASSF1* to Mst1, in conjunction with the binding of Nore1 (a closely related family member of *RASSF1*) to Mst1, appears to activate a proapoptotic pathway [15–17]. It has been proposed that Ras can activate this proapoptotic pathway by utilizing the Ras association domain of RASSF1 and Nore1. Of interest to tumorigenesis is the observation that expression of the splice variant *RASSF1A* is absent in lung cancer lines and in primary tumors [13,14,18,19]. One allele of *RASSF1A* is lost (the 3p21.3 locus) in a high percentage of all lung cancer subtypes and expression of the other allele is inhibited by promoter methylation [18–21]. The *RASSF1A* promoter is hypermethylated in 72% to 79% of small cell lung cancers and in 30% to 40% of NSCLCs [19,21]. Also, expression of *RASSF1A* in lung carcinoma cells was shown to reduce colony formation, suppress anchorage-independent growth, and inhibit tumor formation in nude mice [18]. These data suggest that *RASSF1A* is a tumor suppressor gene and is inactivated in a high percentage of human lung tumors. Other studies have found that RASS1A is a tumor suppressor in numerous other types of

Abbreviations: LOH, loss of heterozygosity; NSCLC, non small cell lung cancer; PCR, polymerase chain reaction; MSP, methylation-specific PCR

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cancers, including breast, ovary [11–14], colon [22], head and neck [23], and prostate [24] cancers.

A recent observation indicates the possibility that activation of the *Ras* gene and hypermethylation of the *RASSF1A* gene are disjointed events. Van Engeland et al. [22] detected oncogenic *Kras2* mutations in 87 of 222 colorectal tumors and *RASSF1A* promoter methylation in 45 of these tumors. Only 11 of these tumors (5%) exhibited both genetic alterations and 110 (50%) contained either *Kras2* mutations or promoter methylation [22]. Similarly, a study of 110 NSCLCs exhibited *Kras2* mutations in 13 tumors and *RASSF1A* promoter methylation in 35 tumors, and only two tumors had the same mutation whereas 46 had either a *Kras2* oncogene or *RASSF1A* promoter methylation [19]. These data suggest that inactivation of *RASSF1A* occurs predominantly in colorectal tumors and adenocarcinomas of the lung without alteration of the *Kras2* gene.

The purpose of this study was to examine the association between *Kras2* mutation and *RASSF1A* methylation in human lung adenocarcinomas, large cell carcinomas, and squamous cell carcinomas, and to determine if there was an inverse correlation between genetic alterations of *RASSF1A* and *Kras2*.

## Materials and Methods

### *Tissues and DNA Isolation*

A total of 65 pathologically documented lung NSCLCs were used in the present study. These tumors and their paired normal tissues were obtained from the Cooperative Human Tissue Network of the Ohio State University Department of Pathology (Columbus, OH). A pathologist classified all tumors histopathologically. High-molecular-weight DNA was isolated from both tumor and normal tissues according to published protocols [25].

### *Analysis of Kras2 Mutations by Polymerase Chain Reaction (PCR) Direct Sequencing*

PCR amplifications of *Kras2* exon 1 from lung tumors were carried out as described previously [26]. The sequences of PCR primers of *Kras2* exon 1 were Kras2-1F: 5′-TTTTT-ATTATAAGGCCTGCT-3′ and Kras2-1R: 5′-GTCCA-CAAAATGATTCTGAA-3′. The 114-bp PCR products were eluted by using QIAquick gel extraction kit (Qiagen, Valencia, CA). The 12th and 13th codon mutations were analyzed on the ABI PRISM 3700 DNA analyzer (Perkin-Elmer/Applied Biosystems, Foster City, CA).

### *Methylation Analyses*

The methylation status of the *RASSF1A* promoter region was determined by chemical modification of genomic DNA with sodium bisulfite and methylation-specific PCR (MSP) was performed. Bisulfite treatment converts cytosine bases to uracil bases but has no effect on methylcytosine bases. The 144-bp fragment of the *RASSF1A* promoter region was amplified using: F: 5′-GTT TAG TTT GGA TTT TGG GGG AG-3′; and R: 5′-CCC RCA ACT CAA TAA ACT CAA ACT C-3′. The reaction was incubated at 95°C for 1 minute, 56°C for 1 minute, 72°C for 1 minute, and 35 cycles. The PCR product was used as a template for the MSP reaction. Primers for the unmethylated reaction were: F: 5′-GGG GTT TGT TTT GTG GTT TTG TTT-3′; and R: AAC ATA ACC CAA TTA AAC CCA TAC TTC A-3′. Primers for the methylated reaction were: F: 5′-GGG TTC GTT TTG TGG TTT CGT TC-3′; and R: 5′-TAA CCC GAT TAA ACC CGT ACT TCG-3′. The annealing temperature was 60°C. DNA from normal lung tissue was used as the control for unmethylated *RASSF1A*, and normal lung DNA treated with Sss1 methyltransferases was used as the control for methylated *RASSF1A*. $H_2O$ was used as negative control. Twenty microliters of each PCR reaction was loaded onto a 6% nondenaturing polyacrylamide gel, stained with ethidium bromide, and pictured under UV light.

## Results

### *Methylation Status of the RASSF1A*

A total of 65 primary NSCLCs including 33 adenocarcinomas, 12 large cell carcinomas, and 20 squamous cell carcinomas were analyzed for *RASSF1A* promoter methylation. As shown in Table 1, 18 of 33 (55%) adenocarcinomas, 3 of 12 (25%) large cell carcinomas, and 5 of 20 (25%) squamous cell carcinomas were detected with *RASSF1A* promoter methylation. Figure 1*A* illustrates the representative MSP analysis on pairs of tumors and normal tissues.

### *Kras2 Gene Mutation*

Using PCR direct sequencing analysis, *Kras2* gene 12th and 13th codon mutations were determined in all the NSCLC DNA. In agreement with previous reports, our data showed that 10 of 33 (30%) adenocarcinomas, 3 of 12 (25%) large cell carcinomas, and only 1 of 20 (5%) squamous cell carcinomas contained the *Kras2* gene mutation at codon

**Table 1.** Correlation Between the *Kras2* Activation and *RASSF1A* Methylation in NSCLCs.

| Tumor Type | Patient Number | *Kras2* Mutation | | | *RASSF1A* Methylation | Tumors Contain *Kras2* Mutations and *RASSF1A* Methylation | Tumors Contain Neither *Kras2* Mutations nor *RASSF1A* Methylation2 |
|---|---|---|---|---|---|---|---|
| | | Codon 12 | Codon 13 | Total | | | |
| Adenocarcinoma | 33 | 8/33 (24%) | 2/33 (6%) | 10/33 (30%) | 18/33 (55%) | 3/33 (9%) | 8/33 (24%) |
| Large cell carcinoma | 12 | 3/12 (25%) | 0/12 (0%) | 3/12 (25%) | 3/12 (25%) | 0/12 (0%) | 6/12 (50%) |
| Squamous cell carcinoma | 20 | 1/20 (5%) | 0/20 (0%) | 1/20 (5%) | 5/20 (25%) | 0/5 (0%) | 14/20 (70%) |

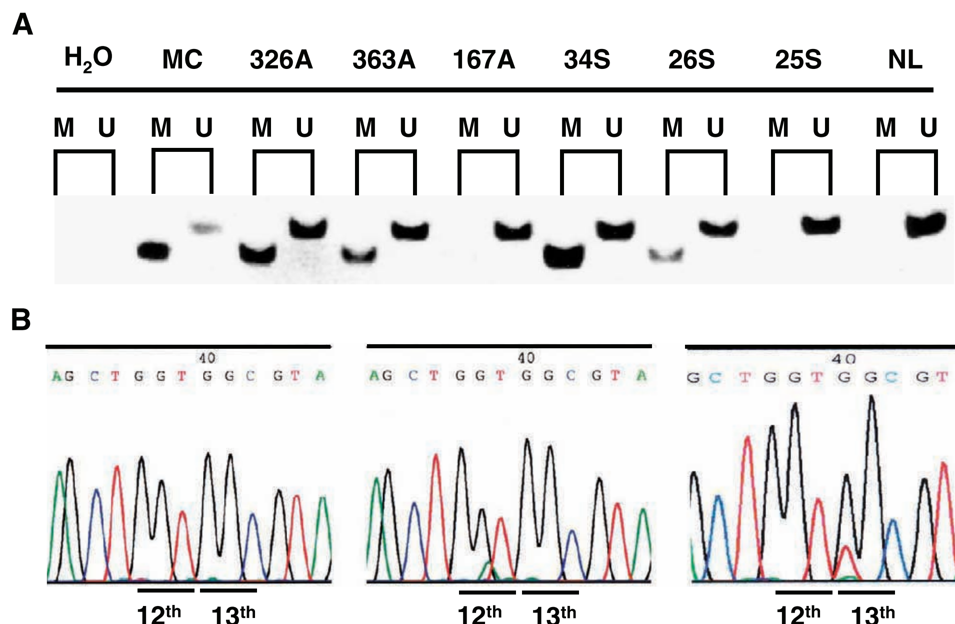


**Figure 1.** *Kras2 mutation analysis and MSP analysis of RASSF1A in NSCLCs. (A) Representative autoradiograph showing RASSF1A promoter hypermethylation in tumors. Tumor identification numbers were indicated above each autoradiograph; the presence of a visible PCR product in those lanes marked M indicated tumor presence of methylated RASSF1A alleles, whereas the presence of a visible PCR product in those lanes marked U indicated tumor presence of unmethylated RASSF1A alleles. Normal lung tissue (NL) and in vitro methylated DNA (MC) were used as negative and positive controls for RASSF1A promoter methylation. $H_2O$ was also included in each reaction as negative control. (B) Representative example of 12th and 13th codon mutations in Kras2. Lung tumor without Kras2 mutation is shown on the left. Lung tumor with Kras2 12th codon mutation (GGT→GAT transition) is shown in the middle. Lung tumor with Kras2 13th codon mutation (GGC→TGC transition) is shown on the right.*

12 (GGT→TGT, CGT, GAT, GCT, and GTT transition) or codon 13 (GGC→GAC, TGC) (Figure 1*B*, Table 1).

*Correlations Between Kras2 Mutation and RASSF1A Inactivation*

By combing *RASSF1A* promoter methylation analysis with *Kras2* mutation data, we found that only 3 of 33 (9%) of the samples contained both a *Kras2* mutation and *RASSF1A* promoter methylation. None of the large cell carcinomas (zero of three) or squamous cell carcinomas (zero of five) with *RASSF1A* promoter methylation contained a *Kras2* mutation (Table 1). Our results indicate that the majority of the adenocarcinomas and large cell carcinomas with *Kras2* mutations lack *RASSF1A* promoter methylation. In addition, 8 of 33 (24%) adenocarcinomas, 6 of 12 (50%) large cell carcinomas, and 14 of 20 (70%) squamous cell carcinomas contain neither *Kras2* mutation nor *RASSF1A* methylation (Table 1). By integrating previous studies [19,22], a comparison of *RASSF1A* methylation and *Kras2* mutation data was complied and summarized in Table 2. These data show that only ~7% adenocarcinomas/large cell carcinomas, 2% stage I lung adenocarcinomas, and 5% colorectal tumors exhibited both *RASSF1A* promoter methylation and *Kras2* mutations.

## Discussion

In the present study, we systematically evaluated the correlation between activating *Kras2* mutations and *RASSF1A* promoter methylation in 33 human lung adenocarcinomas, 12 large cell carcinomas, and 20 squamous cell carcinomas. We have shown that 30% of the adenocarcinomas, 25% of large cell carcinomas, and only 5% of the squamous cell carcinomas contained activating *Kras2* mutation at codon 12 or 13. In addition, we analyzed the methylation status of the promoter region of the *RASSF1A* gene. Our results showed that *RASSF1A* inactivation was observed in 55% of adenocarcinomas, 25% large cell carcinomas, and 25% squamous cell carcinomas. When *Kras2* mutations and *RASSF1A* methylation analysis data were combined, we found that only 9% of lung adenocarcinomas and none of

**Table 2.** *Kras2* Mutation and *RASSF1A* Methylation in Human Lung and Colon Cancers.

| Genetic Changes | Lung Adenocarcinomas and Large Cell Carcinomas* | Stage I Lung Adenocarcinomas† | Colon Cancer‡ |
|---|---|---|---|
| *Kras2* mutation and *RASSF1A* methylation | 3 (7%) | 2 (2%) | 11 (5%) |
| *Kras2* mutation only | 10 (22%) | 11 (10%) | 76 (34%) |
| *RASSF1A* methylation only | 18 (40%) | 33 (30%) | 34 (15%) |
| Neither *Kras2* mutation or *RASSF1A* methylation | 14 (31%) | 64 (58%) | 101 (46%) |
| Total tumors examined | 45 | 110 | 222 |

*The present study.
†Tomizawa et al. [19].
‡Van Engeland et al. [22].

large and squamous cell carcinomas contains both a *Kras2* mutation and *RASSF1A* methylation. These data, together with previous study [19], indicated that the majority of lung adenocarcinomas and large cell carcinomas with *Kras2* mutations lack *RASSF1A* promoter methylation. Our results also showed that 24% adenocarcinomas, 50% large cell carcinomas, and 70% squamous cell carcinomas contain neither a *Kras2* mutation nor *RASSF1A* methylation. These data provide evidence that some other alternative pathways might be involved in human lung tumorigenesis other than *Kras2* mutation and *RASSF1A* hypermethylation.

As shown in Table 2, our results are highly consistent with two recently published studies. Van Engeland et al. [22] evaluated *Kras2* mutations and *RASSF1A* promoter methylation in colorectal cancer. They found that the majority of colorectal cancers with *Kras2* mutation lack *RASSF1A* promoter methylation. Another report by Tomizawa et al. [19] found that only 2% of lung adenocarcinomas with *RASSF1A* hypermethylation showed *Kras2*-activating mutation [19]. They showed that *RASSF1A* methylation occurs more frequently in poorly differentiated tumors than in well-differentiated tumors, suggesting that *RASSF1A* plays an important role in the progression of lung adenocarcinoma and that *RASSF1A* hypermethylation is a marker for prognosis of patients with stage I lung adenocarcinoma [19].

The Ras effector, RASSF1A, suppressed the tumorigenicity of lung cancer cells in a manner similar to wild-type Ras, suggesting that RASSF1A could be an effector of wild-type *Ras* genes, which were recently found to be potent tumor suppressors [27,28]. Thus, loss of *RASSF1A* expression by methylation in human cancer would not require the event of Ras activation. Interestingly, *Kras2* mutations are found in less than 1% of SCLCs, whereas inactivation of *RASSF1A* is close to 80% or 100% by hypermethylation and loss of heterozygosity (LOH) [9]. Therefore, these results raise the possibility that the wild-type *Ras–RASSF1* signaling pathway suppresses lung cancer cell proliferation and malignant transformation, and loss of this controlling function by either *Kras2* point mutation/LOH or *RASSF1A* promoter methylation/LOH may result in tumorigenesis. Because *RASSF1A* has the identical *RAS* associate domain as *RASSF1C*, *RASSF1C* likely binds *RA*S in a GTP-dependent manner. Therefore, it is possible that *RASSF1A* shares the same binding manner as *RASSF1C* [15]. Feig and Buchsbaum [16] and Khokhlatchev et al. [17] suggested that Ras could both promote apoptosis through its association with a Nore1–*RASSF1*–Mst1 complex and suppress apoptosis through its activation of PI3 kinase.

Inverse correlation between tumor suppressor genes involved in the same signaling pathways has been previously noted (e.g., inactivation of both p16 and Rb is rarely observed in the same tumor). The identification of *RASSF1A* as a tumor suppressor gene and a downstream effector of Ras in a proapoptotic pathway suggests that Ras can promote both apoptosis and cell survival (by the previously described PI3-K pathway). With this information on the correlation between genetic alterations of *RASSF1A* and *Kras2* during tumorigenesis, future studies should examine the ability of *RASSF1A* to function as a tumor suppressor gene in experimental lung tumorigenesis and the mechanism through which this novel tumor suppressor gene mediates its effect.

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