Value of $p16^{INK4a}$ and RASSF1A Promoter Hypermethylation in Prognosis of Patients with Resectable Non–Small Cell Lung Cancer

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ABSTRACT

The $p16^{INK4a}$ and RASSF1A are tumor suppressor genes frequently inactivated by de novo promoter hypermethylation in non–small cell lung cancer. We studied 119 patients with non–small cell lung cancer (70 stage I/II and 49 stage IIIA) who had undergone surgery with curative intent. The $p16^{INK4a}$ and RASSF1A promoter methylation statuses were determined by methylation–specific PCR. Statistical analyses, all two–sided, were performed to determine the prognostic effect of hypermethylation on various clinical parameters. Hypermethylation of the $p16^{INK4a}$ and RASSF1A promoters was found in 58 (49%) and 46 (39%) tumors, respectively, and 30 tumors (25%) exhibited hypermethylation of both gene promoters. In patients with stage I/II tumors, only $p16^{INK4a}$ promoter hypermethylation was associated with a poor 5–year overall survival rate ($P = 0.002$). In patients with stage IIIA disease, however, RASSF1A promoter hypermethylation was a stronger predictor of a poor 5–year overall survival rate ($P < 0.0001$) than $p16^{INK4a}$ promoter hypermethylation. Among the 49 patients with stage IIIA tumors, 16 (89%) of the 18 patients whose tumors showed RASSF1A promoter hypermethylation died within 3 years after surgery, as compared with only 12 (39%) of the 31 patients whose tumors had no RASSF1A promoter hypermethylation ($P < 0.0001$). Multivariate analysis indicated that RASSF1A promoter hypermethylation was the stronger independent predictor for survival in patients with locally advanced non–small cell lung cancer. Our results indicate that $p16^{INK4a}$ promoter hypermethylation predicts a poor 5–year survival rates for patients with resectable non–small cell lung cancer, particularly for those with early stage tumors, whereas RASSF1A promoter hypermethylation is a profound prognostic predictor for patients with locally advanced non–small cell lung cancer, suggesting an important role of RASSF1A in non–small cell lung cancer progression.

INTRODUCTION

Non–small cell lung cancer constitutes 80% of all primary lung cancers, which are the leading cause of cancer–related death in both men and women in the United States (1). Despite advances in the treatment of the disease over the past two decades, the prognosis of patients with non–small cell lung cancer has improved only modestly, with the 5–year overall survival rate increasing from 11% in the 1970s to 15% in the late 1990s (2). Patients with early stage non–small cell lung cancer generally have a better survival than those with advanced–stage tumors. For example, patients with stage I non–small cell lung cancer are expected to have an approximate 60% 5–year overall survival rate after surgical resection of their primary tumors, whereas those with stage IIIA disease have an estimated 25% 5–year overall survival rate after surgery followed by radiation with or without chemotherapy.

Biological features of non–small cell lung cancer are determined by underlying molecular alterations of the tumors, including inactivation of the tumor suppressor genes (3–5). Besides mutations and deletions of genes, it is now clear that de novo promoter hypermethylation is a common mechanism to inactivate tumor suppressor genes (6–8). The $p16^{INK4a}$ tumor suppressor gene located on 9p21 encodes a cyclin–dependent kinase inhibitor important for G1 cell cycle arrest (9, 10). Promoter hypermethylation of the gene has been observed frequently early in lung carcinogenesis, including in individuals exposed to tobacco carcinogens but without evidence of cancer (11–13). The RASSF1A tumor suppressor gene is located at 3p21, a region frequently deleted in non–small cell lung cancer (14). Another common mechanism to inactivate RASSF1A is promoter hypermethylation of the gene (15–17). RASSF1A has been shown to bind to the Ras–GTP binding protein Nore1, consistent with its role as a negative effector of Ras oncoprotein (18). In contrast to $p16^{INK4a}$, which is inactivated early in lung carcinogenesis (13, 19), hypermethylation of the RASSF1A promoter occurs relatively late (20), suggesting RASSF1A might be important in non–small cell lung cancer progression.

Because of the difference in timing between methylation of the $p16^{INK4a}$ and RASSF1A promoters in lung carcinogenesis, we wanted to determine the clinical impact of these abnormal-
ities alone or in combination on patients with non-small cell lung cancer. We studied the primary tumors from 119 patients with surgically resectable, stage I-IIIA non-small cell lung cancer for their \( p16^{INK4a} \) and RASSF1A promoter methylation status for associations between the methylation status and clinical parameters.

**MATERIALS AND METHODS**

**Study Population.** One hundred nineteen patients who were diagnosed with pathological stage I to IIIA non-small cell lung cancer and had undergone lobectomy or pneumonectomy for complete resection of their primary tumors at The University of Texas M. D. Anderson Cancer Center between 1994 and 2001 were included in the study. The selection of these patients was based on available fresh tumor tissues and corresponding normal lung tissues. The clinical information and follow-up data were based on chart review and on reports from our Tumor Registry Medical Informatics. Informed consent for the use of residual resected tissues for research was obtained from all of the patients in the study. The study was reviewed and approved by the Surveillance Committee of the institution. None of the patients with stage I or stage II disease received adjuvant chemotherapy or radiotherapy before or after surgery. Among 49 patients with stage IIIA disease, 5 received preoperative chemotherapy or chemoradiotherapy, 20 received postoperative concurrent chemoradiotherapy, 17 received postoperative radiotherapy alone, 2 received postoperative chemotherapy alone, and 5 received no additional treatment.

**DNA Extraction and Methylation-Specific PCR.** Frozen tissues were homogenized, and genomic DNA was extracted by digestion of the homogenized tissues in a buffer containing 50 mmol/L Tris-HCl (pH 8.0), 1% SDS, and 0.5 mg/ml proteinase K at 42°C for 36 hours. The digested tissues were purified with phenyl-chloroform twice. DNA was then precipitated using the EtOH precipitation method and recovered in distilled DNase-free water.

For the methylation-specific PCR, 1 μg of genomic DNA from each tissue sample was used in the initial step of chemical modification. Briefly, DNA was denatured by NaOH and treated with sodium bisulfite (Sigma Chemical Co., St. Louis, MO). After purification with Wizard DNA purification resin (Promega Corp., Madison, WI), the DNA was treated again with NaOH. After precipitation, DNA was recovered in water replacing for DNA was used as blank controls. The hypermethylation status was determined by visualizing a 150-bp PCR product for the \( p16^{INK4a} \) promoter and a 169-bp PCR product for the RASSF1A promoter with the respective methylation-specific primer sets. All PCRs were repeated twice, and the results were reproducible.

**Statistical Analysis.** The \( \chi^2 \) test or Fisher’s exact test were used to test the association between categorical variables. The Cochran-Armitage trend test was used to test the trend of methylation among differentiation levels. Overall survival, disease-specific survival (i.e., survival rates among people who died of lung cancer-related causes specifically), and disease-free survival (i.e., recurrence, metastasis, or cancer death was considered an event) were analyzed. Survival probability was estimated using the Kaplan-Meier method. The log-rank test was used to compare survival times among groups. Cox regression was used to model the risks of \( p16^{INK4a} \) and/or RASSF1A promoter hypermethylation on survival time, with adjustment for clinical and histopathologic parameters (age, sex, tumor histology, tumor size, smoking status, and adjuvant treatment). All statistical tests were two-sided, and \( P < 0.05 \) was considered statistically significant.

**RESULTS**

Clinical characteristics of all patients enrolled in the study are summarized in Table 1. At the censor date of November 14, 2003, the median follow-up period was 51 months (range, 16 to 130 months). Of the 70 patients with stage I or stage II disease, 29 (41%) were still alive, 35 (50%) died of lung cancer, and 6 (9%) died of unrelated causes. No significant difference in 5-year overall, disease-specific, and disease-free survival rates were observed by tumor stage, gender, smoking status, differentiation status, and histologic subtype in this patient group. Among the 49 patients with stage IIIA disease, 18 (37%) were still alive, 29 (59%) died of lung cancer, and 2 (4%) died of unrelated causes. In this group of patients, smokers had significantly poorer 5-year survival rates than the nonsmokers did (\( P = 0.047, P = 0.03, \) and \( P = 0.03 \) for 5-year overall, disease-specific, and disease-free survival rates, respectively). Thirty-five (71%) of the 49 patients received postoperative radiotherapy with (26 patients, including 5 with preoperative chemotherapy) or without (9 patients) concomitant chemotheraphy, whereas 14 patients received no adjuvant therapy after surgery.

Promoter methylation was detected in 58 (49%) and 46 (39%) of the tumor tissue for the \( p16^{INK4a} \) and RASSF1A promoters, respectively, compared with 13 (11%) including 4
samples whose corresponding tumors lacked methylation of the p16\textsuperscript{INK4a} promoter and 4 (3\%) in the corresponding normal-appearing lung tissues (\(P<0.0001\)). Unmethylated promoters of p16\textsuperscript{INK4a} and RASSF1A were detected in all of the normal-appearing lung tissues and in 60\% of tumor tissues, most likely because of the presence of normal cells in the tumor samples. Examples of methylation-specific PCR results are shown in Fig. 1. The undetectable unmethylated promoter in some of the tumors might be because of highly enriched tumor cells in the tissues or the relatively low sensitivity of our assay to pick up small quantities of unmethylated molecules. In patients with stage I or stage II non-small cell lung cancer, tumors with methylation of the p16\textsuperscript{INK4a} promoter had a higher frequency of RASSF1A promoter methylation than those without p16\textsuperscript{INK4a} promoter methylation, 58\% versus 24\% (\(P=0.005\)), suggesting that RASSF1A promoter methylation tends to occur in tumors with p16\textsuperscript{INK4a} promoter methylation because RASSF1A promoter methylation occurs late in lung carcinogenesis (20).\(^4\) However, this association was not significant in tumors from patients with stage IIIA disease (44\% versus 29\%; \(P=0.28\)). Altogether, 30 tumors (25\%; 19 stage I/II stage IIIA) showed concomitant methylation of both p16\textsuperscript{INK4a} and RASSF1A promoters.

We analyzed the potential association between the methylation status of p16\textsuperscript{INK4a} and RASSF1A promoters and sex, age, smoking history, histology, differentiation, and tumor stage. RASSF1A promoter methylation was more frequently observed in poorly differentiated tumors (50\%) than in moderately differentiated (26\%) or in well-differentiated tumors (0\%; \(P=0.04\)) from patients with stage IIIA non-small cell lung cancer, but there was no such association in tumors from patients with stage IIIA non-small cell lung cancer, but there was no such association in tumors from patients with stage II non-small cell lung cancer.

We analyzed the potential association between the methylation status of p16\textsuperscript{INK4a} and RASSF1A promoters and sex, age, smoking history, histology, differentiation, and tumor stage. RASSF1A promoter methylation was more frequently observed in poorly differentiated tumors (50\%) than in moderately differentiated (26\%) or in well-differentiated tumors (0\%; \(P=0.04\)) from patients with stage IIIA non-small cell lung cancer, but there was no such association in tumors from patients with stage II non-small cell lung cancer.

<table>
<thead>
<tr>
<th>Characteristics of patients and tumors</th>
<th>p16\textsuperscript{INK4a} promoter methylation</th>
<th>RASSF1A promoter methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Patients</td>
<td>58 (49%)</td>
<td>61 (51%)</td>
</tr>
<tr>
<td>Gender(^*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>23 (49%)</td>
<td>24 (51%)</td>
</tr>
<tr>
<td>Male</td>
<td>38 (53%)</td>
<td>34 (47%)</td>
</tr>
<tr>
<td>Mean age (±SD) (y)</td>
<td>65.0 ± 10.9</td>
<td>63.5 ± 9.1</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmoker</td>
<td>22 (58%)</td>
<td>16 (42%)</td>
</tr>
<tr>
<td>Smoker</td>
<td>39 (48%)</td>
<td>42 (52%)</td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>34 (57%)</td>
<td>26 (43%)</td>
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<tr>
<td>Squamous cell carcinoma</td>
<td>23 (47%)</td>
<td>26 (53%)</td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>2 (29%)</td>
<td>5 (71%)</td>
</tr>
<tr>
<td>Other</td>
<td>2 (67%)</td>
<td>1 (33%)</td>
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<tr>
<td>Differentiation(^†)</td>
<td></td>
<td></td>
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<tr>
<td>Well</td>
<td>5 (45%)</td>
<td>6 (55%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>25 (50%)</td>
<td>25 (50%)</td>
</tr>
<tr>
<td>Poor</td>
<td>31 (53%)</td>
<td>27 (47%)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I and II</td>
<td>37 (53%)</td>
<td>33 (47%)</td>
</tr>
<tr>
<td>IIIA</td>
<td>24 (49%)</td>
<td>25 (51%)</td>
</tr>
<tr>
<td>Stage I and II, 5-y overall survival</td>
<td>61.7%</td>
<td>28.3%</td>
</tr>
<tr>
<td>Stage III, 5-y overall survival</td>
<td>53.5%</td>
<td>10.8%</td>
</tr>
</tbody>
</table>

\(^*\) Subset analysis indicated that male patients had a higher rate of RASSF1A promoter methylation than females in stage IIIa group (\(P=0.03\)).

\(^†\) Subset analysis indicated that poorly differentiated tumors had a higher rate of RASSF1A promoter methylation than well or moderately differentiated tumors in stage IIIa group (\(P=0.04\)).

\(^4\) L. Mao, unpublished data.

![Fig. 1 Examples of promoter methylation status measured using methylation-specific PCR. A, PCR products of methylated or unmethylated p16\textsuperscript{INK4a} promoter from primary non-small cell lung cancer and corresponding normal lung tissues. B, PCR products of methylated or unmethylated RASSF1A promoter from primary non-small cell lung cancer and corresponding normal lung tissues. Molecular weight markers are listed on left side. (Neg, negative controls using unmethylated DNA; Pos, positive controls using methylated DNA and methylation-specific primer sets; T, primary tumors; N, corresponding normal lung tissues; U, unmethylated promoter; M, methylated promoter.)
stage I/II disease ($P = 0.48$). Additionally, tumors from male patients with stage IIIA non-small cell lung cancer exhibited a significantly higher frequency of RASSF1A promoter methylation (47%) than did those from female patients (13%; $P = 0.03$).

We then analyzed the effect of \( p16^{\text{INK4a}} \) and RASSF1A promoter methylation on the survival of the patient. Because stage IIIA patients often received adjuvant treatment after surgery, whereas stage I/II patients received only surgery, we analyzed the two groups separately. In the stage I/II group, patients whose tumors contained \( p16^{\text{INK4a}} \) promoter methylation had significantly poorer 5-year overall, disease-specific, and disease-free survival rates ($P = 0.002$, $P = 0.0005$, and $P = 0.0006$, respectively) than did patients whose tumors had no \( p16^{\text{INK4a}} \) promoter methylation (Fig. 2, A–C). However, the association between the RASSF1A promoter methylation status and 5-year survival rates was not statistically significant ($P = 0.09$, $P = 0.07$, and $P = 0.07$, respectively; Fig. 2, D–F). Multivariate analysis, including clinical parameters and promoter methylation status, indicated that \( p16^{\text{INK4a}} \) promoter methylation was the only independent predictor of 5-year overall, disease-specific, and disease-free survivals. In patients with stage IIIA disease, in contrast to those with stage I/II tumors, the RASSF1A promoter methylation status was strongly associated with 5-year overall, disease-free, and disease-specific survival ($P < 0.0001$, $P < 0.0001$, and $P = 0.0006$, respectively; Fig. 3, A–C), as was the \( p16^{\text{INK4a}} \) promoter methylation status ($P = 0.003$, $P = 0.002$, and $P = 0.01$, respectively; Fig. 3, D–F). Although both RASSF1A and \( p16^{\text{INK4a}} \) promoter methylation status were independent predictors of survival, RASSF1A was a stronger predictor for 5-year overall, disease-specific, and disease-free survival (hazard ratio = 4.76, $P < 0.0001$; hazard ratio = 6.29, $P < 0.0001$; and hazard ratio = 3.41, $P = 0.0007$, versus hazard ratio = 2.89, $P = 0.007$; hazard ratio = 3.16, $P = 0.005$, and hazard ratio = 2.36, $P = 0.02$, respectively).

To determine whether RASSF1A inactivation might have an added biological value in patients whose tumors also carried \( p16^{\text{INK4a}} \) promoter methylation, we analyzed the 5-year survival rates of the group whose tumors had methylation of both gene promoters. In patients with stage I/II disease, the 5-year survival rates of patients whose tumors had methylation of both promot-

![](image-url)

**Fig. 2** Association between the \( p16^{\text{INK4a}} \) promoter methylation status (A–C) or RASSF1A promoter methylation status (D–F) and overall, disease-specific, and disease-free survival in patients with stage I/II non–small cell lung cancer. (0, groups without methylation of promoter; 1, groups with methylation of promoter; \( E/N \), number of events/total number in each group.)
ers were significantly worse than in patients whose tumors had no promoter methylation or methylation of only one promoter ($P = 0.01$, $P = 0.005$, and $P = 0.005$, respectively, for 5-year overall, disease-specific, and disease-free survival rates; Fig. 4, A–C). Although the number of patients was small in the stage IIIA group, the association between patients whose tumors had methylation of both promoters and poor survivals was striking (Fig. 4, D–F). All 11 patients (100%) in this category died of lung cancer within 3 years after surgery, whereas 13 (62%) of the 21 stage IIIA patients whose tumors had methylation of only one promoter died of lung cancer in 5 years, and only 5 (29%) of the 17 patients whose tumors had no promoter methylation died of lung cancer in 6.5 years ($P < 0.0001$ by log-rank test; Fig. 4F).

Because 35 (71%) of the 49 patients with stage IIIA tumors received postoperative radiotherapy and 26 (53%) of the patients received adjuvant chemotherapy, we wanted to determine whether these treatments had affected the predictive value of the methylation markers. Despite the small sample size, RASSF1A promoter methylation status remained a predictor of overall survival in radiotherapy and nonradiotherapy groups ($P = 0.0004$ and $P = 0.008$, respectively, for overall survival) as well as in chemotherapy and nonchemotherapy groups ($P = 0.001$ and $P = 0.01$, respectively, for overall survival).

**DISCUSSION**

The $p16^{INK4a}$ is frequently inactivated in non-small cell lung cancer through various mechanisms, including promoter hypermethylation (8, 11, 12, 21), but not in small cell lung cancers, which often have an inactivated retinoblastoma tumor suppressor gene. The reported frequencies of $p16^{INK4a}$ promoter hypermethylation in primary non-small cell lung cancer have been 25 to 63% (12, 22–24). The $p16^{INK4a}$ promoter hypermethylation is an early event in lung carcinogenesis even in bronchial epithelial cells chronically exposed to tobacco carcinogens (13). RASSF1A contains an RAS-associated domain, which interacts with the RAS oncprotein to promote cellular apoptosis as well as to inhibit cyclin D1 accumulation (25, 26), and a putative ataxia telangiectasia, mutated kinase phosphoryl-
ation consensus site, which links RASSF1A to DNA-damage response (27). RASSF1A has been found to directly bind and stabilize microtubule structures, suggesting a role of the protein in maintaining genome stability (28). It was shown in a recent study that RASSF1A regulates mitosis by inhibiting the APC-cdc20 complex (29). Although mutation of RASSF1A is rarely found in non-small cell lung cancer, the gene is located at 3p21, a region frequently deleted in non-small cell lung cancer, and its promoter is frequently (30 to 38%) hypermethylated in primary non-small cell lung cancer (17, 30, 31). In contrast with p16INK4a, hypermethylation of the RASSF1A promoter is rarely detected in bronchial epithelial cells chronically exposed to tobacco carcinogens (18), suggesting this is a late event in carcinogenesis. In this study, the much lower frequency (3% versus 11%) of RASSF1A promoter hypermethylation compared with p16INK4a promoter hypermethylation detected in the adjacent normal-appearing lung tissues from patients with non-small cell lung cancer supports this notion.

In patients with early stage non-small cell lung cancer, p16INK4a promoter methylation was a predictor of the clinical outcome of the patient (Fig. 2, A–C), which is consistent with earlier reports analyzing either p16INK4a promoter methylation or p16INK4a protein expression and clinical outcome in patients with early stage non-small cell lung cancer (11, 32, 33). Two previous reports showed that patients with stage I/II non-small cell lung cancer that contained RASSF1A promoter methylation associated with adverse survival (31, 34). Although the association was not statistically significant in this study, patients whose stage I/II tumors carried RASSF1A promoter methylation had poorer 5-year survival rates (Fig. 2, D–F).

One of the interesting findings in our study was that patients with stage IIIA disease whose tumors carried RASSF1A promoter methylation had extremely poor 5-year survival rates compared with those without the abnormality. This was in contrast to the findings in patients with stage I/II disease (Figs. 2 and 3). Interestingly, in the locally advanced tumors, p16INK4a
promoter methylation and RASSF1A promoter methylation were not associated ($P = 0.28$; Table 1), again in contrast with data for stage I/II tumors. These results suggest that inactivation of RASSF1A together with additional molecular alterations that occur between the early stage and locally advanced stage of non-small cell lung cancer renders the tumors extremely aggressive. It should be noted that RASSF1A methylation was not associated with survival in patients with stage III non-small cell lung cancer in a previous report (34). But that study had a smaller sample size and lower rate of methylation frequency compared with stage I/II tumors (34% versus 54%) in the same study (34). Additional studies are therefore needed to validate our findings.

The finding that patients with methylation of both gene promoters had a poorer 5-year overall, disease-specific, and disease-free survival rate than did those with only $p16^{INK4a}$ or RASSF1A promoter methylation (Fig. 4, A–C) suggests that the inactivation of RASSF1A might make the tumor cells more aggressive. This dose-dependent correlation between the methylation status and survivals among patients with no methylation in any of the two promoters, with methylation in only one promoter, and with methylation of both promoters was even more profound (Fig. 4, D–F). If validated, these epigenetic abnormalities may be useful biomarkers for molecular classification of patients with stage IIIA non-small cell lung cancer as well as reasonable therapeutic targets.

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REFERENCES

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