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Featured Article

Detection of Promoter Hypermethylation of Multiple Genes in the Tumor and Bronchoalveolar Lavage of Patients with Lung Cancer

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Abstract

Purpose: Aberrant promoter hypermethylation of several known or putative tumor suppressor genes occurs frequently during the pathogenesis of lung cancers and is a promising marker for cancer detection. We investigated the feasibility of detecting aberrant DNA methylation in the bronchoalveolar lavage (BAL) samples of lung cancer patients.

Experimental Design: We examined the tumor and the matched BAL DNA for aberrant methylation of eight gene promoters (*CDH1, APC, MGMT, RASSF1A, GSTP1, p16, RAR-β2,* and *ARF*) from 31 patients with primary lung tumors by quantitative fluorogenic real-time PCR. BAL from 10 age-matched noncancer patients was used as a control.

Results: Promoter hypermethylation of at least one of the genes studied was detected in all 31 lung primary tumors; 27 (87%) *CDH1*, 17 (55%) *APC*, 14 (45%) *RASSF1A*, 12 (39%) *MGMT*, 7 (23%) *p16*, 3 (10%) *GSTP1*, 3 (10%) *RAR-β2*, and 0 (0%) *ARF*. Methylation was detected in *CDH1* (48%), *APC* (29%), *RASSF1A* (29%), *MGMT* (58%), *p16* (14%), *GSTP1* (33%), *RAR-β2* (0%), and *ARF* (0%) of BAL samples from matched methylation-positive primary tumors, and in every case, aberrant methylation in BAL DNA was accompanied by methylation in the matched tumor samples. BAL samples from 10 controls without evidence of cancer revealed no methylation of the *MGMT,* *GSTP1, p16, ARF,* or *RAR-β2* genes whereas methylation of *RASSF1, CDH1,* and *APC* was detected at low levels. Overall, 21 (68%) of 31 BAL samples from cancer patients were positive for aberrant methylation.

Conclusion: Our findings suggest that promoter hypermethylation in BAL can be detected in the majority of lung cancer patients. This approach needs to be evaluated in large early detection and surveillance studies of lung cancer.

Introduction

Lung cancer is the leading cause of cancer-related death in the United States and other developed countries (1). In 2001, an estimated 169,500 new cases of primary lung cancer were diagnosed, and an estimated 157,400 people died of this disease in the United States (1). The survival rates for lung cancers have changed little over the past two decades. A major factor in the high mortality of lung cancer patients is the presence of metastatic tumors in approximately two-thirds of patients at the time of diagnosis (2).

Detection of lung cancer at earlier stages could potentially increase survival rates by 10- to 50-fold (2). Lung cancer screening by chest X-ray and sputum cytology have proven ineffective in increasing patient survival (3, 4), leading to the search for more sensitive and specific tests. One promising approach is the identification of lung cancer-specific biomarkers and noninvasive methods for the detection of these biomarkers at an early stage.

Aberrant promoter hypermethylation is a major mechanism for silencing tumor suppressor or other cancer-associated genes in many kinds of human cancer (5–9). Thus far, genes such as *APC, CDKN2A, CDH1, RAR-β2,* and *RASSF1A* have been found to harbor hypermethylated promoters in over 30% of lung tumors (10, 11). The development of the real-time methylation-specific PCR, which is more sensitive than conventional methylation-specific polymerase chain reaction (MSP) by 10-fold, has simplified the study of the genes inactivated by promoter hypermethylation in human cancer and has the advantage of increasing specificity attributable to the use of an internally binding fluorogenic hybridization probe for each gene (12, 13). Recent publications have demonstrated the presence of promoter hypermethylation of various genes in bodily fluids, including bronchoalveolar lavage (BAL) DNA of lung cancer patients (14–18).

In the present study, we analyzed the promoter hypermethylation pattern of eight key genes in the primary tumor DNA of 31 lung primary tumors. The methylation pattern found in the primary tumors was compared with the methylation pattern in matched BAL fluid DNA. Approximately two-thirds of the patients with methylated tumors were found to display identical epigenetic changes in the paired BAL fluid.

Materials and Methods

Sample Collection and DNA Preparation. We evaluated tissue samples of 31 patients with lung cancer, who underwent curative surgery between 1995 and 1999 at the Johns Hopkins University, School of Medicine. Detailed information on these patients is summarized in Table 1. Obtained tissue specimens were immediately snap frozen in liquid nitrogen and stored at −80°C. H&E-stained sections were histologically examined every 20 sections for the presence or absence of tumor cells, as well as for tumor density. Only sections that showed >70% of tumor cells were used for DNA extraction. Under the proper consent, BAL fluid was collected during flexible bronchoscopy in the operating room for the patient undergoing resection of non-small cell lung carcinoma before thoracotomy. BAL fluids from the 10 age-matched individuals with nonmalignant lung diseases were also collected during bronchoscopy under standard procedures. Briefly, after the initial examinations were performed with bronchoscopy, four 30-ml (6 teaspoons) washes of germ-free i.v. salt solution were sequentially installed and retrieved by gentle suction, pooled, and placed on ice and were then transported to the laboratory and centrifuged at $1800 \times g$ for 10 min at 4°C. The cell pellet was then collected and stored at −80°C. DNA isolation was performed as described previously (19).

Bisulfite Treatment. DNA from tumor and BAL fluid was subjected to bisulfite treatment, as described previously (20). Briefly, 2 μg of genomic DNA was denatured in 0.2 M NaOH for 20 min at 50°C. The denatured DNA was diluted in 500 μl of freshly prepared solution of 10 mM hydroquinone and 3 M sodium bisulfite and incubated for 3 h at 70°C. After incubation, the DNA sample was desalted through a column (Wizard DNA Clean-Up System, Promega), treated with 0.3 M NaOH for 10 min at room temperature, and precipitated with ethanol. The bisulfite-modified genomic DNA was resuspended in 120 μl of H_2O and stored at −80°C.

Methylation Analysis. Templates were amplified by a fluorescence-based real-time PCR (Taqman) as described previously (21). In brief, primers and probes were designed to specifically amplify the bisulfite-converted promoter of the gene of interest. The ratios between the values of the gene of interest and the internal reference gene, β-actin, obtained by Taqman analysis were used as a measure for representing the relative level of methylation in the particular sample. Fluorogenic PCRs were carried out in a reaction volume of 25 μl consisting of 600 nM each primer, 200 nM probe, 5 units of *Taq* polymerase, 200 μM each of dATP, dCTP, and dGTP; 400 μM dTTP; and 5.5 mM $MgCl_2$. Three microliters of treated DNA solution were used in each real-time MSP reaction. Amplifications were carried out in 384-well plates in a 7700 Sequence detector (Perkin-Elmer Applied Biosystems). Each plate consisted of patient samples and multiple water blanks, as well as positive and negative controls. Leukocytes from a healthy individual were methylated *in vitro* with excess SssI methyltransferase (New England Biolabs Inc., Beverly, MA) to generate completely methylated DNA, and serial dilutions of this DNA were used for constructing the calibration curves on each plate.

Statistical Analysis. All statistical tests were performed using Excel software (Microsoft).

Results

We tested the promoter methylation pattern of *CDH1*, *APC*, *MGMT*, *RASSF1A*, *GSTP1*, *p16*, *RAR-β2*, and *ARF* in 31 primary lung tumors and corresponding matched BAL DNA. The methylation frequencies of these eight individual genes are listed in Table 2. *APC*, *MGMT*, *RASSF1A*, and *CDH1* gene methylation ratios in primary tumor tissue, BAL, and control BAL are shown in Fig. 1 as scatter plots.

Frequency of Methylation in Primary Lung Tumor. Aberrant promoter hypermethylation of at least one of the genes investigated was detected in all 31 lung primary tumors (100%; Table 2; Fig. 2). The median methylation values (gene/β-actin X 100) for each gene in tumor and BAL DNA are shown in Table 2. The frequency of aberrant methylation of each gene is listed in Table 2. In the tumor samples, frequent methylation was detected in *CDH1* (87%), *APC* (55%), *RASSF1A* (45%), and *MGMT* (39%). Methylation of p16 was detected in 23% of cases. Methylation of *GSTP1* (10%) and *RAR-β2* (10%) were much less common. Methylation of the *ARF* promoter DNA was not detected in any of the 31 cases.

Aberrant methylation in primary lung carcinoma had no correlation with patient demographic data, including age and

Table 1 Gene promoter hypermethylation of multiple genes in primary tumor tissue from lung cancer patients

	n^a	CDH1	APC	RASSF1A	GSTP1	p16	MGMT	RAR-β2	ARF
Age									
50>	29	26	16	12	3	7	11	3	0
50<	2	1	1	2	0	0	1	0	0
Histological type									
Adeno	21	20	12	11	3	4	8	2	0
SCCA	7	5	4	2	0	3	2	1	0
NSCLC	2	1	0	1	0	0	2	0	0
Large cell									
Carcinoma	1	1	1	0	0	0	0	0	0
Stage									
I	17	17	9	8	1	3	5	2	0
II	9	7	5	5	2	2	3	1	0
III	4	2	2	1	0	2	3	0	0
IV	1	1	1	0	0	0	1	0	0

[a] *n*, number of cases examined.
SCCA, squamous cell carcinoma; NSCLC, non-small cell lung cancer.

Table 2 Frequency of methylation in primary tumor and corresponding BAL[a] fluid samples of lung cancer patients

Gene aberrantly methylated	Tumor methylation positive % (number of methylation positive/ number of total case)	BAL methylation positive % (number of methylation positive/ number of methylated tumor tissue)	Cutoff value	Median tumor	Median BAL
CDH1	87% (27/31)	48% (13/27)	0.22	1.52	0.14
APC	55% (17/31)	29% (5/17)	1.7	30.04	12.83
RASSF1A	45% (14/31)	29% (4/14)	0.05	6.79	0.17
MGMT	39% (12/31)	58% (7/12)	0	0.13	0.40
p16	23% (7/31)	14% (1/7)	0	11.99	0
GSTP1	10% (3/31)	33% (1/3)	0	0.08	0
RAR-β2	10% (3/31)	0% (0/3)	0	0	0
ARF	0% (0/31)	0% (0/31)	0	0	0

[a] BAL, bronchoalveolar lavage.

gender, histological subtype, and staging of the tumor (data not shown).

Epigenetic Alterations in BAL DNA from Lung Cancer Patients. The matching 31 BAL samples from these lung cancer patients were then tested for epigenetic alterations. Overall, 21 of 31 (68%) patients were methylation positive for at least one of the eight genes tested (Table 2; Fig. 2). BAL DNA from 0 of the 10 controls with non-neoplastic lung disease displayed promoter hypermethylation at five genes examined. *CDH1*, *RASSF1A*, and *APC* showed very low levels of methy-lation in three control BAL samples; however, the median methylation levels in these control samples for *CDH1*, *APC*, and *RASSF1A* were 0 in each gene and the range was 0.000–0.022, 0.147–1.760, and 0.000–0.048, respectively. For these three genes, we set the highest methylation level in normals as the cutoff value to determine methylation-positive samples for lung cancer tissue and BAL fluid. With this cutoff, the frequency of aberrant promoter methylation detected in matched BAL fluid for each marker was 48% (13 of 27) for *CDH1*, 58% (7 of 12) for *MGMT*, 29% (4 of 14) for *RASSF1A*, 29% (5 of 17) for *APC*,
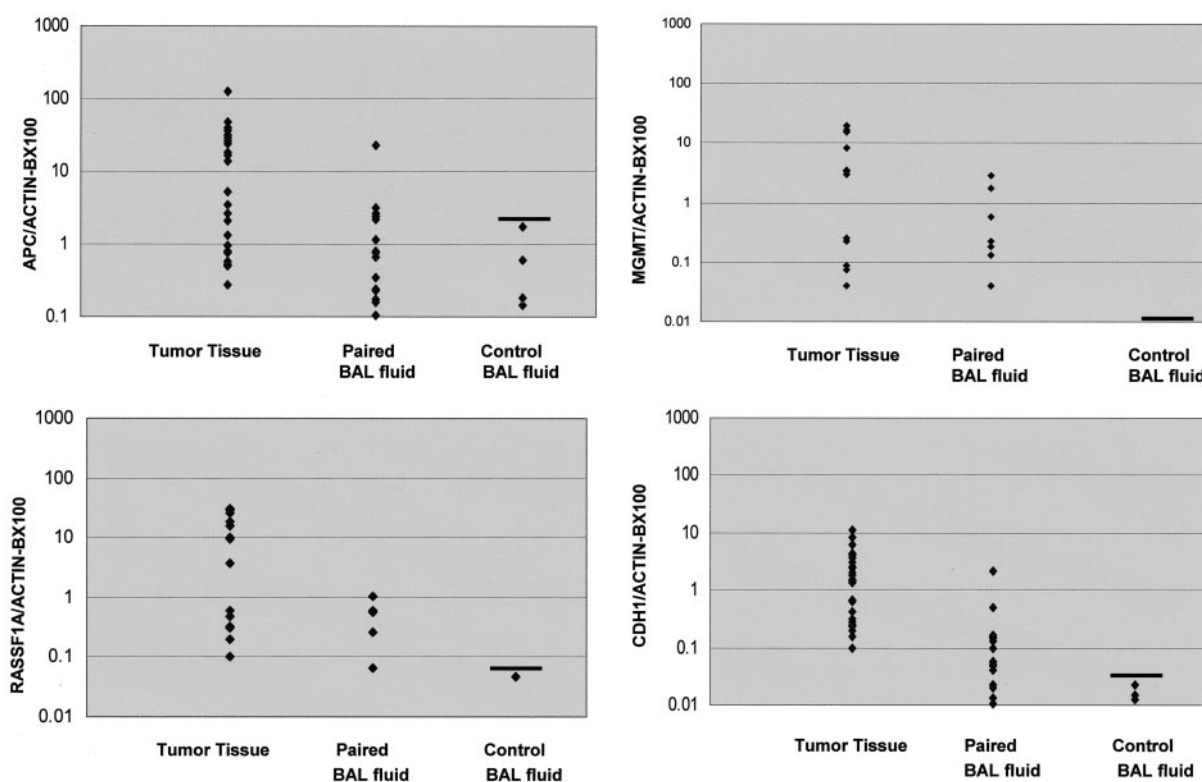

Fig. 1 Representative examples of methylation levels of *APC*, *MGMT*, *RASSF1A*, and *CDH1* in lung cancer tissue matching bronchoalveolar lavage (BAL) samples from lung cancer patients and BAL from noncancer patients. Calculation of the gene of interest:β-actin ratio was based on the fluorescence emission intensity values for both the gene of interest and β-actin obtained by quantitative real-time PCR analysis. The relative amount of methylated promoter DNA is much higher in tumor tissue than the corresponding BAL sample. Zero values are plotted on the X-ordinate for simplicity, although they do not exist on a log scale.

Primary Tumor Tissue   **Paired BAL**

Fig. 2 Summary of methylation of *CDH1*, *RASSF1A*, *APC*, *MGMT*, *GSTP1*, *RAR*-β2, *p16*, and *ARF* in 31 corresponding tumor tissue (*left*) and bronchoalveolar lavage (BAL) samples (*right*). *Black boxes* represent samples that are methylated; *white boxes* represent samples devoid of methylation.

33% (1 of 3) for *GSTP1*, 14% (1 of 7) for *p16*, and 0% (0 of 3) for *RAR*-β2 (Table 2). The identical methylation pattern was found between primary tumor and matched BAL samples as shown in Fig. 2. As seen in tumor samples, the median methylation level for *APC* was the highest (median, 12.83; range, 0.000–23.282) in BAL samples (Fig. 1). Overall, we found no correlation between the methylation index (MI = total number of genes methylated/total number of genes analyzed) and any of the clinicopathological characteristics, *i.e.,* tumor type, grade and stage in BAL samples (data not shown).

Discussion

In this study, all 31 of primary lung tumors harbored CpG island hypermethylation of at least one of eight cancer-related genes. Three commonly methylated genes (*CDH1*, *APC*, and *RASSF1A*) accounted for 80% of our positive cases. Sixty-eight percent of patients with aberrant methylation in primary tumors also exhibited hypermethylation in BAL DNA. It is noteworthy that detection of promoter methylation in BAL is a specific event; *i.e.,* (*a*) aberrant methylation was not detected in any of the 10 control BAL samples with the exception of negligible levels in three genes for which a cutoff was developed; (*b*) the identical methylation profiles were found in the corresponding tumor; and (*c*) aberrant methylation was not detected in the BAL of lung cancer patients without methylation in the corresponding tumor.

The development of real-time PCR has simplified the study of genes inactivated by promoter hypermethylation in human cancer. It is a highly sensitive assay that is capable of detecting methylated alleles in the presence of a 1000-fold excess of unmethylated alleles. However, it is more stringent and maybe more specific because in addition to the two PCR primers, the

fluorescent-labeled hybridization probe has to anneal correctly between the two primers. The quantitative MSP is often more sensitive than conventional MSP but varies based on the promoter, primers, and condition. In a previous study, based on conventional MSP, we detected methylated *p16* alleles in the BAL fluid from 63% of the patients with a methylated primary tumor (18). In the present study, *p16* was methylated in only 14% of BAL samples with a methylated primary tumor. The reason for this discrepancy may be attributable to primer design, but it should be noted that the sample size was different in both studies, and tumor stages and grade also differed.

Several studies using different approaches have demonstrated promoter hypermethylation of *CDH1* (40–55%), *APC* (46–96%), *RASSF1A* (30–40%), *p16* (25%–41%), *MGMT* (16–27%), *RAR*-β (40–43%), *GSTP1* (7–12%), and *ARF* (6–8%) in lung cancer (11, 22). We observed a higher frequency of methylation for *CDH1* (87%), *RASSF1A* (45%), and *MGMT* (39%) in primary lung tumors. Again, these results may reflect a different sensitivity for our assay. Consistent with our findings, a recent study demonstrated *MGMT* promoter methylation in 38% of their cases that were examined by quantitative MSP (23). Although, methylation of *p16*, *GSTP1*, *ARF*, and *APC* were similar to previous reports (11), we found methylation for *RAR*-β2 in 10% of primary tumors in contrast to a higher frequency in another study (24). We do not have a clear explanation for this discrepancy, but assay sensitivity and small sample size may be the causes.

To our knowledge, there have been only two studies (18, 25) using limited number of methylation markers for the detection of lung cancer using BAL samples. Thus, our methylation assay using eight different genes in the same BAL DNA confirms and extends previous observations. The specificity of four

genes (*MGMT*, *RAR*-β*2*, *GSTP1*, and *p16*) was 100% with undetectable methylation levels in all normal samples. The detection of tumor molecular signatures in body fluids has implications for the identification of high-risk subjects, patients with preinvasive or early-stage lesions, and for monitoring residual disease. Molecular approaches characterized by a high specificity have variable sensitivity, perhaps because of the presence of only minimal tumor DNA in BAL or because of a high level of contamination with normal DNA. Several approaches to improve assay sensitivity have been applied to tumor tissue, plasma, sputum, stool, bronchoalveolar lavage, and bronchial brushings. Sensitivity was improved over conventional MSP (20) by performing a semi-nested MSP after a DNA preamplification step (26) or a nested two-stage PCR (17). We can improve the sensitivity of quantitative MSP in BAL fluid by the following: (*a*) isolating aberrant cells from BAL before DNA extraction; (*b*) adding nested or seminested PCR before quantitative MSP; and (*c*) increasing the number of lung cancer-specific markers. More sensitive assays, however, generally result in imperfect specificity and must be validated in clinical samples.

Exfoliative material (present in sputum, bronchoalveolar lavage, and bronchial brushings) offers diagnostic access, but the sensitivity of current cytological tests is low. Diagnostic tools that would provide high specificity and sensitivity would clearly be of enormous benefit to patients, particularly if the specimens could be obtained by noninvasive means. To this end, the detection of aberrant methylation in sputum in addition to BAL DNA may offer a promising approach for the noninvasive diagnosis of lung cancer. As seen in saliva for the detection of head and neck cancer (27), these approaches are highly specific and correlate with tumor methylation status. Apart from lung cancer detection, it would be interesting to see whether the detection of aberrant methylation in the sputum or BAL can be used in the monitoring of disease progress after curative surgery. If methylated DNA disappears shortly in these samples after curative surgery, the reappearance of these markers may suggest recurrence of disease that may require more intensive screening and aggressive treatment. Additional studies are necessary to elucidate the role of detecting aberrant methylation in such samples as a tool for early detection and surveillance of lung cancer.

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