Molecular Detection of Tumor Cells in Bronchoalveolar Lavage Fluid From Patients With Early Stage Lung Cancer

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Background: **Conventional cytologic analysis of sputum is an insensitive test for the diagnosis of non-small-cell lung cancer (NSCLC). We have recently demonstrated that polymerase chain reaction (PCR)-based molecular methods are more sensitive than cytologic analysis in diagnosing bladder cancer. In this study, we examined whether molecular assays could identify cancer cells in bronchoalveolar lavage (BAL) fluid.** ***Methods:*** **Tumor-specific oncogene mutations, CpG-island methylation status, and microsatellite alterations in the DNA of cells in BAL fluid from 50 consecutive patients with resectable (stages I through IIIa) NSCLC were assessed by use of four PCR-based techniques.** ***Results:*** **Of 50 tumors, 28 contained a p53 mutation, and the identical mutation was detected with a plaque hybridization assay in the BAL fluid of 39% (11 of 28) of the corresponding patients. Eight of 19 adenocarcinomas contained a K-ras mutation, and the identical mutation was detected with a mutation ligation assay in the BAL fluid of 50% (four of eight) of the corresponding patients. The p16 gene was methylated in 19 of 50 tumors, and methylated p16 alleles were detected in the BAL fluid of 63% (12 of 19) of the corresponding patients. Microsatellite instability in at least one marker was detected with a panel of 15 markers frequently altered in NSCLC in 23 of 50 tumors; the identical alteration was detected in the BAL fluid of 14% (three of 22) of the corresponding patients. When all four techniques were used, mutations or microsatellite instability was detected in the paired BAL fluid of 23 (53%) of the 43 patients with tumors carrying a genetic alteration.** ***Conclusion:*** **Although still limited by sensitivity, molecular diagnostic strategies can detect the presence of neoplastic cells in the proximal airway of patients with surgically resectable NSCLC. [J Natl Cancer Inst 1999;91:332–9].**

Lung cancer is the leading cause of cancer-related deaths among both males and females in the United States *(1)*. It was estimated that more than 170 000 new cases of primary lung cancer would be diagnosed in this country in 1998 and that more than 160 000 people will die of the disease *(1)*. Surgical resection remains the most effective form of treatment for non-small-cell lung cancer (NSCLC); however, at the time of diagnosis, more than 65% of all patients will have advanced disease that is no longer amenable to curative therapy. In addition, a large percentage of patients undergoing surgical resection ultimately die of recurrent NSCLC, demonstrating the frequent presence of occult metastatic disease at the time of diagnosis *(2)*. Attempts to improve lung cancer survival have focused on eliminating the cause (cigarette smoking), preventing the disease in high-risk groups (chemoprevention trials), diagnosing the disease at an early curable stage, and developing new adjuvant and neoadjuvant protocols. Each of these strategies has met with limited success.

Conventional cytologic analysis of sputum has not improved overall survival when added to a screening program of annual chest radiography for the early diagnosis of lung cancer *(3,4)*. Molecular techniques have identified tumor-specific oncogene mutations in cytologically negative sputum samples obtained from patients before the diagnosis of lung cancer *(5)*. However, the impressive sensitivities achieved by molecular techniques in this series were obtained in patients with dysplasia found by cytologic analysis of sputum.

In the present study, we examined the frequency of tumor-specific oncogene mutations, CpG-island methylation status, and microsatellite alterations in 50 consecutive, prospectively collected bronchoalveolar lavage (BAL) samples from patients with resectable NSCLC.

Materials and Methods

Sample Collection

Primary tumor, blood, and BAL fluid were collected prospectively from 50 consecutive patients undergoing surgical resection of NSCLC by a single surgeon at The Johns Hopkins Hospital or the Johns Hopkins Bayview Medical Center. BAL fluid was collected during flexible bronchoscopy performed at the time of pulmonary resection. Lavage was performed after guiding the bronchoscope into the segmental bronchus of the pulmonary lobe most likely to contain the tumor on the basis of earlier bronchoscopic and/or radiographic evaluation. Aliquots of 20 mL of warm saline were injected into the lobe until a volume of at least 30 mL was collected in a specimen trap. The BAL fluid was then transported to the laboratory on ice and centrifuged at 1800*g* for 10 minutes at 4 °C. The cell pellet was then collected and stored at −80 °C. Lymphocytes were collected from blood and used as a source of normal DNA. Tumor samples were promptly frozen at −80 °C after initial gross pathologic examination.

This research protocol was approved by the Joint Committee on Clinical Investigation of The Johns Hopkins School of Medicine in accord with an

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See "Notes" following "References."

assurance filed with the U.S. Department of Health and Human Services. Written informed consent was obtained from all patients.

Pathologic stage was determined by the revised International System for Staging Lung Cancer *(6)*. Tumors were classified as peripheral, parenchymal, or central on the basis of preoperative radiographic studies, bronchoscopic or operative findings, and pathologic analysis. Peripheral tumors were located at or within 1 cm of the visceral pleura. Central tumors were visible on bronchoscopy or involved the main lobar bronchus upon pathologic examination. The remaining tumors were classified as parenchymal.

Portions of the primary tumor were cut into 7-μm sections, stained with hematoxylin–eosin, and examined by light microscopy. Additional 12-μm sections were cut and placed in a mixture of 1% sodium dodecyl sulfate and proteinase K (0.5 mg/mL) at 48 °C overnight. Tumors with a low neoplastic cellularity (<70%) were further microdissected to remove contaminating normal cells. The BAL cell pellet was also digested in 1% sodium dodecyl sulfate/proteinase K (0.5 mg/mL) as described above. DNA was then extracted from either type of sample with phenol/chloroform and precipitated with ethanol.

p53 Sequencing

A 1.8-kilobase fragment of the p53 gene (also known as TP53) (exons 5 through 9) was amplified from primary tumor DNA in all 50 patients by polymerase chain reaction (PCR) as described previously *(7,8)*. The PCR products were purified and sequenced directly by cycle sequencing (Amplicycle sequencing kit; The Perkin-Elmer Corp., Branchburg, NJ) by use of appropriate sequencing primers *(7,8)*. The products of the sequencing reactions were then separated by electrophoresis in 8 *M* urea/6% polyacrylamide gels, fixed, and exposed to film.

In addition, all 50 tumors were sequenced by use of the GeneChip® p53 assay (Affymetrix Inc., Santa Clara, CA) by the manufacturer's protocol. Exons 2 through 11 of the p53 gene from each tumor and the normal reference DNA were amplified as 10 amplicons in a single PCR. Each PCR mixture contained 250 ng of genomic DNA, 5 μL of the p53 primer set (Affymetrix Inc.), 10 U of AmpliTaq Gold (The Perkin-Elmer Corp.), PCR buffer II (The Perkin-Elmer Corp.), 2.5 m*M* $MgCl_2$, and all four deoxynucleoside triphosphates (dNTPs) (each at 0.2 m*M*) in a final volume of 100 μL. The reaction tubes were heated to 95 °C for 10 minutes and then subjected to 35 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 45 seconds and then to a final extension of 10 minutes at 72 °C. Forty-five microliters of amplified tumor and reference DNA was then fragmented with 0.25 U of fragmentation reagent (Affymetrix Inc.) at 25 °C for 18 minutes in a solution of 2.5 U of calf intestine alkaline phosphatase, 0.4 m*M* EDTA, and 0.5 m*M* Tris acetate (pH 8.2), followed by heat inactivation at 95 °C for 10 minutes.

The fragmented amplicons were then 3′ end labeled with fluorescein-labeled dideoxyadenosine 5′-monophosphate. Fifty microliters of fragmented DNA was incubated at 37 °C for 45 minutes in 100 μL containing 25 U of terminal deoxynucleotidyltransferase (Boehringer Mannheim Biochemicals, Indianapolis, IN), terminal deoxynucleotidyltransferase buffer, and 10 μ*M* fluorescein-N6-ddATP (where ddATP is dideoxyadenosine 5′-triphosphate), followed by heat inactivation at 95 °C for 5 minutes. The fluorescein-labeled sample was then hybridized in 0.5 mL containing 6× SSPE (1× SSPE = 0.15 *M* NaCl/10 m*M* sodium phosphate [pH 7.4]/1 m*M* EDTA), 0.05% Triton X-100, 1 mg of acetylated bovine serum albumin, and 2 n*M* control oligonucleotide F1 (Affymetrix Inc.) to the p53 probe array for 30 minutes at 45 °C. The probe array was washed four times with wash buffer A and then scanned by laser (HP GeneArray Scanner; Hewlett Packard, Wilmington, DE). The emitted light intensity is proportional to bound tumor DNA at each location on the probe array and was used by the GeneChip software to compare the nucleotide sequence of the tumor DNA to the reference sequence. Intensity patterns differing from the reference sample were quantified, and sites containing mutant bases were then displayed by the GeneChip software with an assigned score (range, 0–32) corresponding to the difference in intensity. The magnitude of the score also varied with the number of oligonucleotide probe sets present at a specific site. All sites were covered by two probe sets (one sense and one antisense). In addition, 300 known missense mutations in the p53 gene were also covered by an additional 14 probe sets. Scores exceeding an empirically determined threshold (a score of 13) were designated as a mutation. Samples with mutations detected by the GeneChip p53 assay that were not present on the direct sequencing gel were further confirmed by repeating the direct sequencing of the involved exon.

Oligonucleotide Hybridization

BAL samples from patients found to have a p53 mutation in their primary tumor were analyzed for the presence of tumor-specific p53 mutations with oligonucleotide plaque hybridization. The exon containing the p53 mutation was amplified for 35 cycles (94 °C for 30 seconds, 58 °C for 1 minute, and 70 °C for 1 minute) from the primary tumor, lymphocytes, and BAL fluid with primers containing *Eco*RI sites (exon 5, 4S [5′-GTAGGAATTCACTTGTGCCCTGACTT-3′] and 5ASECO [5′-ATCGAATTCAGACCTAAGAGCAAT-3′]; exon 6, 6SECO [5′-ATCGAATTCCCCAGGCCTCTGATT-3′] and 6ASECO [5′-ATCGAATTCGAGACCCAGTTGCAA-3′]; exon 7, 7SECO [5′-ATCGAATTCTCCCCAAGGCGCACT-3′] and 7ASECO [5′-ATCGAATTCGGTAAGAGGTGGGCC-3′]; exon 8, 8SECO [5′-ATCGAATTCAAATGGACAGGTAGA-3′] and 8ASECO [5′-ATCGAATTCTTGTCCTGCTTGCTT-3′]; and exon 9, 9SECO [5′-ATCGAATTCTCTGTTGCTGCAGAT-3′] and 9ASECO [5′-ATCGAATTCTGAGGTCACTCACCTGGA-3′]). The PCR products were then cloned into a λ bacteriophage vector (Stratagene Cloning Systems, La Jolla, CA) and amplified further in *Escherichia coli* cells *(7)*. Between 500 and 1000 clones were transferred to nylon membranes (NEN Research Products, Boston, MA) and hybridized with ^{32}P-end-labeled oligonucleotide probes specific for the p53 mutation identified in each patient's primary tumor as previously described (theoretical sensitivity = 1 : 1000 to 1 : 10 000) *(5)*. Hybridizing plaques signified the presence of a mutant p53 gene. After stripping, all filters were hybridized with a wild-type p53 oligonucleotide to identify plaques containing the inserted p53 fragments. All assays included both positive (tumor DNA with the mutation) and negative (tumor DNA with a different mutation and water alone) controls.

K-ras Mutation Ligation Assay

A 270-base-pair fragment containing exon 1 of the K-ras gene was amplified from the tumor and BAL fluid DNA of 27 patients with nonsquamous NSCLC (adenocarcinoma [n = 19], bronchoalveolar carcinoma [n = 6], adenosquamous carcinoma [n = 1], and large-cell carcinoma [n = 1]) as described previously *(9)*. The K-ras gene was also amplified from the tumor and BAL fluid DNA of 17 of the 23 patients with squamous cell cancer even though these tumors have been demonstrated previously to only rarely harbor K-ras gene mutations *(10)*. This fragment was used as the template for four separate mutation ligation assays (theoretical sensitivity = 1 : 100 to 1 : 1000) to detect all possible mutations at K-ras codon positions 12a, 12b, 13a, and 13b, as previously described *(11)*.

K-ras-Enriched PCR

A sensitive mutant-enriched PCR technique was also used to screen the tumor DNA of 27 patients with nonsquamous NSCLC and all 50 BAL samples for K-ras mutations *(12)*. In the first round of amplification, 1 μg of tumor DNA was amplified in 25 μL containing 60 ng of primers, 2.5 U of *Taq* polymerase (Sigma Chemical Co., St. Louis, MO), all four dNTPs (each at 0.2 m*M*), 50 m*M* KCl, 10 m*M* Tris–HCl (pH 8.3), and 1.5 m*M* $MgCl_2$ for 20 cycles (94 °C for 1 minute, 56 °C for 1 minute, and 72 °C for 1 minute). Five microliters of the first amplification products was then digested for 3 hours at 60 °C with *Bst*NI. The digested PCR products were then subjected to a second round of amplification (20 cycles) and digestion with *Bst*NI as described above. The products of the second *Bst*NI digestion were then separated by electrophoresis on nondenaturing polyacrylamide gels and stained with ethidium bromide [theoretical sensitivity = $1 : 10^4$ to $1 : 10^6$ *(12,13)*].

p16 Methylation-Specific PCR

Methylation-specific PCR was used to determine the methylation status of the CpG island of p16 in all 50 tumors and in the matched BAL fluid from 49 patients as described *(14)*. One microgram of tumor DNA or BAL-fluid DNA was modified with sodium bisulfite and precipitated with ethanol. The modified DNA was then amplified by use of both methylated- and unmethylated-specific primers as described *(14)*. PCR products were loaded directly onto nondenaturing 6%–8% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination [theoretical sensitivity = 1 : 1000 *(14)*].

Microsatellite Analysis

Fifteen markers for microsatellite analysis were selected on the basis of previous work (our unpublished results), demonstrating frequent microsatellite al-

terations (expansion or deletion of a repeat unit) in NSCLC *(15)*. Tumor DNA and normal lymphocyte DNA from all 50 patients were examined with the entire panel of 15 microsatellite markers. BAL samples from 24 patients were analyzed with the 15-marker panel, whereas the remaining 26 BAL samples were examined only when the corresponding tumor demonstrated microsatellite instability. Oligonucleotides were obtained from Research Genetics (Huntsville, AL) or synthesized from sequences in the Genome Database (D3S1340, D3S1351, D8S321, D9S242, D11S488, D20S82, D20S85, CSFIR-1, and ACTβ-2) with the exception of the following loci: UT5307 (5′-GGATATAGCTGGCAATGGC-3′ [sense] and 5′-TCGGAATGCCTACTTCCCAG-3′ [antisense]), UT5320 (5′-ACCGACAGACTCTTGCCTC-3′ [sense] and 5′-TTGAGATGACCCTGAGACTG-3′ [antisense]), L17686 (5′-GCACCAATGCTCCAGAAATG-3′ [sense] and 5′-TCATGGTGCCATGATAGGAG-3′ [antisense]), L17835 (5′-TTGCACCACTATACTCCAGC-3′ [sense] and 5′-TCAGTTTAAGGTTCTCACCTG-3′ [antisense]), G29028 (5′-GCAGTGAGCTGAGATAATGC-3′ [sense] and 5′-TCACTAGCAGATGCGATAATG-3′ [antisense]), and G08460 (5′-TGGCGCTGATGCTCCACATTC-3′ [sense] and 5′-CTGGCTGACAGATAAAGCACT-3′ [antisense]). One marker from each primer pair was labeled with T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA). PCR amplification was performed with 60 ng of DNA isolated from the tumor, normal lymphocytes, and BAL fluid as described above. Products were separated in 8% denaturing urea/polyacrylamide/formamide gels, followed by autoradiography [theoretical sensitivity = 1 : 100 to 1 : 200 *(16)*].

Statistical Analysis

Groups were compared by use of the Fisher exact test (two-tailed). All *P* values are two-sided.

Results

Primary Tumor

The clinical characteristics of 50 patients undergoing pulmonary resection for NSCLC are shown in Table 1. Twenty-eight patients had stage I disease (18 with stage IA and 10 with stage IB), 15 patients had stage II NSCLC (two with stage IIa and 13 with stage IIb), and seven patients had stage IIIa disease. Mean tumor size was 3.7 cm. The histologic type of the 50 tumors included squamous cell cancer (n = 23), adenocarcinoma (n = 19), bronchoalveolar carcinoma (n = 6), adenosquamous carcinoma (n = 1), and large-cell carcinoma (n = 1). p53 mutations were detected in tumors from 28 (56%) of 50 patients with NSCLC and were found statistically significantly ($P = .014$) more often in squamous cell cancer (19 [83%] of 23 patients) than in adenocarcinoma (eight [42%] of 19 patients). Nineteen (68%) of 28 p53 mutations in tumors were detected with direct cycle sequencing, and 22 (81%) of 27 mutations in tumors were detected with the p53 GeneChip. Mutations in the K-ras gene were identified in 10 (37%) of 27 patients with non-squamous-cell NSCLC, including eight (42%) of 19 patients with adenocarcinoma and two (33%) of six patients with bronchoalveolar carcinoma. Identical results were obtained with the mutation ligation assay and the K-ras mutation-enriched PCR. K-ras mutations were detected in two (12%) of 17 squamous cell cancers analyzed with the mutation ligation assay.

Methylated p16 alleles were present in 19 (38%) of 50 tumors tested. Microsatellite instability was identified in 23 (46%) of 50 tumors by use of the panel of microsatellite markers. These microsatellite "shifts" were seen in seven tumors (14%) with L17686; five tumors (10%) with D20S82; four tumors (8%) with UT5320; three tumors (6%) with L17835; two tumors (4%) with D8S321, D20S85, D9S242, and D3S1351; and one tumor (2%) with each of the following sequences, UT5307, G29028, D11S488, ACTβ-2, or G08460. The relationship between pathologic stage and cell type and the presence of p53 mutations, K-ras mutations, microsatellite instability, and methylated p16 alleles is shown in Table 1.

BAL Fluid

p53 mutant cells were detected in the BAL fluid of 11 (39%) of the 28 patients with p53 mutations in their primary tumor (Table 2 and Fig. 1). Four of the 12 tumors with K-ras mutations had the identical mutation detected in the BAL fluid (Fig. 2). The mutation ligation assay detected mutations in four samples, whereas the mutant-enriched PCR technique detected a mutation in only one sample (also detected by the ligation assay). None of the BAL samples from the 32 patients with a wild-type K-ras tumor contained a K-ras mutation when the mutation ligation assay was used. Only one of these 32 BAL samples contained a

Table 1. Frequency of p53 and K-ras mutations, any microsatellite instability, and p16 gene methylation status in the primary tumor from patients with resectable non-small-cell lung cancer

		No. of patients/total No. (%)			
Parameter	n*	p53 mutations	K-ras mutations†	Microsatellite instability‡	p16 methylation status
Pathologic stage§					
IA	18	11/18 (61)	2/15 (13)	7/18 (39)	8/18 (44)
IB	10	5/10 (50)	4/8 (50)	4/10 (40)	4/10 (40)
IIA	2	1/2 (50)	1/2 (50)	0/2	2/2 (100)
IIB	13	7/13 (54)	4/12 (33)	7/13 (54)	3/13 (23)
III	7	4/7 (57)	1/7 (14)	5/7 (71)	2/7 (29)
Total samples	50	28/50 (56)	12/44 (27)	23/50 (46)	19/50 (38)
Tumor cell type					
Squamous cell	23	19/23 (83)	2/17 (12)	14/23 (61)	9/23 (39)
Adenocarcinoma	19	8/19 (42)‖	8/19 (42)	7/19 (37)	9/19 (47)
Bronchoalveolar	6	0/6¶	2/6 (33)	1/6 (17)	1/6 (17)
Other	2	1/2 (50)	0/2	1/2 (50)	0/2
Total samples	50	28/50 (56)	12/44 (27)	23/50 (46)	19/50 (38)

*n = total number of patients with tumor of given stage or cell type.

†K-ras mutation analysis performed on 17 of 23 squamous cell cancers.

‡Number of tumors containing at least one microsatellite alteration in panel of 15 markers tested.

§Staging using the revised International System for Staging Lung Cancer *(6)*.

‖Two-sided $P = .014$ versus squamous cell cancers.

¶Two-sided $P = .0008$ versus squamous cell cancers.

Table 2. Frequency of tumor-specific p53 and K-ras mutations, any microsatellite instability, and p16 gene methylation status in the bronchoalveolar lavage (BAL) fluid from patients with resectable non-small-cell lung cancer

Parameter	n*	No. of BAL samples with detectable alteration/No. of BAL samples analyzed with corresponding alteration in primary tumor (%)				
		p53 plaque hybridization assay [1 : 1000]†	K-ras mutation ligation assay [1 : 200]†	Microsatellite instability [1 : 20]†	p16 methylation status [unknown]†	Any assay
Pathologic stage						
IA	18	1/11 (9)	1/2 (50)	1/7 (14)	5/8 (63)	7/17 (41)
IB	10	3/5 (60)	2/4 (50)	0/4	2/4 (50)	5/8 (63)
IIA	2	1/1 (100)	1/1 (100)	0/0	2/2 (100)	2/2 (100)
IIB	13	4/7 (57)	0/4	1/6 (17)	2/3 (67)	7/11 (64)
III	7	2/4 (50)	0/1	1/5 (20)	1/2 (50)	2/5 (40)
Total samples	50	11/28 (39)	4/12 (33)	3/22 (14)	12/19 (63)	23/43 (53)
Tumor cell type						
Squamous cell	23	6/19 (32)	0/2	3/13 (23)	5/9 (56)	11/21 (52)
Adenocarcinoma	19	4/8 (50)	4/8 (50)	0/7	7/9 (78)	11/17 (65)
Bronchoalveolar	6	0/0	0/2	0/1	0/1	0/4
Other	2	1/1 (100)	0/0	0/1	0/0	1/1 (100)
Total samples	50	11/28 (39)	4/12 (33)	3/22 (14)	12/19 (63)	23/43 (53)
Tumor site						
Peripheral	27	5/16 (31)	3/9 (33)	2/12 (17)	6/10 (60)	11/25 (44)
Parenchymal	13	2/8 (25)	1/2 (50)	0/6	3/5 (60)	6/11 (55)
Peripheral/parenchymal	40	7/24 (29)‡	4/11 (36)	2/18 (11)	9/15 (60)	17/36 (47)
Central	10	4/4 (100)	0/1	1/4 (25)	3/4 (75)	6/7 (86)
Total samples	50	11/28 (39)	4/12 (33)	3/22 (14)	12/19 (63)	23/43 (53)

*n = total number of patients with tumor of given stage, cell type, or site.

†Working limit of sensitivity defined as "proven" upper limit of sensitivity (i.e., detection of the molecular alteration in BAL fluid compared with presence of mutant alleles by quantitative p53 plaque hybridization assay) (*see* Table 3).

‡Two-sided $P = .032$ versus central tumors.

K-ras mutation when the mutant-enriched PCR was used. Methylated p16 alleles were detected in 12 of the 19 samples from patients with a methylated primary tumor (Fig. 3). None of the 30 BAL samples analyzed from patients with p16 unmethylated tumors contained methylated p16 alleles.

Tumor-specific microsatellite alterations (instability) were detected in only three (14%) of the 22 BAL fluids from patients with alterations present in the primary tumor (Fig. 4). We were unable to amplify a PCR product from one BAL sample with microsatellite instability detected in the corresponding tumor with marker D8S321. The BAL fluid from 24 patients was analyzed with the entire panel of 15 microsatellite markers. Microsatellite instability was present in 10 of these 24 tumors. However, tumor-specific microsatellite alterations were detected in the BAL fluid from only one (10%) of these 10 patients. Four (17%) of the 24 BAL samples contained a microsatellite alteration not present in the corresponding tumor.

One or more of the molecular assays were used to examine the BAL fluid from the 43 patients whose tumor displayed at least one molecular marker. Tumor-specific mutations or microsatellite instability was detected with at least one assay in 23 (53%) of these 43 BAL fluid samples.

Tumor location strongly influenced the ability to detect molecular alterations in BAL fluid. Tumor-specific p53 mutations were detected significantly more often in BAL fluid from patients with centrally located tumors than in BAL fluid from patients with tumors located in the parenchyma or peripherally (100% versus 29%; $P = .032$). Tumor size also played a significant role in detecting molecular alterations in the BAL fluid. Only one of 11 stage IA tumors had a detectable p53 mutation in the BAL fluid, and this tumor was 3.0 cm in size. The p53 plaque assay detected molecular alterations in the BAL fluid from patients with stage II tumors significantly more often than in the BAL fluid from patients with stage IA tumors (62% versus 9%; $P = .045$). The detection rate for tumor-specific p53 mutations in BAL fluid was similar between samples obtained from patients with squamous cell carcinoma (32%) and samples obtained from patients with adenocarcinoma (50%).

The ratio of mutant clones to total clones in the BAL fluid examined with the oligonucleotide plaque hybridization ranged from one in seven clones to one in 400 clones (Table 3). The ratio of mutant to normal clones was lower than one in 100 clones in all three positive BAL fluid specimens from patients with adenocarcinoma of the lung. In contrast, in five of the six patients with squamous cell carcinoma and in the one patient with large-cell carcinoma, the ratio of mutant to total clones in the BAL fluid was greater than one in 100 clones. The K-ras mutation ligation assay detected mutant cells to one in 200 cells. Methylated p16 alleles were detected in BAL fluid from 12 of the 19 patients with a methylated primary tumor. The p53 plaque assay was also positive (sample 971, one in seven clones; sample 1174, one in 400 clones) in just two of these samples (Table 3), giving a wide range for the sensitivity limit of the methylation assay. The sensitivity of the microsatellite assay was considerably lower as expected, detecting tumor-specific alterations in the BAL fluid only when the number of tumor cells was greater than one in 20 (5%) (Table 3).

Discussion

There is no effective test available to screen high-risk groups for lung cancer despite being the leading cause of cancer deaths among both men and women in the United States *(1,3,4)*. Conventional cytologic analysis of sputum was no more effective

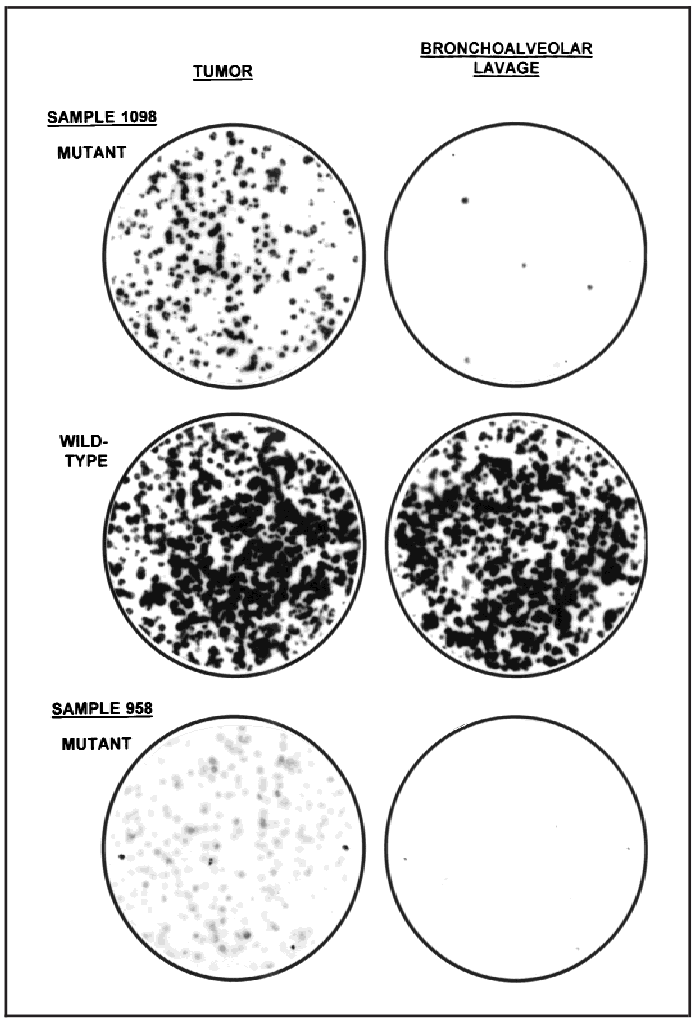

Fig. 1. Detection of mutant p53 alleles in bronchoalveolar lavage (BAL) fluid from patients with non-small-cell lung cancer. Nylon membranes were hybridized with both mutant-specific and wild-type oligonucleotides. A fragment of the p53 gene was amplified from DNA from the clinical samples as indicated, cloned, and transferred to nylon membranes. BAL fluid contained a few mutant p53 alleles (1 : 170) in sample 1098 but not in sample 958.

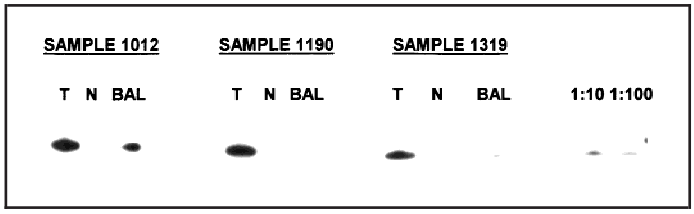

Fig. 2. Detection of mutant K-ras alleles in bronchoalveolar lavage (BAL) fluid with a mutation ligation assay. Mutant K-ras alleles (Cys^{12}) detected in BAL fluid in samples 1012 and 1319 but not in sample 1190. T = tumor; N = normal (wild-type). Lanes 1 : 10 and 1 : 100 contain a dilution of tumor DNA with K-ras mutation (Cys^{12}) with wild-type DNA from same patient **(far right).** Cys = cysteine (codon).

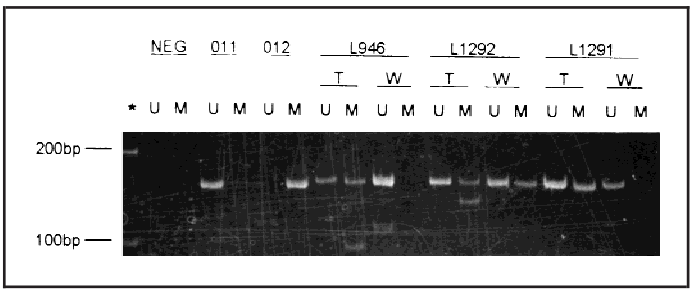

Fig. 3. Detection of methylated p16 alleles in tumor (T) and corresponding bronchoalveolar lavage (BAL) fluid (W) with methylation-specific polymerase chain reaction (PCR). Primer sets used for amplification are designated as unmethylated (U) or methylated (M). NEG shows the products of a negative control reaction devoid of DNA, and 011 and 012 show the products from positive control cell lines with unmethylated and methylated p16 alleles, respectively. Methylated p16 alleles were detected in the BAL fluid in sample L1292 but not in the BAL fluid in samples L946 and L1291. Additional bands represent nonspecific PCR products. * = marker lane; bp = base pairs.

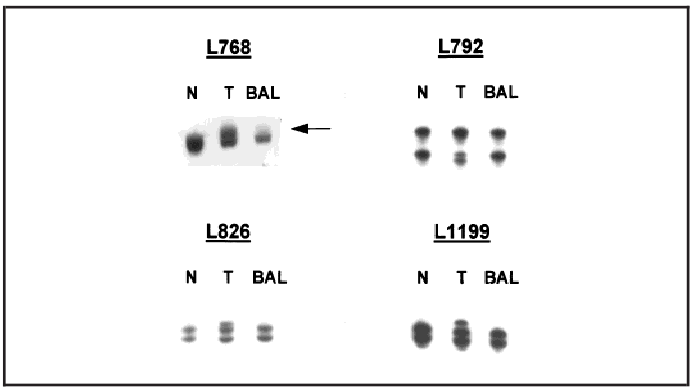

Fig. 4. Identification of microsatellite instability in clinical samples. Microsatellite instability (deletion of repeat units) in both tumor (T) and bronchoalveolar lavage (BAL) fluid in a patient with non-small-cell lung cancer (sample L768). Microsatellite alterations in primary tumor but not in BAL fluid (samples L792, L826, and L1199) were identified. N = normal lymphocyte DNA.

than annual chest radiographs in detecting lung cancer in several large prospective randomized trials *(3,4).* Periodic screening with chest radiographs has not been demonstrated to decrease lung cancer mortality; however, their efficacy in lung cancer screening remains controversial *(17).* In our study, four of the most promising molecular assays were used to evaluate BAL fluid obtained from 50 patients with resectable NSCLC and detected cancer cells in 53% of samples when the patient's primary tumor was positive for a molecular marker.

Cytologic analysis of sputum is currently used clinically to screen for or diagnose lung cancer; however, this test has several limitations. The majority of cancers detected by cytologic analysis are squamous cell cancers, whereas the most common cell type in NSCLC is adenocarcinoma *(18).* Moreover, cytologic analysis is much more accurate at detecting central lesions than peripheral cancers, which also occur more commonly *(19).* Bechtel et al. *(19)* reported on 51 patients with NSCLC detected by sputum cytologic testing. Eighty-six percent of the lesions were squamous cell cancer, and all but one of the tumors were centrally located and visible by bronchoscopic examination *(19).* BAL has been used to improve the detection rate for peripheral lesions. Thirty-three percent of patients with peripheral NSCLC were detected by BAL in a recent series (tumor stage unknown in this group), and the sensitivity of BAL for diagnosing disseminated NSCLC may be as high as 88% *(20,21).*

Several recent innovations, including the automated image cytometer or staining for heterogeneous ribonuclear protein overexpression, may improve the detection rate of cytologic techniques *(22,23).* We elected to evaluate the ability of current molecular techniques at detecting tumor-specific molecular al-

Table 3. Comparison of p53 mutant alleles present in bronchoalveolar lavage (BAL) fluid and corresponding results of molecular assays with variable sensitivities

Sample No.	p53 mutation*	Cell type	Tumor site	No. of mutant alleles/ total No. of clones in BAL	Microsatellite instability†	K-ras mutation ligation assay†	p16 methylation assay†
971	Intron 6, A to G at 846	Squamous	Central	1/7	+		+
768	CGC to CAC at 175	Squamous	Peripheral	1/15	+		
847	ATC to TTC at 195	Squamous	Peripheral	1/20			
1011	GAG to TAG at 339	Large cell	Peripheral	1/24	−		
775	AGG to AGT at 249	Squamous	Parenchymal	1/45	−		
1216	CAG to TAG at 165	Squamous	Central	1/45	−		
1049	ACC to ATC at 253	Adenocarcinoma	Central	1/115			
1098	CGC to CTC at 158	Adenocarcinoma	Peripheral	1/170		+	
826	TGC to GGC at 176	Squamous	Central	1/200	−		
1174	GCC to CCC at 159	Adenocarcinoma	Parenchymal	1/400	−	−	−

*For all samples except 971, the numbers at right refer to nucleotide numbers of the published p53 complementary DNA sequence; for sample 971, the number at right refers to the intron 6 nucleotide number downstream from the 3′-end of exon 6 in the p53 gene.

†+ = positive; − = negative. If blank, the depicted molecular alteration was absent in the primary tumor and could not be evaluated.

terations in the BAL fluid from a group of patients with predominantly peripheral resectable NSCLC. In most cases, the entire BAL sample was used for molecular analysis to enable the completion of all four assays. One limitation to the current study is the unknown diagnostic sensitivity of cytologic analysis in this group of 50 patients to provide a direct comparison between cytologic and molecular techniques in detecting tumor cells in BAL fluid.

Oncogene or tumor suppressor gene mutations have been detected by several investigators *(5,12,13)* in the sputum of patients with primary adenocarcinoma of the lung. We identified K-ras and p53 mutations in eight of 10 sputum samples from patients who were participating in the Johns Hopkins Lung Project and who later developed adenocarcinoma *(5)*. However, each of these samples was selected from the 3% of samples collected in that trial that already had at least mild dysplasia upon cytologic examination. Several additional studies *(12,13)* have reported that K-ras mutations are detectable in the sputum of a high percentage of patients with adenocarcinoma when the primary tumor contains a K-ras mutation. Although K-ras mutations were frequently detected despite negative cytologic findings in most cases, each of these studies included a large number of patients with advanced unresectable lung cancer.

Several techniques have been used to detect K-ras mutations in sputum and stool samples; these techniques include plaque hybridization, the mutation ligation assay, and various mutant-enriched PCR techniques *(5,12,13,24)*. The mutation ligation assay is rapid, requires a single PCR amplification, and has a sensitivity approaching that of the plaque hybridization *(11)*. In this study, the mutation ligation assay was able to detect tumor cells with a sensitivity of at least one in 200 normal cells. Mutant-enriched PCR techniques have a reported sensitivity of at least one in 10^4; however, false-positive results are possible as the error rate of *Taq* polymerase or other thermostable enzymes is surpassed. Yakubovskaya et al. *(12)* used this technique to identify K-ras mutations in normal lung tissue from patients with lung cancer as often as from the primary cancer itself. In addition, 49% of patients with squamous cell cancer and 12% of control patients without cancer had K-ras mutations in their sputum. With a mutant-enriched PCR protocol designed to limit false-positive results, we were unable to improve upon the detection rate compared with the mutation ligation assay.

Although we detected K-ras mutant cells in 50% of patients with adenocarcinoma, molecular screening may still have a role in the early detection of patients with adenocarcinoma. Cytologic screening is notoriously poor in patients with adenocarcinoma, because adenocarcinoma cells are shed into the airway in low numbers *(18,19)*. In the Johns Hopkins Lung Project trial, the lung cancers detected by cytologic screening alone were almost exclusively of the squamous cell type *(18)*. In our study, all of the adenocarcinomas detected by the plaque assay contained fewer than 1% tumor cells in the BAL.

Tumor-specific microsatellite alterations have been used to identify tumor cells in the urine of patients with bladder cancer and in the serum of patients with head and neck and small-cell lung cancer *(25,26)*. In addition, tumor-specific microsatellite alterations have been demonstrated previously in the sputum of several patients, each with small-cell lung cancer and NSCLC *(15,16)*. We reported that this approach could detect approximately one cancer cell against a background of 200 normal cells, suggesting that the use of microsatellite alterations might prove useful as a diagnostic tool in NSCLC *(16)*. The reported frequency of microsatellite instability in NSCLC varies considerably from 2% to 55% in large part depending on the specific microsatellite loci examined *(27–29)*. We previously examined 73 microsatellite markers in 47 patients with NSCLC to find loci frequently altered in NSCLC and identified a panel of 12 markers that was able to detect at least one microsatellite alteration in 55% of the primary tumors (unpublished results). When we used the same panel and several additional markers found by other investigators to demonstrate microsatellite instability in NSCLC, we identified microsatellite alterations in a similar number of tumors (46%).

Microsatellite DNA markers are significantly more accurate than conventional cytology in identifying cancer cells in the urine of bladder cancer patients *(25)*. In contrast to molecular analysis of urine, we were able to detect tumor-specific microsatellite alterations only in a small percentage (14%) of BAL samples examined. In addition, microsatellite alterations not present in the corresponding tumor were detected in 17% of the BAL samples examined with the entire panel of 15 markers. A similar observation was reported by Miozzo et al. *(15)*, who examined histologically normal bronchial biopsy specimens taken from a site distant from a primary lung cancer for microsatellite alterations and identified microsatellite alterations that were not present in the primary tumor in 24% of these patients.

The results of the quantitative p53 plaque hybridization assay suggest that the threshold for detecting microsatellite alterations in BAL fluid was between a neoplastic cellularity of 5% and 7%. The percentage of tumor cells in the BAL fluid in this study was 10-fold to 100-fold lower than that in the urine of patients with bladder cancer and explains the low sensitivity of the microsatellite panel at detecting alterations in the BAL fluid.

Methylation-specific PCR has a sensitivity approaching one in 1000 in dilution experiments and may be useful in detecting clonal cell populations with methylated p16 genes in clinical samples *(14)*. In the current study, we were able to detect methylated p16 alleles in the BAL fluid from 63% of the patients with a methylated primary tumor. The quantitative p53 plaque assay was also positive in just two BAL samples with methylated p16 alleles, suggesting that the threshold for detecting methylated p16 alleles was between a tumor-to-normal cell ratio of one in seven and one in 400. Our results suggest that this approach may be useful in detecting cancers where the frequency of p16 inactivation by methylation is high *(30)*. However, unlike the primary tumor-specific oncogene mutations or microsatellite instability described above, p16 methylation is not necessarily specific. p16 methylation is a common molecular alteration and may not be limited to one neoplastic clone or may be present in other preneoplastic epithelial patches. Thus, although p16 methylation testing lends itself to prospective analysis without prior knowledge of the molecular alteration, it must be surveyed and carefully analyzed in normal control subjects and in cigarette smokers without cancer. Preliminary evidence from a pilot study on lung cancer suggests that p16 methylation may be detected in sputum in the absence of clinically evident cancer *(31)*. On the other hand, methylated p16 alleles were not observed in the BAL fluid from any of the patients with p16 unmethylated tumors in this study.

The molecular detection of lung cancer by testing BAL fluid (and certainly sputum) remains challenging. Although still limited by sensitivity, molecular approaches can detect tumor-specific mutations or molecular alterations in the proximal airway in resectable NSCLC. Further studies are needed to define the clinical significance of each of these mutations or alterations in patients at high risk of developing lung cancer. The more sensitive oligonucleotide plaque hybridization, mutation ligation, and p16 methylation assays detected these alterations in BAL fluid with similar frequency. Further improvements in the sensitivity of all of these assays may be achieved through enriching the epithelial cell component of the BAL fluid or sputum or by collecting multiple samples, an approach that has been successful at increasing the sensitivity of cytologic techniques. In addition, broad technical improvements in molecular detection assays and high through-put automation are necessary for eventual clinical implementation.

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