Prognostic implication of aberrant promoter hypermethylation of CpG islands in adenocarcinoma of the lung

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Objectives: DNA hypermethylation in promoter regions has been studied for various types of cancer. However, there is no clear evidence that shows whether methylation status can predict long-term survival in patients with lung cancer.

Methods: We collected tissues from 72 patients with lung adenocarcinomas. The cancer and normal lung tissues were tested for DNA hypermethylation by using methylation-specific polymerase chain reaction. The genes investigated were *p16INK4a* (*p16*), retinoic acid receptor β -promoter (*RAR β P2*), death-associated protein kinase (*DAPK*), O⁶-methylguanine-DNA-methyltransferase (*MGMT*), and glutathione-S-transferase P1 (*GSTP1*). The status of the DNA methylation was analyzed, and we focused on long-term outcomes, as well as other clinical variables.

Results: DNA hypermethylation was observed in 83% for *p16*, 63% for *RAR β P2*, 32% for *DAPK*, 17% for *MGMT*, and 46% for *GSTP1* from the cancer tissue. From normal lung tissue, the results of methylation were positive in 75% for *p16*, 24% for *RAR β P2*, 10% for *DAPK*, 6% for *MGMT*, and 33% for *GSTP1*. During the mean follow-up period of 18 ± 11 months (1-40 months), 25 (35%) patients experienced recurrence, and 13 died. In multivariable analysis, old age (>60 years, $P = .007$), male sex ($P = .004$), unmethylation of *DAPK* from cancer tissue ($P = .045$), and hypermethylation of *RAR β P2* from normal tissue ($P = .000$) were risk factors for poor survival. Pathologic stage ($P = .023$), unmethylation of *DAPK* from normal tissue ($P = .043$), and hypermethylation of *RAR β P2* from normal tissue ($P = .030$) were risk factors for disease-free survival.

Conclusions: DNA methylation status of CpG islands seems to be a useful predictor of long-term outcome for adenocarcinoma of the lung. However, because the predictive power is still low, further studies, including those with multiple genes, are necessary to increase its usefulness in the clinical setting.

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Various studies have been performed to find ideal molecular markers to predict the long-term outcomes of lung cancer, such as long-term survival and recurrence. Among these efforts, DNA hypermethylation in promoter regions has been studied for various types of cancer. The inactivation of the tumor suppressor gene *p16INK4a* (*p16*),¹ the retinoic acid receptor β -promoter gene (*RAR β P2*),² the DNA repair gene O⁶-methylguanine-DNA-methyltransferase (*MGMT*),³ the detoxifying gene glutathione-S-transferase P1 (*GSTP1*),⁴ and the death-associated protein kinase gene (*DAPK*)⁵ by promoter hypermethylation has been well described in primary lung cancer. Our previous study demonstrated that *RAR β P2* and *DAPK* might affect the recurrence pattern in non-small cell lung cancer (NSCLC) after surgical resection.⁶ However, there has been no clear evidence that demonstrated correlation between methylation status and long-term survival in patients with lung cancer.

TABLE 1. Summary of primer sequences, annealing temperatures, and PCR product sizes used for methylation-specific polymerase chain reaction

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Annealing temperature (°C)	Product size (bp)
<i>p16</i>	M: TTATTAGAGGGTGGGGCGGATCGC	M: GACCCCGAACCGCGACCGTAA	62	150
	U: TTATTAGAGGGTGGGGTGGATTGT	U: CAACCCCAAACCAACCATAA	60	151
<i>RARβP2</i>	M: TCGAGAACGCGAGCGATTCTG	M: GACCAATCCAACCGAAACGA	59	146
	U: TTGAGAATGTGAGTGATTGA	U: AACCAATCCAACCAAAACAA	55	146
<i>DAPK</i>	M: GGATAGTCGGATCGAGTTAACGTC	M: CCCTCCCAAACGCCGA	57	98
	U: GGAGGATAGTTGGATTGAGTTAATGTT	U: CAAATCCCTCCCAAAACACCAA	55	106
<i>MGMT</i>	M: TTTTCGACGTTCTGAGTTTTCGC	M: GCACTCTTCCGAAAACGAAACG	62	81
	U: TTTGTGTTTTGATGTTGTAGGTTTTGT	U: AACTCCACACTCTTCCAAAAACAAACA	59	93
<i>GSTP1</i>	M: TTCGGGGTGTAGCGGTCGTC	M: GCCCAATACTAAATCAGGACG	57	91
	U: GATGTTGGGGTGTAGTGTTGTT	U: CCACCCAATACTAAATCACAACA	55	97

RARβP2, Retinoic acid receptor β-promoter; *DAPK*, death-associated protein kinase; *MGMT*, O⁶-methylguanine-DNA-methyltransferase; *GSTP1*, glutathione-S-transferase P1.

We examined whether aberrant DNA hypermethylation could be used to predict the clinical outcomes of patients with primary adenocarcinoma of the lung after surgical resection.

Materials and Methods

Sample Collection

Among the 653 patients who underwent surgical resection for primary lung cancer between December 2000 and April 2004 at Seoul National University Hospital, we selected 72 patients whose pathologic diagnoses were adenocarcinoma and whose frozen specimens were available from our Lung Cancer Tissue Bank. Informed consent for tissue collection and gene analysis for research purposes were obtained from individual patients preoperatively according to the policy of the Lung Cancer Tissue Bank, Cancer Research Institute, Seoul National University. Twenty-nine subjects were men, and 43 were women (mean age, 59 years; range, 38-85 years). Ten patients were actively smoking within 3 months before the operation, and 20 were ex smokers. Forty-two patients denied having a history of active smoking. Seven patients received preoperative chemotherapy. Lobectomy was performed in 65 patients, pneumonectomy was performed in 6 patients, and wedge resection was performed in 1 patient. Radical mediastinal lymph node dissection was performed in every patient. Fifteen patients received adjuvant chemotherapy, 17 received adjuvant irradiation, and 1 received both treatments postoperatively. Tissue sampling was performed immediately after lung resection. The cancer mass was cut in half, and the tissue from the most solid part was sampled. The normal lung tissues were obtained from the normal-appearing portion of the lung in the resected specimen, far apart from the cancer mass. The tissues were quickly frozen in liquid nitrogen and stored at -80°C until analysis.

DNA Preparation and Bisulfite Modification

Genomic DNA samples from frozen lung cancer tissue were isolated by use of the QIAmp Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. One microgram

of genomic DNA was digested with restriction enzyme (*HindIII*; Intron Biotechnology, Kyungki-Do, Korea), and bisulfite conversion was carried out with reagents provided in the CpGenome DNA Modification Kit (Intergen, Purchase, NY).

Methylation-specific Polymerase Chain Reaction

Polymerase chain reaction (PCR) primers that distinguish between these methylated and unmethylated DNA sequences were then used. Primer sequences of all genes for both the methylated and unmethylated forms, annealing temperatures, and the expected PCR product sizes are summarized in Table 1.

Methylation-specific PCR (MSP) amplification for the 5 genes was carried out with the hot start method by using the Qiagen HotStart Master Mix Kit (Qiagen, Hilden, Germany). Thermocycling conditions were initial denaturation and hot start at 95°C for 15 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at a specific temperature for 1 minute, and extension at 72°C for 45 seconds. Normal lung tissue DNA treated in vitro with an excess of *SssI* methyltransferase (New England Biolabs, Ipswich, Mass) was used as a positive control for methylated alleles of each gene. Water blanks were used as a negative control.

Clinical Data

All 72 patients had follow-up visits at the clinic every 3 months. The mean follow-up period was 18 months (range, 1-40 months). On every visit, chest radiograms and sputum cytology examinations were performed, and chest computed tomographic scans were obtained on an annual basis. If there were any symptoms or observations suggesting recurrence, additional evaluations, such as bone scanning, brain magnetic resonance imaging, or positron emission tomographic/computed tomographic scanning, were performed depending on the suspected site of the recurrence.

The relationship between the clinical outcomes (overall survival and disease-free survival) and DNA hypermethylation patterns were analyzed, along with clinical variables, such as sex, age, and pathologic TNM stage.

TABLE 2. Risk factors for long-term survival rates: Univariable and multivariable analysis of various clinical factors and gene hypermethylation

Factors	Log rank P value	Cox regression	
		Odds ratio (95% CI)	P value
Age (>60 y)	.1073	9.500 (1.837-49.127)	.007
Sex (female)	.0020	10.042 (2.116-47.667)	.004
Stage (>II)	.0544	—	—
<i>p16</i>			
Cancer	.3014	—	—
Normal	.5740	0.176 (0.029-1.074)	.060
<i>RARβP2</i>			
Cancer	.7642	—	—
Normal	.0002	35.559 (4.901-257.981)	.000
<i>DAPK</i>			
Cancer	.0664	0.244 (0.062-0.967)	.045
Normal	.4118	—	—
<i>MGMT</i>			
Cancer	.4454	—	—
Normal	.3592	—	—
<i>GSTP1</i>			
Cancer	.8436	—	—
Normal	.0300	—	—

CI, Confidence interval; *RARβP2*, retinoic acid receptor β-promoter; *DAPK*, death-associated protein kinase; *MGMT*, O⁶-methylguanine-DNA-methyltransferase; *GSTP1*, glutathione-S-transferase P1.

Statistical Analysis

The frequency of methylation in the normal tissue and the cancer tissue was calculated for each considered gene and then tested by using the McNemar χ^2 test for paired proportion differences. Because the distribution of patients' number in TNM stage was not appropriate for analysis, we merged the category into early ($\leq$ stage II) and late stage ($>$ stage II), and an association of methylation with TNM stage was investigated by using the χ^2 test. Overall survival and recurrence patterns were investigated by using the Kaplan-Meier method, and the differences between groups determined on the basis of risk factors were tested by using the log-rank test. Cox regression analysis was used to explore the influence of independent prognostic factors in a multivariable model. The factors were chosen by using a stepwise forward method with criteria for variable inclusion of 0.06 and that for variable exclusion of 0.10.

Results

Frequency of Methylation in Lung Cancer Tissue

DNA hypermethylation was observed in 83% (60/72) for *p16*, 63% (45/72) for *RARβP2*, 32% (23/72) for *DAPK*, 17% (12/72) for *MGMT*, and 46% (33/72) for *GSTP1* from the cancer tissue. From normal lung tissue, methylation was positive in 75% (54/72) for *p16*, 24% (17/72) for *RARβP2*, 10% (7/72) for *DAPK*, 6% (4/72) for *MGMT*, and 33% (24/72) for *GSTP1* (Table E1). The frequencies of individual gene hypermethylation between cancer tissue and normal tissue were significantly different in *RARβP2*, *DAPK*,

and *MGMT*. Abnormal promoter hypermethylation in at least one gene was found in 63 (87.5%) patients at the cancer tissue and observed in 57 (79.2%) patients at the normal tissue. The unmethylated form of all genes, which resulted in an unmethylated band, was detected in 100% of the samples, suggesting coexistence with noncancer cells. We also conducted a test for normal lung tissue from nonsmoking benign lung disease and found no band for methylation in each gene (Figure E1).

Clinicopathologic Correlation

Pathologic TNM stages included stage Ia in 13, stage Ib in 19, stage IIa in 0, stage IIb in 11, stage IIIa in 22, stage IIb in 6, and stage IV in 1 patient. Methylation frequencies of individual genes were not associated with the TNM staging. There were no operative deaths. During the mean follow-up period of 18 months (standard deviation, 11; range, 1-40 months), 25 (35%) patients experienced recurrence, and 13 died. Clinical variables, such as sex, age, TNM stage, and gene methylation patterns, were tested as to whether they affect overall survival. When exploring each factor at a time, male sex ($P = .002$), higher TNM stage ($>$ II, $P = .054$), hypermethylation of *RARβP2* in normal tissue ($P = .000$), and hypermethylation of *GSTP1* in normal tissue ($P = .030$) turned out to be significant variables that affected poor long-term survival (Table 2 and Figure 1). Although unmethylation of *DAPK* in cancer tissue seemed to affect long-term survival, it did not result in statistical significance ($P = .066$). In the Cox regression model we considered multifactors chosen by using a stepwise forward method. Male sex ($P = .004$), old age ($>$ 60 years, $P = .007$), unmethylation of *DAPK* from cancer tissue ($P = .045$), and hypermethylation of *RARβP2* from normal tissue ($P = .000$) were the risk factors for poor survival (Table 2 and Figure E2). Only advanced TNM stage ($>$ II, $P = .052$) was a risk factor for disease-free survival in univariable analysis. However, unmethylation of *DAPK* ($P = .043$) from normal tissue and hypermethylation of *RARβP2* from normal tissue ($P = .030$), as well as advanced TNM stage ($>$ II, $P = .023$), were risk factors for disease-free survival (Table 3 and Figure E3).

Discussion

A significant amount of time has been spent searching for prognostic markers that predict survival for patients with cancer. Gene-silencing events that involve transcriptional inactivation associated with abnormally methylated promoter CpG islands are a fundamental feature of human cancer. By using MSP assays developed by Herman and coworkers,⁷ the methylation status of the promoter region for various tumor suppressor genes have been extensively studied by many researchers. Hypermethylation of promoter CpG islands for *p16* has been reported to be a marker for

Figure 1. Kaplan-Meier curves for overall survival according to the methylation status for the normal tissue *RARβP2* (A) and the cancer tissue *DAPK* (B). The numbers on the graph represent patients remaining at risk. *DAPK*, Death-associated protein kinase; *RARβP2*, retinoic acid receptor β -promoter.

poor survival in peripheral-type small adenocarcinoma⁸ and NSCLC.^{9,10} On the contrary, however, others reported no significant correlation between hypermethylation for *p16* and survival.¹¹ Promoter hypermethylation of *DAPK*,¹² fragile histidine triad (*FHIT*),¹³ Ras association domain family 1 (*RASSF1A*),^{10,14,15} insulin-like growth factor-binding protein 3 (*IGFBP*),¹⁶ and *MGMT*¹⁷ have been reported as prognostic markers for NSCLC.

In the present study we demonstrated that hypermethylation of *RARβP2* in normal tissue ($P = .000$) and unmethyl-

TABLE 3. Risk factors for recurrence-free survival: Univariable and multivariable analysis of various clinical factors and gene hypermethylation

Factors	Log rank P value	Cox regression	
		Odds ratio (95% CI)	P value
Age (>60 y)	.4207	—	—
Sex (female)	.0920	—	—
Stage (>II)	.0515	2.493 (1.132-5.490)	.023
<i>p16</i>			
Cancer	.5974	—	—
Normal	.0674	—	—
<i>RARβP2</i>			
Cancer	.2597	—	—
Normal	.0946	2.642 (1.098-6.360)	.030
<i>DAPK</i>			
Cancer	.3530	—	—
Normal	.0943	0.121 (0.016-0.9529)	.043
<i>MGMT</i>			
Cancer	.9179	—	—
Normal	.1507	—	—
<i>GSTP1</i>			
Cancer	.8376	—	—
Normal	.1173	—	—

CI, Confidence interval; *RARβP2*, retinoic acid receptor β -promoter; *DAPK*, death-associated protein kinase; *MGMT*, O⁶-methylguanine-DNA-methyltransferase; *GSTP1*, glutathione-S-transferase P1.

lation of *DAPK* in cancer tissue ($P = .045$) were significant variables that affected long-term survival. The study of retinoids in lung cancer dates back to seminal observations by Wohlbach and Howe,¹⁸ who discovered that vitamin A deprivation in cattle led to an increased incidence of lung and upper aerodigestive tract cancers. A gradual loss of *RARβ* is seen in normal tissue, damaged epithelium, areas of dysplasia, squamous cell carcinomas, and adenocarcinomas, which supports the hypothesis that the *RARβ* gene might exert tumor-suppressive effects in lung cancer. However, there are still debates regarding the role of *RARβ* for lung cancer development.

We had previously reported there was no significant relationship between gene methylation and long-term survival in our series of surgically resected NSCLC.⁶ In that cohort, however, unmethylation of *DAPK* and hypermethylation of *RARβP2*, as well as advanced T stage and preoperative chemotherapy, were significant risk factors for early recurrence in the remaining lung. Interestingly, Kim and associates¹⁹ found that hypermethylation of the *RARβP2* gene has a differential effect on the development of second primary lung cancers in patients with NSCLC, depending on their smoking status. Specifically, they reported second primary lung cancer developed more frequently when *RARβP2* was unmethylated than when it was hypermethylated in current smokers. In contrast, the development of second primary lung cancer in former smokers was more prevalent in pa-

tients with hypermethylated *RARBP2*.¹⁹ Their report suggested the possible explanation for our previous result, as well as a different effect of retinoids on survival. In this study we did not find any correlation between the *RARBP2* methylation in cancer tissue and survival. Moreover, the *RARBP2* methylation in cancer tissue did not show a specific pattern of recurrence in the remaining tracheobronchial tree. The explanation for these observations is not clear. It might be because we excluded squamous cell carcinoma, which has been known to be more related to smoking compared with adenocarcinoma. Our series included an unusually high number of women and nonsmokers. It has been suggested that adenocarcinoma in nonsmoking women might exhibit different biologic behavior from lung cancer in male smokers with squamous cell histology. For example, nonsmoker female adenocarcinoma has been known to have a higher rate of epidermal growth factor receptor (*EGFR*) mutation. Indeed, in our study samples, as many as 31 (43%) patients were found to have some form of *EGFR* mutation, which is a much higher rate than would be expected (unpublished data). Another reason might be because our series did not have enough cases or an appropriate follow-up period to demonstrate the development of second primary lung cancer. Although we performed DNA sequencing of bisulfite PCR product for the *RARBP2* gene in 3 samples and verified correct sequences, we did not conduct tests for all 5 genes in every sample. The lack of confirmatory sequencing experiments prohibited us from being able to deny any chance of false-negative or false-positive results, which might have affected our results.

Many researchers are investigating the methylation status of normal tissue adjacent to the tumor.^{20,21} It is relevant to think that hypermethylation of normal tissue might suggest the presence of a premalignant area and might have an effect on the prognosis. In our previous report we were unable to find any significance between hypermethylation of the normal tissue and long-term survival. In the present study the hypermethylation of normal tissue *RARBP2* was a clear prognostic factor for poor survival in both univariable and multivariable analyses. We analyzed our data, stratifying by smoking history. However, it did not affect the result at all. Because our institution has a strict guide for smoking cessation for patients who undergo lung resection, none of our patients smoked postoperatively, and we did not have current smokers at the time of follow-up, for whom the methylation of *RARBP2* might have worked as a good prognostic factor, as Kim and associates have suggested.¹⁹

DAPK is a proapoptotic serine-threonine kinase involved in apoptosis. Recent experiments established that *DAPK* functions as a tumor suppressor in at least 2 different stages of tumorigenicity, namely an apoptotic checkpoint functioning early during cell transformation and a second one that occurs later in cancer development (ie, during metastasis).

For lung cancer, Kim and colleagues²² reported that increased tumor size, pathologic stage, and lymph node involvement correlated with *DAPK* loss. However, others reported that *DAPK* promoter methylation was detected without any association with the stage of disease.^{20,23,24} Our series did not show any correlation between *DAPK* methylation status and stage of cancer either. Our result is interesting because unmethylation, instead of hypermethylation, of *DAPK* was a risk factor for poor survival. We do not have an appropriate explanation for this phenomenon. There might be another factor that causes *DAPK* methylation to affect survival in different ways, or it could be only a false-positive result. Because this is the first report analyzing pure adenocarcinoma of the lung for the *DAPK* gene, further study for this specific gene is mandatory. Another explanation can be addressed on the basis of statistical analysis method. We primarily used a conventional 5% significance level for each test. However, we might also need to consider a more conservative significance level for the univariable Cox regression analysis, considering a possible chance effect to find a significant result caused by multiple testing. If we use a 1% significance level, most of the candidate factors turn out to be nonsignificant, but hypermethylation of *RARBP2* in normal tissue would still be a highly significant predictor of overall survival, whereas *DAPK* would not. We also note the limited size of this study and that associations identified might have occurred by chance. Therefore the findings suggested should be interpreted with caution and in a less conclusive but exploratory way. Although we were not able to demonstrate the functional mechanism, we observed that the search for the DNA methylation status of CpG islands in both cancer tissue and adjacent normal tissue showed promise as a predictor of long-term outcome for adenocarcinoma of the lung. However, because the predictive power is still low, further studies, including those with multiple genes, are necessary to increase its usefulness in the clinical setting.

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Discussion

David S. Schrump (Bethesda, Md). Dr Kim, I would like to congratulate you on a very elegant study.

Similar to other neoplasms, lung cancers exhibit a DNA methylation paradox, which is manifested as a derepression of multiple imprinted genes, upregulation of cancer-testis antigens, and a paradoxical inactivation of tumor suppressor genes. The fact that not all of the imprinted alleles or the cancer-testis antigens are upregulated and that the tumor suppressor genes are not simultaneously repressed during malignant transformation suggests that the mechanisms underlying the methylation paradox are extremely complex.

A number of tumors, including colorectal carcinomas and leukemias, exhibit a methylation profile (methylator phenotype) that seems to correlate with patient outcome. To date, a methylator phenotype has not been identified in lung cancers. Published studies from numerous laboratories, including our own, demonstrate considerable heterogeneity regarding gene-expression profiles among different patients with lung cancer.

In your study you examined the methylation status of 5 genes that are commonly methylated in human lung cancers. This work appears to extend your previous studies pertaining to methylation analysis of 4 of these genes, namely *p16*, *RARβ*, *DAPK*, and methylguanine-methyltransferase in 61 patients with lung cancer. In that study DNA methylation was not associated with any clinicopathologic characteristics and did not appear to coincide with patient survival. In that study you demonstrated that unmethylation of *DAPK* in the tumor and hypermethylation of *RARβ* in normal tissues coincided with increased risk of recurrence. In the current study involving 72 patients with adenocarcinomas, you have substantiated your previous findings. In all likelihood, the phenomenon might indicate that the inhibition of apoptotic pathways is not contingent on methylation of *DAPK*. Furthermore, the methylation of *RARβ* in the normal tissues might actually be a surrogate marker of more profound epigenetic changes that are relevant in the tumor itself.

I have several questions concerning this article. The methylation analysis that you performed involved methylation-specific PCR techniques, which have been fairly well standardized; however, there are some difficulties with this technique, particularly in terms of analysis of primary tumor specimens because of the kinetics of the PCR reactions and false-positive, as well as false-negative, results. Whereas the frequency of methylation of *DAPK* observed in your study was consistent with that seen in reported studies, the methylation frequencies for the other 4 genes were considerably higher than typically reported in the literature. To date, there has been no significant difference in the methylation status of these genes in squamous versus adenocarcinoma histology. Could you comment or speculate as to why the frequency of methylation was so much higher in your study relative to what has been reported in the literature with patients who were also from Asia.

Dr Kim. For the first question, that is one of the problems we have with the MSP method in terms of a high possibility of having a false-positive rate. It can happen because it is a PCR that has a high false-positive rate. Also, if the bisulfite modifications happen to be insufficient, we will have a false-positive rate as well. Our previous study showed that there was a high frequency of *MGMT* methylation compared with that of the current study. To decrease the false-positive rate, we changed our primers and the PCR conditions. However, we discovered that our population had a

relatively high frequency of methylation, especially for *p16*. This result might possibly be related to ethnic differences. For example, for *EGFR* mutation, Asian persons have been known to have a higher frequency of mutation. In conclusion, one possibility might be a false-positive result, and the other might be related to ethnic differences.

Dr Schrump. Were any of the PCR results confirmed by bisulfite sequencing, once again to get at the issue of either false-positive or false-negative results here?

Dr Kim. We have been doing the PCR sequencing, and it is consistent at least for *RAR β* . I personally think that the more important thing is immunohistochemistry to explain the mechanism of carcinogenesis. Histone deacetylation is likely one of the mechanisms of controlling functional expression. However, methylation results do not consistently match with the immunohistochemistry in our experience.

Dr Schrump. I have 2 other brief questions. Do you have any insight regarding the K-ras mutation status of these tumors? As you know, K-ras mutations have been associated with aberrant methylation of certain genes. Also, have you observed any association between smoking exposure and particular methylation profiles?

Dr Kim. This population is made up of patients with adenocarcinoma, and more than 50% of the patients were women; women generally do not smoke in Korea. Additionally, I stratified the patients according to smoking status but could not find any difference. Regarding the K-ras mutation, I have been working on it with the same cohort of patients. We found about 20% of the patients had mutations for K-ras, but we have not matched those data with this study.

Dr Schrump. Forty-three of the 72 patients in the present study had early-stage tumors (stage I or stage II). As we look at methylator phenotypes in more advanced tumor stages, nodal status emerges as the predominant predictor of prognosis and survival. Have you had an opportunity or do you intend to return to your data to analyze specifically the early-stage tumors? Conceivably, patients with these tumors did not receive adjuvant chemotherapy or radiation that might have confounded your analysis.

Dr Kim. That finding is research I would like to continue in the future. Unfortunately, the study population is small in the present study, and stratifying a limited population for early lung cancer might not be possible. However, because we have been collecting our specimens since 1996, we might be able to perform that kind of analysis in the future.

Dr Schrump. I congratulate you on an excellent study.

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TABLE E1. Frequency of methylation in 72 tissue samples of adenocarcinoma of the lung

Gene	Frequency of methylation (%)		P value*
	Lung cancer tissue	Normal lung tissue	
<i>p16</i>	60 (83%)	54 (75%)	.238
<i>RARβP2</i>	45 (63%)	17 (24%)	.000
<i>DAPK</i>	23 (32%)	7 (10%)	.000
<i>MGMT</i>	12 (17%)	4 (6%)	.039
<i>GSTP1</i>	33 (46%)	24 (33%)	.064

RARβP2, Retinoic acid receptor β-promoter; *DAPK*, death-associated protein kinase; *MGMT*, O⁶-methylguanine-DNA-methyltransferase; *GSTP1*, glutathione-S-transferase P1. *P values were calculated by using the McNemar χ² test for paired proportion differences.

Figure E1. Representative methylation-specific polymerase chain reaction product of the promoter for *p16*, *RARβP2*, *DAPK*, *MGMT*, and *GSTP1* in tumor and normal tissue from patients with adenocarcinoma of the lung (A) and the result in normal tissue from nonsmoking patients with benign lung disease (B). The presence of product in lane M indicates the presence of methylated genes; the presence of product in lane U indicates the presence of unmethylated genes. *DAPK*, Death-associated protein kinase; *GSTP1*, glutathione-S-transferase P1; *H₂O*, water control; *IVM*, in vitro methylated control; *MGMT*, O⁶-methylguanine-DNA-methyltransferase; *Nor*, normal tissue; *Normal*, nonsmoking benign patients; *Patient*, patient with adenocarcinoma; *RARβP2*, retinoic acid receptor β-promoter; *Tum*, tumor tissue; *U*, unmethylation.

Figure E2. Cox regression cumulative hazard function for overall survival according to the methylation status for the normal tissue *RARβP2* (A) and the cancer tissue *DAPK* (B). *DAPK*, Death-associated protein kinase; *RARβP2*, retinoic acid receptor β -promoter.

Figure E3. Cox regression cumulative hazard function for disease-free survival according to methylation status for the normal tissue *RARβP2* (A) and *DAPK* (B). *DAPK*, Death-associated protein kinase; *RARβP2*, retinoic acid receptor β -promoter.