

ORIGINAL ARTICLE

DNA Methylation Markers and Early Recurrence in Stage I Lung Cancer

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ABSTRACT

BACKGROUND

Despite optimal and early surgical treatment of non–small-cell lung cancer (NSCLC), many patients die of recurrent NSCLC. We investigated the association between gene methylation and recurrence of the tumor.

METHODS

Fifty-one patients with stage I NSCLC who underwent curative resection but who had a recurrence within 40 months after resection (case patients) were matched on the basis of age, NSCLC stage, sex, and date of surgery to 116 patients with stage I NSCLC who underwent curative resection but who did not have a recurrence within 40 months after resection (controls). We investigated whether the methylation of seven genes in tumor and lymph nodes was associated with tumor recurrence.

RESULTS

In a multivariate model, promoter methylation of the cyclin-dependent kinase inhibitor 2A gene *p16*, the H-cadherin gene *CDH13*, the Ras association domain family 1 gene *RASSF1A*, and the adenomatous polyposis coli gene *APC* in tumors and in histologically tumor-negative lymph nodes was associated with tumor recurrence, independently of NSCLC stage, age, sex, race, smoking history, and histologic characteristics of the tumor. Methylation of the promoter regions of *p16* and *CDH13* in both tumor and mediastinal lymph nodes was associated with an odds ratio of recurrent cancer of 15.50 in the original cohort and an odds ratio of 25.25 when the original cohort was combined with an independent validation cohort of 20 patients with stage I NSCLC.

CONCLUSIONS

Methylation of the promoter region of the four genes in patients with stage I NSCLC treated with curative intent by means of surgery is associated with early recurrence.

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SURGERY WITH CURATIVE INTENT IS THE standard of care for patients with stage I non-small-cell lung cancer (NSCLC), yet notwithstanding advances in treatment, the dissemination of tumor cells outside the area of curative resection is a leading cause of relapse.¹⁻³ Despite surgery, approximately 30 to 40% of patients with NSCLC who have discrete lesions and histologically negative lymph nodes (stage I cancer; T1-2N0, according to the tumor-node-metastasis [TNM] classification criteria) die of recurrent disease.⁴⁻⁶ Many of these recurrences are systemic, making it likely that such patients had occult micrometastases beyond the margins of surgical resection.⁴

Epigenetic gene silencing is a molecular mechanism of silencing a gene by methylating its promoter region. Epigenetic silencing is involved in the initiation and progression of several types of cancer, including lung cancer.^{7,8} The detection of epigenetic alterations with the use of a method like the methylation-specific polymerase-chain-reaction (PCR) assay⁹ may allow for the molecular staging of cancer. With the methylation-specific PCR assay, relatively few genes are required to analyze each type of cancer.⁹ The method can detect occult micrometastases in lymph nodes from patients with esophageal,¹⁰ colorectal,¹¹ gastric,¹⁰ prostate,¹² or lung cancer,^{10,13} but its value for predicting the recurrence of early-stage, resected NSCLC has not been examined.

We designed a nested case-control study of early-stage NSCLC (T1-2N0) to test the association between clinical outcome and the DNA methylation status of tumor, regional lymph nodes confined to the pleural space, and mediastinal lymph nodes. We studied seven genes: the cyclin-dependent kinase inhibitor 2A gene *p16*, the H-cadherin gene *CDH13*, the adenomatous polyposis coli gene *APC*, the *Ras* association domain family 1 gene *RASSF1A*, the O⁶-methylguanine-DNA methyltransferase gene *MGMT*, the PYD and CARD domain-containing gene *ASC*, and the death-associated protein kinase 1 gene *DAPK*. These genes are thought to be important in the biologic development of lung cancer and are frequently methylated in lung cancer.¹⁴⁻¹⁸ We hypothesized that the methylation-specific PCR assay could be used to define patterns of DNA methylation that can delineate the behavior of the primary tumor and to detect micrometastases in histologically negative lymph nodes. Our results show that aberrant pat-

terns of promoter methylation in the primary tumor, and in regional and mediastinal lymph nodes, can be used to identify patients with stage I NSCLC who have an increased risk of recurrence.

METHODS

PATIENTS

Evidence of recurrent disease was evaluated in 715 patients with pathologically verified stage I (T1-2N0) cancer who received a diagnosis of NSCLC (codes 162.3 to 162.9 of the *International Classification of Diseases, Ninth Revision, Clinical Modification*) and who underwent lobectomy or greater resections at the Johns Hopkins Hospital between January 1, 1986, and July 31, 2002. The case patients were 71 patients at our institution in whom the tumor recurred within 40 months after surgery, by which time approximately 80% of NSCLC recurrences occur.⁴ Follow-up of all 71 case patients was performed at Johns Hopkins Hospital with the use of radiographic imaging and, usually, histologic verification of recurrence. On the basis of age, NSCLC stage, date of surgery (± 5 years), and sex, we matched the case patients to 158 controls with stage I NSCLC in whom there was no recurrence during the 40-month follow-up period. In this phase of the study, tissue samples from 51 of the 71 case patients and 116 of the 158 matched controls were available for methylation analysis. Seven of the 116 controls had a recurrence more than 40 months after surgery. Neither case patients nor controls received adjuvant chemotherapy; between 1986 and 2002, guidelines did not recommend adjuvant therapy for patients with stage IB NSCLC.^{19,20}

All cases of cancer were staged according to the revised TNM classification criteria,⁵ including the histologic status of mediastinal lymph nodes sampled from levels II, IV, VII, VIII, IX, and X on the right side and levels V, VI, VII, VIII, and IX on the left side. Regional lymph nodes confined to the pleural space were resected en bloc with the tumor. Samples from case patients had no macroscopically or microscopically positive surgical margins, and the patients had had a lobectomy or greater resection.²¹ We also evaluated 162 paraffin-embedded tissue blocks from the 20 patients in the validation cohort (11 case patients and 9 matched controls). Of these 20 patients, 18 had undergone resection at our institution after

August 2002. This study was approved by the Johns Hopkins Institutional Review Board. The requirement of written informed consent was waived.

PREPARATION OF TUMOR AND LYMPH-NODE SPECIMENS

All specimen blocks were procured from pathology archives en masse in a blinded fashion with regard to whether they were obtained from case patients or controls. There were no differences in the distribution of tumor and lymph nodes or number of samples per patient between the case patients and controls. Specimens were labeled with study-specific coded identifiers only; laboratory investigators had no knowledge of the patient's group or the source tissue of the DNA. DNA was extracted from a pool of three sequential sections, each 10 μm in thickness, from unstained, paraffin-embedded slides of resected tumors, regional lymph nodes, or mediastinal lymph nodes. For each sample of tumor or lymph-node tissue, adjacent sections were stained with hematoxylin and eosin for histologic confirmation of the presence or absence of cancer. Unstained tissue sections were deparaffinized, and DNA was extracted as described previously.²² The concentration of DNA was measured spectrophotometrically, and 1 μg of DNA was denatured with the use of sodium hydroxide and modified with the use of sodium bisulfite. We then purified the DNA samples by using Wizard DNA purification resin (Promega), treated them again with sodium hydroxide, precipitated them with ethanol, and resuspended them in water.

METHYLATION-SPECIFIC PCR ASSAY

DNA methylation was evaluated with the use of the methylation-specific PCR assay, performed by three persons working independently. Each of them extracted DNA and performed all steps of the assay separately.²³ A combined total of 889 samples of tumor and lymph-node tissues were examined. A multiplex-nested methylation-specific PCR assay was used for all samples, as described previously.²⁴ The nested method initially amplifies bisulfite-modified DNA with the use of flanking PCR primers, without preferentially amplifying methylated or unmethylated DNA. The resulting fragment is then used as the template for the methylation-specific PCR assay. Primer sequences and PCR conditions for *p16*, *MGMT*, *DAPK*, *RASSF1A*, *CDH13*, *ASC*,

and *APC* have been described previously,^{15,17,24-26} including conditions optimized to achieve specific detection of methylation in tumor tissue but not in normal lymphocytes (Table 1 in the Supplementary Appendix, available with the full text of this article at www.nejm.org).^{15,17,24-26} Placental DNA treated with *SssI* methyltransferase (New England Biolabs) was used as a positive standard. DNA from normal lymphocytes and water (bisulfite-modified water and unmodified water) were used as negative standards. PCR products were separated on 2% agarose gel or 6% nondenaturing polyacrylamide gel and were visually scored as methylated or unmethylated according to the presence or absence of a PCR product, respectively (Fig. 1 in the Supplementary Appendix).^{23,25,27} If any tumor block or lymph-node specimen was positive for methylation, all of the primary tumor or all associated lymph nodes in that nodal basin, respectively, were scored as positive.

STATISTICAL ANALYSIS

We verified histologic results and deaths or recurrent disease during the follow-up period by reexamining the original hospital records. The primary end point was time to recurrent local or distant disease, measured from the date of surgery to the time of cancer-related death or censoring. Data for controls who were alive and had no evidence of disease at the end of the study were censored for recurrence or death. All deaths of case patients were cancer-related, and no controls were lost to follow-up. Associations among prognostic factors, presence or absence of recurrence, and patient group were assessed by means of univariate and multivariate logistic-regression analysis. The association of risk factors with time-to-event or time-to-censoring end points was analyzed with the use of the log-rank test. Results of all models are reported as odds ratios with 95% confidence intervals. All statistical calculations were performed with the use of Stata statistical software. Two-sided P values of less than 0.05 were considered to indicate statistical significance.

We hypothesized that 40% or more of the case patients would have microscopic disease in the resected lymph nodes, as compared with 20% or less of controls, yielding an odds ratio of 2 for case patients. Under these assumptions, the study would have a statistical power of 80% to detect a

Table 1. Baseline Characteristics of the 187 Patients.*

Characteristic	Original Cohort (N=167)		Validation Cohort (N=20)
	Case Patients (N=51)	Controls (N=116)	
Age — yr			
Median	64	67	66
Interquartile range	58–71	60–72	57–72
Sex — no. (%)			
Male	24 (47.1)	54 (46.6)	8 (40.0)
Female	27 (52.9)	62 (53.4)	12 (60.0)
Race — no. (%)†			
White	43 (84.3)	96 (82.8)	15 (75.0)
Black	6 (11.8)	19 (16.4)	5 (25.0)
Other	2 (3.9)	1 (0.9)	0
Stage — no. (%)			
IA (T1N0)	26 (51.0)	75 (64.7)	9 (45.0)
IB (T2N0)	25 (49.0)	41 (35.3)	11 (55.0)
Tumor diameter — no. (%)			
≤3 cm	25 (49.0)	72 (62.1)	13 (65.0)
>3 cm	26 (51.0)	44 (37.9)	7 (35.0)
Surgical procedure — no. (%)			
Lobectomy	46 (90.2)	95 (81.9)	20 (100)
Pneumonectomy or bilobectomy	4 (7.8)	4 (3.4)	0
Sublobar resection	1 (2.0)	17 (14.7)	0
Histologic characteristics — no. (%)‡			
Adenocarcinoma	30 (58.8)	62 (53.4)	15 (75.0)
Squamous-cell	15 (29.4)	42 (36.2)	4 (20.0)
Other	6 (11.8)	12 (10.3)	1 (5.0)
Median ASA physical-status score§	3	3	3
Smoking status — no. (%)			
Current or former smoker	43 (84.3)	102 (87.9)	20 (100)
Nonsmoker	8 (15.7)	12 (10.3)	0
Unknown	0	2 (1.7)	0

* The case patients were matched with the controls on the basis of age, sex, NSCLC stage, and date of surgery (± 5 years). Because of rounding, percentages may not total 100.

† Race was self-reported.

‡ “Adenocarcinoma” includes bronchioloalveolar carcinoma and adenosquamous histologic features. “Other” includes large-cell, basaloid, and mucoepidermoid histologic features.

§ Physical status was graded according to the American Society of Anesthesiologists (ASA) Physical Status Classification System; scores range from 1 to 6, with higher scores indicating more severe disease. A score of 3 corresponds to a patient with severe systemic disease.

significant effect among 168 case patients and controls, matched in a two-to-one ratio.

The authors designed the study; gathered, interpreted, and held the data; wrote the paper; made

the decision to publish; and vouch for the completeness and accuracy of the data. There were no agreements concerning confidentiality of data between OncoMethylome Sciences and the authors.

Table 2. Prevalence of Gene Methylation in Tumor or Regional or Mediastinal Lymph Nodes in the Original Cohort, According to Gene.*

Methylated Gene	Tumor		P Value	Regional Lymph Nodes		P Value	Mediastinal Lymph Nodes		P Value
	Controls (N=104)	Case Patients (N=50)		Controls (N=82)	Case Patients (N=41)		Controls (N=56)	Case Patients (N=34)	
	percent		percent		percent				
<i>MGMT</i>	36.1	34.7	0.87	29.5	37.5	0.38	35.8	44.1	0.44
<i>ASC</i>	34.9	38.8	0.65	27.2	29.3	0.81	42.9	44.1	0.91
<i>DAPK</i>	35.3	36.0	0.93	41.5	42.5	0.91	30.8	39.4	0.41
<i>APC</i>	34.0	36.0	0.81	18.7	13.5	0.48	13.0	29.4	0.06
<i>RASSF1A</i>	35.9	50.0	0.10	16.5	12.8	0.61	9.6	20.6	0.15
<i>p16</i>	26.0	52.0	0.001	13.7	35.0	0.009	16.7	48.5	0.001
<i>CDH13</i>	22.8	38.8	0.04	19.5	32.5	0.12	25.0	46.9	0.04

* P values were calculated with the use of the chi-square test for homogeneity.

RESULTS

CHARACTERISTICS OF THE PATIENTS

Clinical and demographic variables were similar in case patients and controls (Table 1). On the basis of the American Society of Anesthesiologists Physical Status Classification System, case patients and controls were found to be equally fit for surgery. The most frequent site of recurrence was the ipsilateral lung (in 45.1% of patients), followed by metastasis to bone (13.7%), brain (11.7%), and mediastinum (11.7%). Although 14.7% of controls underwent sublobar resections, all pulmonary resections in these patients were done with curative intent.

RISK OF RECURRENCE ACCORDING TO CLINICAL PREDICTORS

The covariates of pathological stage, age, sex, histologic characteristics of the tumor, smoking status, and race were not associated with the risk of recurrence in patients with histologically negative lymph nodes (Table 2 in the Supplementary Appendix). Although pathological tumor stage showed the strongest association with recurrence, independently of other covariates, the association was not significant. For example, patients with stage IB disease (according to the 1986 classification of the American Joint Committee on Cancer²⁸) had an adjusted odds ratio for recurrence of 1.71 (95% confidence interval [CI], 0.86 to 3.41; $P=0.13$), as compared with patients with smaller tumors and no pleural invasion (stage IA disease) (Table 2 in the Supplementary Appendix).

GENE METHYLATION AND RECURRENCE

Methylation profiles of the seven genes were obtained from 727 of the 731 paraffin blocks, from 167 cases and controls. As compared with controls, the largest differences in the univariate distribution among case patients of the frequency of methylation in any type of tissue were found in four genes — *p16*, *CDH13*, *RASSF1A*, and *APC* — especially in tumors or mediastinal lymph nodes (Table 2). When *p16* or *CDH13* was methylated in the primary tumor, the adjusted odds ratio for recurrence was 3.50 (95% CI, 1.65 to 7.41; $P=0.001$) and 2.12 (95% CI, 0.98 to 4.59; $P=0.06$), respectively (Fig. 1 and Table 3). When these same genes were methylated in regional lymph nodes, the odds ratio was 3.62 (95% CI, 1.41 to 9.32; $P=0.008$) and 1.99 (95% CI, 0.81 to 4.88; $P=0.13$), respectively. If methylation of *p16* or *CDH13* was found in mediastinal lymph nodes, the odds of recurrence was 4.67 (95% CI, 1.53 to 14.42; $P=0.007$) and 3.98 (95% CI, 1.22 to 13.01; $P=0.02$), respectively (Fig. 1 and Table 3). Methylation of *RASSF1A* or *APC* in the tumor or mediastinal nodes was not significantly associated with recurrence (Fig. 1).

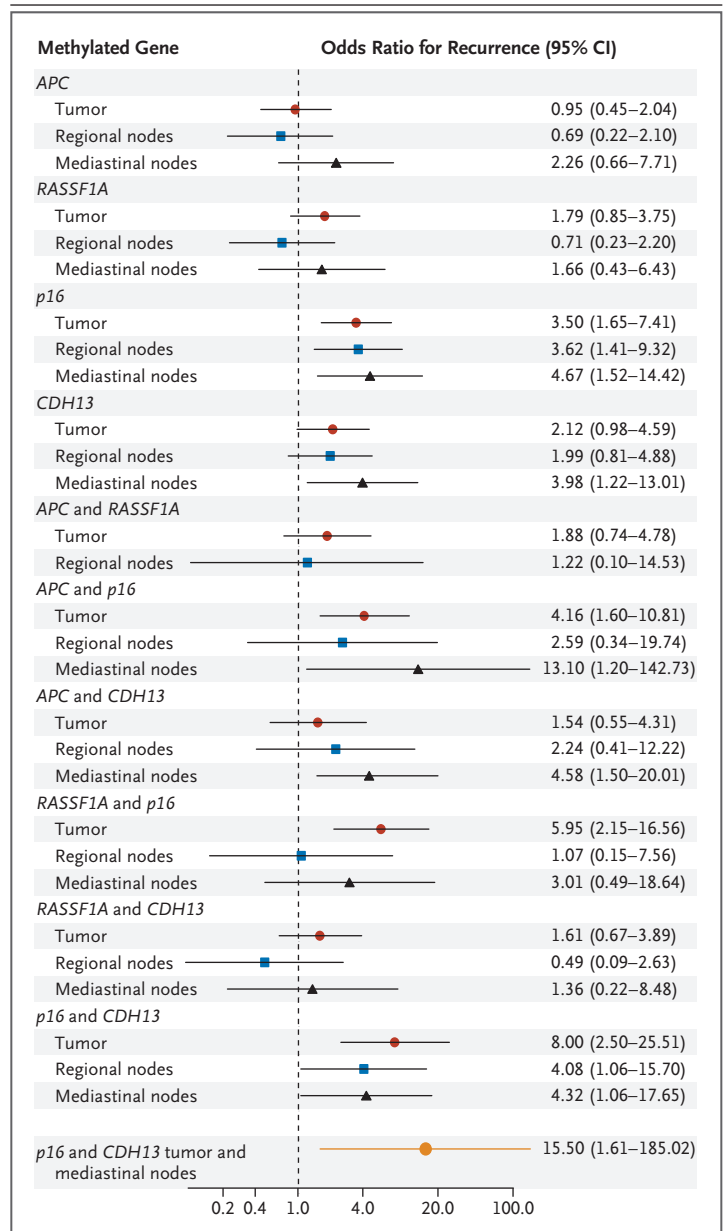
The six possible pairs of these four genes were examined for an association with recurrence. Among these pairs, four had a significant association with recurrence in at least one type of tissue: *p16* and *CDH13*, *CDH13* and *APC*, *APC* and *p16*, and *RASSF1A* and *p16* (Table 3 and Fig. 1). Methylation of the gene pair *p16* and *CDH13* in the primary tumor alone was associated with an odds ratio for recurrence of 8.00 (95% CI, 2.50 to 25.51; $P<0.001$) for the case patients as

Figure 1. Odds Ratios for Recurrence among Case Patients as Compared with Controls, According to Methylated Gene and Site.

Multivariate logistic-regression analysis was performed with the use of data for the four genes that had the largest univariate differences in distribution with regard to methylation: the adenomatous polyposis coli gene *APC*, the *Ras* association domain family 1 gene *RASSF1A*, the cyclin-dependent kinase inhibitor 2A gene *p16*, and the H-cadherin gene *CDH13*. The prognostic value of each gene was adjusted for stage (IA or IB), age, sex, race (white or black), histologic feature of the tumor (adenocarcinoma, squamous-cell, or other), and smoking status (current or former smoker or non-smoker) and then graphed as a forest plot. Among single genes, the methylation of either *p16* or *CDH13* was associated with the most significant odds ratios of recurrence, regardless of the type of tissue. Methylation of either *RASSF1A* or *APC* in the primary tumor or in mediastinal lymph nodes was associated with an elevation in the odds of recurrence that was not significant. Patients with methylation of both *p16* and *CDH13* in both tumor and mediastinal lymph nodes had a significantly higher odds of lung-cancer recurrence than those without methylation of this pair of genes ($P=0.03$).

compared with the controls; when methylation of the two genes was found in both the tumor and the mediastinal lymph nodes, the estimated odds ratio for recurrence was 15.50 (95% CI, 1.61 to 185.02; $P=0.03$) (Fig. 1 and Table 3). Methylation of *p16* and either *CDH13*, *RASSF1A*, or *APC* in paired tumor and mediastinal lymph-node samples from the 51 case patients was associated with early recurrence (median, 9 months; range, 5 to 30), whereas in the absence of methylation of these markers, the median time to recurrence was 25 months after surgery (range, 6 to 40; $P=0.04$).

We examined the methylation status of *p16*, *CDH13*, *APC*, and *RASSF1A* in an independent validation cohort of 20 patients (11 case patients and 9 controls) with stage I NSCLC (Table 1). In this cohort of limited size, we validated the gene pair in the original cohort with the highest odds ratio of recurrence among case patients, as compared with controls, when methylated in both tumor and lymph nodes — *p16* and *CDH13* — using univariate analysis (Table 3 in the Supplementary Appendix). Multivariate analyses of data in the combined original and validation cohorts showed that the estimated odds of recurrence associated with methylation of *p16* and *CDH13* in tumor and mediastinal lymph nodes was 25.25 (95% CI, 2.53 to



252.35; $P=0.006$) for case patients as compared with controls (Table 3).

KAPLAN–MEIER ESTIMATES

Kaplan–Meier plots indicated that methylation of one or more of four genes — *p16*, *CDH13*, *RASSF1A*, and *APC* — in any sample from the patient was related to the duration of recurrence-free survival (Fig. 2A through 2D). For example, the 5-year recurrence-free survival rates for no methylated genes, one to two methylated genes, and three to four methylated genes in the mediastinal lymph nodes

Table 3. Multivariate Odds Ratios and 95% Confidence Intervals for the Estimated Risk of Recurrence among Case Patients as Compared with Controls, According to the Methylation Status of Four Genes.*

Gene	Original Cohort (N=167)		Original and Validation Cohorts (N=187)	
	Odds Ratio (95% CI)	P Value	Odds Ratio (95% CI)	P Value
Unmethylated gene†	1.00		1.00	
Methylated APC				
Tumor	0.95 (0.45–2.04)	0.90	1.31 (0.67–2.58)	0.43
Regional nodes	0.69 (0.22–2.10)	0.51	0.78 (0.32–1.91)	0.59
Mediastinal nodes	2.26 (0.66–7.71)	0.19	1.87 (0.65–5.56)	0.25
Tumor and mediastinal nodes	2.37 (0.52–10.83)	0.27	2.00 (0.55–7.33)	0.30
Methylated RASSF1A				
Tumor	1.79 (0.85–3.75)	0.12	1.86 (0.94–3.68)	0.07
Regional nodes	0.71 (0.23–2.20)	0.55	0.82 (0.31–2.15)	0.68
Mediastinal nodes	1.66 (0.43–6.43)	0.46	2.13 (0.65–6.96)	0.21
Tumor and mediastinal nodes	0.66 (0.11–3.88)	0.65	0.97 (0.23–3.98)	0.96
Methylated p16				
Tumor	3.50 (1.65–7.41)	0.001	3.55 (1.77–7.13)	<0.001
Regional nodes	3.62 (1.41–9.32)	0.008	4.14 (1.81–9.49)	0.001
Mediastinal nodes	4.67 (1.53–14.42)	0.007	5.09 (1.96–13.18)	0.001
Tumor and mediastinal nodes	5.23 (1.33–20.46)	0.02	8.41 (2.42–29.20)	0.001
Methylated CDH13				
Tumor	2.12 (0.98–4.59)	0.06	2.33 (1.16–4.69)	0.02
Regional nodes	1.99 (0.81–4.88)	0.13	2.67 (1.20–5.93)	0.02
Mediastinal nodes	3.98 (1.22–13.01)	0.02	4.04 (1.53–13.63)	0.005
Tumor and mediastinal nodes	6.89 (1.36–34.87)	0.02	7.55 (1.99–28.60)	0.003
Methylated APC and RASSF1A				
Tumor	1.88 (0.74–4.78)	0.18	2.25 (1.02–5.00)	0.046
Regional nodes	1.22 (0.10–14.53)	0.88	2.34 (0.46–11.75)	0.30
Mediastinal nodes	—	—	3.49 (0.30–40.75)	0.32
Tumor and mediastinal nodes	—	—	2.37 (0.17–33.12)	0.52
Methylated APC and p16				
Tumor	4.16 (1.60–10.81)	0.004	4.48 (1.91–10.51)	0.001
Regional nodes	2.59 (0.34–19.74)	0.36	2.43 (0.59–9.94)	0.22
Mediastinal nodes	13.10 (1.20–142.73)	0.04	7.46 (1.35–41.20)	0.02
Tumor and mediastinal nodes	5.27 (0.38–73.57)	0.22	7.70 (0.70–84.88)	0.10
Methylated APC and CDH13				
Tumor	1.54 (0.55–4.31)	0.41	2.14 (0.94–4.91)	0.07
Regional nodes	2.24 (0.41–12.22)	0.35	3.30 (0.88–12.40)	0.08
Mediastinal nodes	4.58 (1.50–20.01)	0.04	3.13 (0.89–11.01)	0.08
Tumor and mediastinal nodes	11.79 (0.85–163.34)	0.07	9.48 (0.87–103.18)	0.07
Methylated RASSF1A and p16				
Tumor	5.95 (2.15–16.56)	0.001	5.26 (2.13–13.00)	<0.001
Regional nodes	1.07 (0.15–7.56)	0.94	1.92 (0.39–9.45)	0.42
Mediastinal nodes	3.01 (0.49–18.64)	0.24	4.60 (0.85–25.03)	0.08
Tumor and mediastinal nodes	2.91 (0.24–35.36)	0.40	3.67 (0.32–41.69)	0.29

Table 3. (Continued.)

Gene	Original Cohort (N=167)		Original and Validation Cohorts (N=187)	
	Odds Ratio (95% CI)	P Value	Odds Ratio (95% CI)	P Value
Methylated <i>RASSF1A</i> and <i>CDH13</i>				
Tumor	1.61 (0.67–3.89)	0.29	1.71 (0.78–3.74)	0.18
Regional nodes	0.49 (0.09–2.63)	0.41	0.88 (0.25–3.04)	0.84
Mediastinal nodes	1.36 (0.22–8.48)	0.75	1.91 (0.40–9.21)	0.42
Tumor and mediastinal nodes	2.49 (0.18–34.29)	0.50	3.51 (0.32–38.57)	0.30
Methylated <i>p16</i> and <i>CDH13</i>				
Tumor	8.00 (2.50–25.51)	<0.001	6.71 (2.50–18.00)	<0.001
Regional nodes	4.08 (1.06–15.70)	0.04	6.13 (1.99–18.89)	0.002
Mediastinal nodes	4.32 (1.06–17.65)	0.04	4.66 (1.53–14.16)	0.007
Tumor and mediastinal nodes	15.50 (1.61–185.02)	0.03	25.25 (2.53–252.35)	0.006

* Odds ratios are reported on the basis of the multivariate logistic-regression model adjusted for NSCLC stage (IA or IB), age, sex, race (white or black), histologic feature (adenocarcinoma, squamous-cell, or other), and smoking status (current or former smoker or nonsmoker). Dashes indicate that no methylation was found among samples from controls.

† This is the reference category.

were 77.3% (95% CI, 61.9 to 87.1), 51.4% (34.0 to 66.4), and 30.0% (7.1 to 57.8), respectively ($P<0.001$) (Fig. 2C). Among patients with stage I NSCLC, the 5-year recurrence-free survival rate in the group of patients with two or more of the four methylated genes in the primary tumor and mediastinal lymph nodes was 27.3% (95% CI, 6.5 to 53.9), as compared with 65.3% (53.1 to 75.0) in the group with fewer than two methylated genes at those sites ($P<0.001$) (Fig. 2D).

Methylation of both *p16* and *CDH13* in tumor and mediastinal lymph nodes was associated with a 5-year recurrence-free survival rate of 14.3% (95% CI, 0.7 to 46.5), as compared with 63.1% (95% CI, 50.2 to 73.5) in the absence of methylation of these genes ($P<0.001$) (Fig. 2H). This association between methylation of both *p16* and *CDH13* and survival was also found in the independent validation cohort of 20 case patients and controls (Fig. 3B).

In the validation cohort, the methylation of two or more of the four genes in tumors and mediastinal lymph nodes was associated with a lower 5-year rate of recurrence-free survival (16.7% of patients; 95% CI, 0.8 to 51.7) than that for fewer than two genes (53.8%; 95% CI, 24.8 to 76.0; $P=0.04$) (Fig. 3A). Similarly, methylation of *p16* and *CDH13* in tumors and mediastinal nodes resulted in a worse 5-year rate of recurrence-free survival (0.0%; 95% CI, 0.0 to 0.0) than if this pair of genes was unmethylated (53.3%; 95% CI, 26.3 to 74.4; $P<0.001$) (Fig. 3B).

In the original and validation cohorts combined (total, 187 patients), the methylation status of *p16* and *CDH13* was assayed in primary tumor and mediastinal lymph-node specimens from 91 patients (41 case patients and 50 controls). Of the 11 patients in whom methylation of the two genes was found in both tissue types, 10 had recurrence of the tumor within 30 months after resection, 9 within 17 months, and 8 within 12 months. Patients with two or more methylated genes in tumor and mediastinal nodes also had a worse 5-year rate of recurrence-free survival (23.5% of patients; 95% CI, 7.3 to 44.9) than those with fewer than two methylated genes (63.5%; 95% CI, 52.4 to 72.7; $P<0.001$) (Fig. 3C). When both *p16* and *CDH13* were methylated in the tumor and mediastinal nodes, there was a significantly lower rate of recurrence-free survival (9.1%; 95% CI, 0.5 to 33.3) than if *p16* and *CDH13* were unmethylated (61.2%; 95% CI, 49.7 to 70.9; $P<0.001$) (Fig. 3D).

DISCUSSION

Our study indicates that methylation of the promoter regions of certain genes in a resected NSCLC specimen is associated with recurrence of the tumor. A relation between gene methylation and tumor recurrence has been shown.^{29,30} The four genes of interest in our study are *p16*, *CDH13*, *APC*, and *RASSF1A*. They are involved in cell-cycle control (*p16*), invasion and metastasis (*CDH13*, *APC*), and *Ras* signaling (*RASSF1A*). Other studies of *p16*

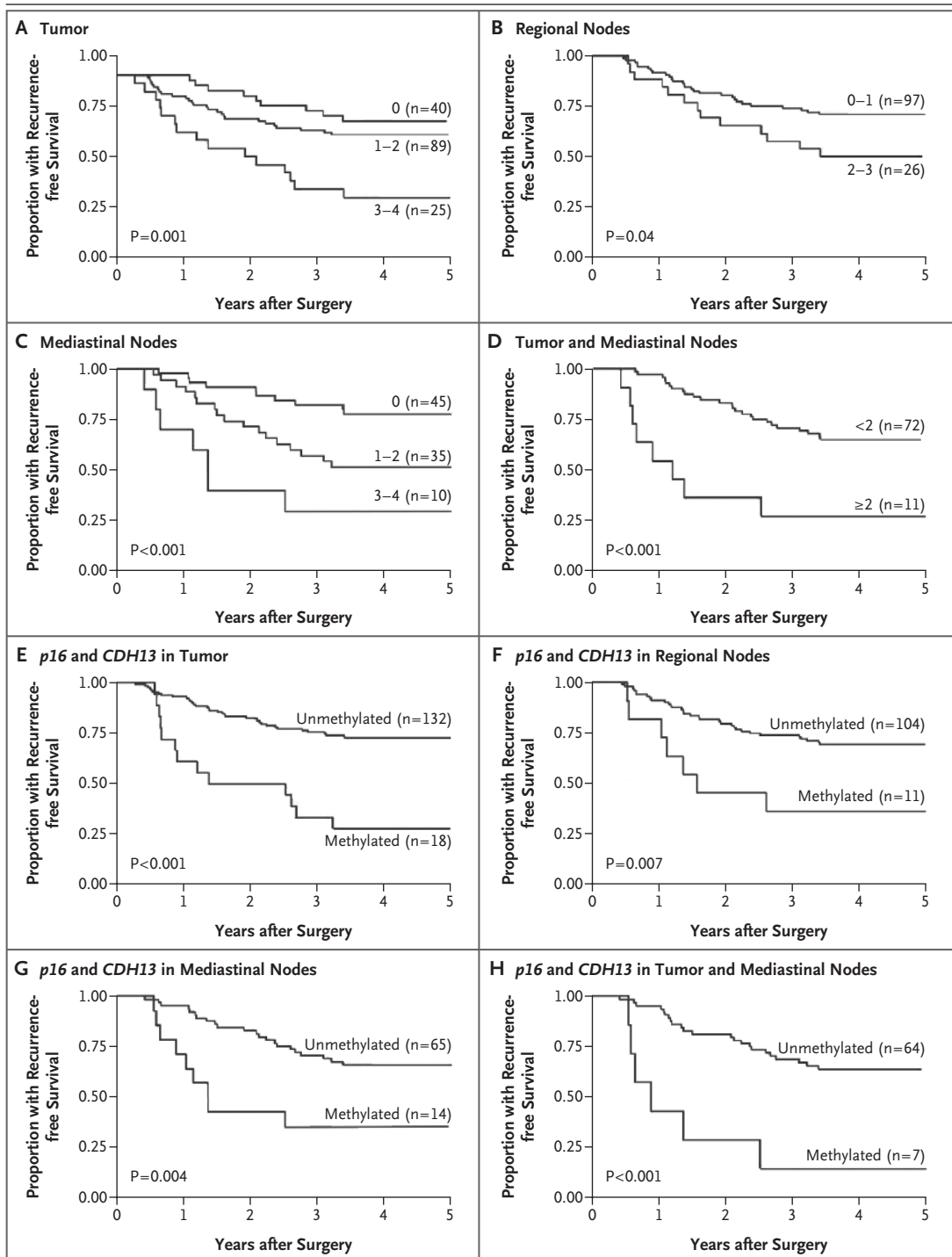
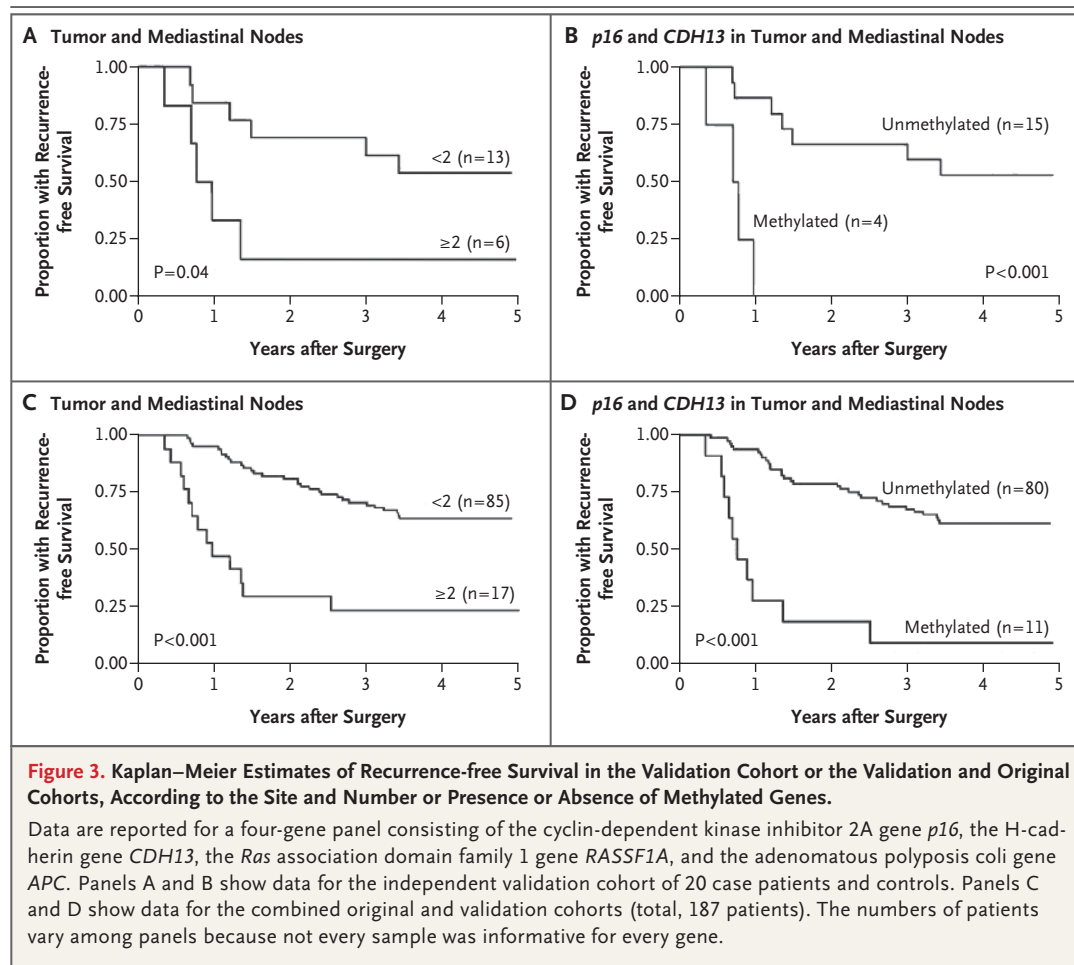


Figure 2. Kaplan–Meier Estimates of Recurrence-free Survival among 167 Case Patients and Controls with Stage I Non–Small-Cell Lung Cancer from the Original Cohort, According to the Site and Number or Presence or Absence of Methylated Genes.

Data are reported for a four-gene panel consisting of the cyclin-dependent kinase inhibitor 2A gene *p16*, the H-cadherin gene *CDH13*, the *Ras* association domain family 1 gene *RASSF1A*, and the adenomatous polyposis coli gene *APC*. In all three types of tissue, the recurrence-free survival rates decrease with an increasing number of genes (Panels A, B, and C) and when the genes are methylated (Panels E, F, and G). This same effect on recurrence-free survival is evident when the tumor and mediastinal lymph nodes are considered together (Panels D and H). The numbers of patients vary among panels because not every sample was informative for every gene.



expression or promoter-region methylation in lung cancer have focused mostly on the primary tumors,^{10,13,18,31–33} but we found that molecular examination of lymph nodes improves the assessment of risk of recurrence. The methylation of these genes in histologically normal regional lymph nodes probably indicates the presence of microscopically undetectable micrometastases. Immunohistochemical analyses, for example, may miss a rare cell in the background of normal tissue, whereas the methylation-specific PCR assay is sufficiently sensitive to detect a signal of DNA methylation.

The current method of assessment of the risk of recurrence in patients with stage I (T1–N0) NSCLC is imprecise — one third of such tumors recur after curative surgery. Our results suggest that the detection of promoter methylation of certain genes may identify cells with a potential for metastatic spread not only within NSCLC but also in lymph nodes. It is possible that the methylated

genes in lymph nodes represent tumor DNA that drained to the nodes through the lymphatic system, but this is unlikely because mediastinal nodes are located far from the lung, in the mediastinum. The correlation between short survival and the number of methylated genes in the regional and mediastinal lymph nodes supports the presence of micrometastases in those sites. Recent promising results for predicting the risk of lung cancer³⁴ or its recurrence¹⁷ have been obtained by examining changes in gene methylation in sputum.³⁴ Our study was retrospective and conducted at a single institution, and the number of patients studied was small. For these reasons, replication of our findings in a large, prospectively studied cohort is essential before the four-gene panel we investigated can be used in clinical practice.

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REFERENCES

- Hellman S. Stopping metastases at their source. *N Engl J Med* 1997;337:996-7.
- Riethmüller G, Johnson JP. Monoclonal antibodies in the detection and therapy of micrometastatic epithelial cancers. *Curr Opin Immunol* 1992;4:647-55.
- Zhu JJ, Maruyama T, Jacoby LB, et al. Clonal analysis of a case of multiple meningiomas using multiple molecular genetic approaches: pathology case report. *Neurosurgery* 1999;45:409-16.
- Martini N, Bains MS, Burt ME, et al. Incidence of local recurrence and second primary tumors in resected stage I lung cancer. *J Thorac Cardiovasc Surg* 1995;109:120-9.
- Mountain CF. Revisions in the International System for Staging Lung Cancer. *Chest* 1997;111:1710-7.
- Hoffman PC, Mauer AM, Vokes EE. Lung cancer. *Lancet* 2000;355:479-85. [Erratum, *Lancet* 2000;355:1280.]
- Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;349:2042-54.
- Baylin SB, Ohm JE. Epigenetic gene silencing in cancer — a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer* 2006;6:107-16.
- Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. *Cancer Res* 2001;61:3225-9.
- Pellisè M, Castells A, Ginès A, et al. Detection of lymph node micrometastases by gene promoter hypermethylation in samples obtained by endosonography-guided fine-needle aspiration biopsy. *Clin Cancer Res* 2004;10:4444-9.
- Sanchez-Cespedes M, Esteller M, Hibi K, et al. Molecular detection of neoplastic cells in lymph nodes of metastatic colorectal cancer patients predicts recurrence. *Clin Cancer Res* 1999;5:2450-4.
- Köller mann J, Müller M, Goessl C, et al. Methylation-specific PCR for DNA-based detection of occult tumor cells in lymph nodes of prostate cancer patients. *Eur Urol* 2003;44:533-8.
- Harden SV, Tokumaru Y, Westra WH, et al. Gene promoter hypermethylation in tumors and lymph nodes of stage I lung cancer patients. *Clin Cancer Res* 2003;9:1370-5.
- Belinsky SA, Nikula KJ, Palmisano WA, et al. Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc Natl Acad Sci U S A* 1998;95:11891-6.
- Toyooka KO, Toyooka S, Virmani AK, et al. Loss of expression and aberrant methylation of the CDH13 (H-cadherin) gene in breast and lung carcinomas. *Cancer Res* 2001;61:4556-60.
- Toyooka S, Toyooka KO, Maruyama R, et al. DNA methylation profiles of lung tumors. *Mol Cancer Ther* 2001;1:61-7.
- Machida EO, Brock MV, Hooker CM, et al. Hypermethylation of ASC/TMS1 is a sputum marker for late-stage lung cancer. *Cancer Res* 2006;66:6210-8.
- Brock MV, Kim MP, Hooker CM, et al. Pulmonary resection in octogenarians with stage I nonsmall cell lung cancer: a 22-year experience. *Ann Thorac Surg* 2004;77:271-7.
- Ettinger DS, Cox JD, Ginsberg RJ, et al. NCCN non-small-cell lung cancer practice guidelines: the National Comprehensive Cancer Network. *Oncology (Williston Park)* 1996;10:Suppl:81-111.
- Johnson BE. NCCN: small cell lung cancer. *Cancer Control* 2001;8:Suppl 2:32-43.
- Ginsberg RJ, Rubinstein LV. Randomized trial of lobectomy versus limited resection for T1 N0 non-small cell lung cancer. *Ann Thorac Surg* 1995;60:615-22.
- House MG, Guo M, Efron DT, et al. Tumor suppressor gene hypermethylation as a predictor of gastric stromal tumor behavior. *J Gastrointest Surg* 2003;7:1004-14.
- Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci U S A* 1996;93:9821-6.
- van Engeland M, Weijenberg MP, Roemen GM, et al. Effects of dietary folate and alcohol intake on promoter methylation in sporadic colorectal cancer: the Netherlands cohort study on diet and cancer. *Cancer Res* 2003;63:3133-7.
- Guo M, Ren J, House MG, Qi Y, Brock MV, Herman JG. Accumulation of promoter methylation suggests epigenetic progression in squamous cell carcinoma of the esophagus. *Clin Cancer Res* 2006;12:4515-22.
- van Engeland M, Roemen GM, Brink M, et al. K-ras mutations and RASSF1A promoter methylation in colorectal cancer. *Oncogene* 2002;21:3792-5.
- Esteller M, Garcia-Foncillas J, Andion E, et al. Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N Engl J Med* 2000;343:1350-4. [Erratum, *N Engl J Med* 2000;343:1740.]
- Mountain CF. A new international staging system for lung cancer. *Chest* 1986;89:Suppl:225S-233S.
- Brock MV, Gou M, Akiyama Y, et al. Prognostic importance of promoter hypermethylation of multiple genes in esophageal adenocarcinoma. *Clin Cancer Res* 2003;9:2912-9.
- Lu C, Soria JC, Tang X, et al. Prognostic factors in resected stage I non-small-cell lung cancer: a multivariate analysis of six molecular markers. *J Clin Oncol* 2004;22:4575-83.
- Gu J, Berman D, Lu C, et al. Aberrant promoter methylation profile and association with survival in patients with non-small cell lung cancer. *Clin Cancer Res* 2006;12:7329-38.
- Wang J, Lee JJ, Wang L, et al. Value of p16INK4a and RASSF1A promoter hypermethylation in prognosis of patients with resectable non-small cell lung cancer. *Clin Cancer Res* 2004;10:6119-25.
- Safar AM, Spencer H, Su X, Cooney CA, Shwaikei A, Fan CY. Promoter hypermethylation for molecular nodal staging in non-small cell lung cancer. *Arch Pathol Lab Med* 2007;131:936-41.
- Belinsky SA, Liechty KC, Gentry FD, et al. Promoter hypermethylation of multiple genes in sputum precedes lung cancer incidence in a high-risk cohort. *Cancer Res* 2006;66:3338-44.

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