Detection by Denaturant Gradient Gel Electrophoresis of Tumor-specific Mutations in Biopsies and Relative Bronchoalveolar Lavage Fluid from Resectable Non-Small Cell Lung Cancer

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ABSTRACT

A PCR-denaturant gradient gel electrophoresis (DGGE) method was developed for the detection of *p53* and K-*ras* mutations in primary operable tumors and paired BAL samples of non-small cell lung cancer. Among 36 patients, 9 showed *p53* exon V mutations in biopsies and in three paired bronchoalveolar lavage (BAL) specimens with a 33% concordance. Five patients presented *p53* exon VI mutations in biopsies and in two paired BALs with a 40% concordance. No mutations were found in *p53* exon VII either in biopsies or in paired BAL samples with 100% concordance. Exon VIII mutations were found in six primary tumors and in two BALs with a 33% concordance. Of 36 patients, we detected 7 (19.4%) with K-*ras* exon I mutations on tumor samples. DGGE analysis of DNA from BAL samples revealed three mutations distributed on K-*ras* exon I with a 42% overall concordance with respect to tumor tissue. Molecular screening by DGGE of *p53*-amplified DNA from BAL had cumulative 46.6% sensitivity, 100% specificity, and 77.7% accuracy. DGGE K-*ras* detection showed 43% sensitivity, 100% specificity, and 88.8% test accuracy. The method proposed demonstrated to be specific, accurate, and at relatively low cost but limited by low sensitivity in detecting the presence of neoplastic cells in patients with resectable non-small cell lung cancer.

INTRODUCTION

Lung cancer is one of the major causes of cancer-related deaths among both males and females in western countries. Prognosis of patients with lung cancer is primarily dependent on stage at diagnosis. Presently, only 25–40% of all lung tumors are considered resectable at the time of initial assessment, and only 20% are found to have limited disease at surgery (1). Patients with stage I tumors could have a 75% 5-year survival and 67% 10-year survival after surgical resection (2). Although surgical treatment remains the most effective form of treatment for NSCLC,[3] >65% of all patients will have advanced disease that is no longer amenable to curative therapy at the time of diagnosis (3). In addition, a large percentage of patients undergoing surgical resection ultimately die of recurrent disease, probably because of occult metastatic disease at the time of diagnosis (4). Thus, because present clinical techniques are not capable in the majority of lung cancer cases to detect tumors before they have already progressed beyond effective treatment, the development of tests aimed to allow early diagnosis and treatment of lung cancer is an important goal for lung cancer research (5–7).

Recent advances in molecular biology might have enabled the identification of oncogenes and tumor suppressor genes involved in carcinogenesis and progression of cancer. Molecular methods have proven to be suitable for identification of oncogene mutations in cytologically negative specimens obtained from patients before the diagnosis of NSCLC. These tests showed an impressive sensitivity and diagnostic accuracy in detection of different tumor cells exfoliated in human fluids (8).

Genetic changes may represent early activation events, whereas others are more likely to accompany late events related to invasion and metastasis. On the basis of limited evidence, some investigators have suggested that in some human tumor types, including lung cancer, *ras* mutations may fall into the former category (9). A *ras* gene mutation is detected in 10–30% of NSCLC cases, and 80% of *ras* mutations occur at codon 12 of the K-*ras* gene (10). K-*ras* mutations are more frequently observed in adenocarcinoma than in squamous cell carcinoma, are infrequent in small-cell lung cancer, and are not of prognostic significance for patients with resected stage I and II NSCLC

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; BAL, bronchoalveolar lavage; DGGE, denaturant gradient gel electrophoresis; CT, computed tomography; Tm, theoretical melting temperature; wt, wild type; mut, mutant.

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(11). In NSCLC, *p53* mutations are found in about 50% of the tumors, being more frequent in squamous cell carcinoma than in adenocarcinoma (12, 13). In a cohort of 244 consecutive stage I NSCLC patients, *p53* expression and K-*ras* codon 12 mutation had independent prognostic importance with respect to cancer-free survival (14).

Body fluids sometimes contain cells or cell debris bearing the related tumor gene mutations. Detection of oncogene mutations in tumor cells obtained from urine (15), stool (16), BAL (3, 17), and sputum (1, 18) was feasible in patients with, respectively, bladder, colon, and lung cancers. Cancer cells in bronchoalveolar washing fluid are always mixed with a large number of genetically normal cells, including alveolar macrophage, WBCs, and normal bronchoalveolar cells (3). Field *et al.* (19) showed that a substantial proportion of cells in the bronchial lavage from suspected lung cancer patients (unresolved chest infection, abnormal chest X-ray, cough, weight loss, stridor) carry identifiable genetic alterations. However, the presence of genetic alterations in the BAL of individuals with no clinical or radiological evidence of lung cancer (*i.e.,* from chronic smokers) raises the question of whether instability is a phenomenon associated with cancer or represents a feature of nonneoplastic diseases.

The goal of our study was to determine the efficacy and diagnostic accuracy of PCR-DGGE molecular for early diagnosis of a primary lung tumor or of a local recurrence.

MATERIALS AND METHODS

Patients. From April 1995 to December 1997, we selected 36 patients candidated to pulmonary resection with no previous adjuvant therapy or history of lung cancer. Preoperative evaluation included: chest radiography, fiberoptic bronchoscopy with BAL and fluid collection from the segmental bronchus of the pulmonary lobe most likely to contain the tumor, biopsy when possible, CT-guided fine needle biopsy when the tumor was not seen at bronchoscopy, chest and upper abdominal CT, cranial CT when neurological symptoms were present, forced expiratory volume in 1 s, vital capacity, and tumor markers.

We observed 28 (77%) males and 8 (23%) females. Median age of patients was 65 years (range, 53–77 years) with 16 patients (44%) <65 and 20 (55%) patients >65 years old. Tumors were pathologically classified as squamous cell carcinoma ($n = 18$), adenocarcinoma ($n = 13$), large-cell carcinoma ($n = 4$), and adenosquamous carcinoma ($n = 1$). All tumors were pathologically graded as grade 1 ($n = 4$), 2 ($n = 10$), and 3 ($n = 22$). According to TNM classification, 19 (52.7%) patients were staged as I, 2 (5.6%) as IIA, 3 (8.3%) as IIB, 10 (27.7%) as stage IIIA, and 2 (5.6%) as stage IIIB. In Table 1, all of the clinicopathological parameters of the patients are summarized.

Information on ever/never smoking was abstracted from the medical charts. Patients were classified as smokers if they smoked for a defined period during their lifetime and were presently smoking at the time of diagnosis ($n = 23$). Patients smoking <200 cigarettes during their lifetime and who never used any other tobacco-related products were classified as nonsmokers ($n = 13$). No information about type of tobacco was available.

Tumor and BAL Samples. Fresh tumor samples and correspondent BAL fluids were obtained from 36 patients. Tumor samples were collected by biopsy on macroscopic tumor-like lesions. Tumors with a low neoplastic cellularity (<60%) were further microdissected to remove contaminating normal cells, as previously reported (3). One part of the tumor was immediately frozen and stored in liquid nitrogen for PCR-DGGE (3).

The BAL fluid was transported to the lab on ice and centrifuged 1800 g for 10 min at 4°C. The cell pellet was then collected and stored at −80°C. BAL sample collections were performed after guiding the bronchoscopy into the segmental bronchus of the pulmonary lobe most likely to contact the tumor.

DNA Extraction, PCR, and Denaturing Gradient Gel Electrophoresis. Tumor homogenates were used for DNA preparation. DNA was isolated from tumor samples by SDS-proteinase K treatment and phenol-chloroform extraction, followed by ethanol precipitation. We handled tissue specimens and purified the DNA with disposable equipment in a sequestered area to minimize possible contamination during subsequent amplification procedures. BAL samples were centrifuged for 5 min at 1000 g. DNA was purified from the pellets or sediments with the same procedure of tissue samples. DNA from 36 tumor tissue samples and corresponding BAL specimens was studied for mutational analysis using PCR followed by DGGE. PCR was performed for *p53* exons V (fragments Va and Vb), VI, VII, and VIII and K-*ras* exon I (20, 21). Reaction mixture included 100–300 ng of template DNA in 50 mM Tris-HCl (pH 8.6) with 10 mM KCl, 1.5 mM $MgCl_2$, for exon Va, 1.0 mM for exon Vb, 1.0 mM for exons VI, VII, and VIII, and 1.5 mM for K-*ras* exon I, 0.25 mM each dNTP, 25 pmol of each primer, and 1.5 units of Taq polymerase (AmpliTaq, Perkin-Elmer). The 100-μl mixture was incubated in a 9600 Perkin-Elmer thermocycler for 35 cycles of 94°C (45 s), 57°C for exon VIII, 68°C for exon VII, 56°C for exon VI (45 s), 54° for exon Va, 53° for exon Vb, and 56°C for K-*ras* exon I, and 72°C (45 s). The reaction was initiated with one 7-min incubation cycle at 94°C and ended with a 10-min incubation at 72°C. The primers were synthesized by an automatic synthesizer. A "GC clamp" was attached to one of the primers in each set. The following primer pairs were constructed: Va sense: (5′-T-TCCTCTTCCTGCAGTACTC-3′); Va antisense GC-clamp: (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCG-CGCCCG- TGGCGCGGACGCGGGTGCCG-3′); Vb sense GC-clamp: (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGC-CGCCCCGCGCCCGTTCCACACCCCCGCCCGGCA-3′); Vb antisense: (5′-GCCCCAGCTGCTCACCATC-3′); VI sense GC-clamp: (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCG-CCGCCCCCGCGCCCGCACTGATTGCTCTTAGGT-3′); VI antisense: (5′-AGTTGCAAACCAGACCTC-3′); VII sense GC-clamp: (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCG-CCGCCCCGCGCCCGTGTGTTATCTCCTAGGTTGGC-3′); VII antisense: (5′-CAAGTGGCTCCTGACCTGG-3′); VIII sense: (5′-ATCCTGAGTAGTGGTAATCT-3′); VIII antisense GC-clamp: (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGC-CGCCCCGCGCCCG-TTACCTCGCTTAGTGCTCCCT-3′);

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Table 1 Clinicopathological parameters of patients with NSCLC

Patient	Age (yr)	Sex	Smoke	Histotype	Grade	T	N	Stage	Site
1	70	M[a]	Y	E	3	3	0	IIB	P
2	60	M	N	A	3	3	3	IIIB	P
3	63	M	Y	E	2	2	0	I	P
4	67	M	Y	E	1	2	0	I	C
5	64	M	Y	E	3	2	0	I	P
6	66	F	N	A	2	2	0	I	P
7	58	M	Y	E	2	2	0	I	C
8	72	F	N	A	3	2	0	I	P
9	53	M	N	A	3	2	2	IIIA	P
10	77	M	Y	E	3	2	0	I	P
11	59	F	Y	A	1	3	3	IIIB	P
12	71	M	N	A	3	2	2	IIIA	C
13	63	M	Y	E	2	2	0	I	P
14	67	F	Y	A	3	2	2	IIIA	P
15	65	M	N	L	3	1	0	I	P
16	63	M	N	A	1	2	1	IIA	P
17	60	F	Y	E	3	1	2	IIIA	P
18	66	M	Y	E	2	1	2	IIIA	C
19	66	M	Y	A	1	2	0	I	P
20	62	M	N	E	2	3	0	IIB	C
21	71	M	Y	E	3	3	1	IIIA	C
22	53	M	Y	L	3	2	2	IIIA	C
23	73	M	Y	L	3	2	1	IIA	P
24	67	F	N	E	3	2	2	IIIA	P
25	72	M	Y	L	3	1	2	IIIA	P
26	53	M	N	E	3	2	0	I	P
27	59	M	Y	E	3	1	2	IIIA	C
28	68	M	Y	E	3	2	0	I	P
29	58	M	Y	A	3	2	0	I	C
30	71	F	N	E	3	3	0	IIB	P
31	74	M	N	E	3	2	0	I	P
32	65	M	Y	A	2	2	0	I	P
33	58	F	N	A	2	1	0	I	C
34	72	M	Y	AS	2	2	0	I	P
35	66	M	Y	A	3	1	0	I	P
36	61	M	Y	E	2	2	0	I	C

[a] M, male; F, female; Y, yes; N, no; E, epidermoid carcinoma; A, adenocarcinoma; L, large cell carcinoma; AS, adenosquamous carcinoma; C, central; P, peripheral.

K-*ras* exon I sense (5′-ATGACTGAATATAAACTTGT-3′); and K-*ras* exon I antisense GC-clamp: (5′-CGCCCGCCGCGC-CCCGCGCCCGTCCCGCCGCCCCCGCGCCCG-CTCTATT-GTTGGATCATATT-3′).

The theoretical melting profiles of the amplified fragments were produced by the software program MacMelt by Bio-Rad, based on the statistical mechanical principles and algorithms developed by Poland (22) and the nearest-neighbor bp doublet parameters introduced by Gotoh and Tagashira (23). Parallel denaturing gradient gels (16 × 20 × 0.1 cm) contained 10% acrylamide in TAE buffer [40 mM Tris acetate/1 mM EDTA (pH 8.0)] with *N*-methylene-bis-acrylamide as the cross-linker and varying denaturant concentration consisting of urea and formamide. The gels were polymerized with ammonium persulfate (200 μl/gel) and *N,N,N′,N′*-tetramethylethylenediamine (20 μl/gel). Different ranges of gradient concentrations were chosen for each exon in accord to Tm: (*a*) exon Va: Tm, 74°C (range of gradient, 40–80%); (*b*) exon Vb: Tm, 79°C (range of gradient, 40–80%); (*c*) exon VI: Tm, 74°C (range of gradient, 40–80%); (*d*) exon VII: Tm, 74°C (range of gradient, 30–80%); (*e*) exon VIII: Tm, 77°C (range of gradient, 40–80%); and (*f*) K-*ras* exon I: Tm, 76°C (range of gradient, 45–75%). The boundary of denaturant concentration was determined to be above and below the melting of a given domain.

Amplified DNA from human cancer cell lines with specific *p53* mutations on different codons were used as positive controls, and human breast carcinoma cell line MCF-7 with no *p53* mutation was used as the negative control. For exon Va, we used a DNA sample of known mutation (codon 141, TGC-TAC) from a patient with Li-Fraumeni syndrome; for exon Vb, we used the human acute lymphoblastic leukemia Cem cell line with mutation on codon 175 (CGC-CAC); for exon VI, we used human acute T-cell leukemia cell line Jurkat with known mutation on codon 196 (CGA-TGA); for exon VII, we used the Cem cell line with known mutation on codon 248 (CGG-CAG); for exon VIII, we used human small cell lung cancer cell line NCI-H510 with known mutation on codon 282 (CGG-GGG); and for K-*ras,* we used the Colo 205 cell line (wt) and human colorectal cancer SW-480 (mut codon 12) cell line. Neoplastic cells carrying a somatic mutation are often heterozygous for the mutation itself, allowing the formation of the heteroduplex molecules on the denaturant gel. We created heteroduplexes by mixing the mut and the wt PCR fragment in a microfuge tube and denaturing the DNA by heating at 95°C for 2 min, followed by an incubation

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at 65°C for 1 h, and finally incubation at room temperature for 2–20 h. Into the prep well of the denaturing gradient gel, we loaded tumor DNA with paired BAL-DNA and, for each gel, wt control, mut control, and mixture of wt and mut control. Gels were run submerged in TAE Buffer at 56°C at 80 V constant in the D-Gene System electrophoresis-cell (Bio-Rad). Extensive circulation of the buffer was provided during the runs. The running time was 4–6 h. After electrophoresis, the gels were stained for a few minutes with ethidium bromide (2 mg/liter of TAE) and photographed on a UV transilluminator.

Statistical Analysis. To evaluate PCR-DGGE diagnostic accuracy in the BALs collected, we considered four events: A, positive test: presence of mutation in the primitive tumor and in the relative BAL (true positive); B, positive test: absence of mutation in tumor but presence of mutation in BAL (false positive); C, negative test: presence of mutation in tumor but absence in paired BAL (false negative); and D, negative test: absence of mutation either in tumor or in BAL (true negative). Decision diagnostic criteria were obtained from the results A, B, C, and D. Test sensitivity is equal to A/A+C. Test specificity is equal to D/D+B. Test accuracy is equal to: A+D/A+B+C+D. Predictive positive result or positivity prediction is equal to A/A+B. Predictive negative result or negativity prediction is equal to D/D+C.

The correlation between mutational DGGE analysis results and clinicopathological parameters was assessed by the Pearson χ^2 test. We considered as statistical significant *P*s < 0.05. All statistics were carried out by STATISTICS software package.

RESULTS

DGGE Analysis

***p53* DGGE Mutational Analysis.** Before DGGE analysis, theoretical melting maps were calculated for the different exons in *p53*. We calculated the theoretical melting profiles of previously identified wt and mut samples. Tissues and BAL specimens from 36 NSCLC patients were screened for K-*ras* exon I and *p53* mutations by DGGE analysis. Our study concentrated on *p53* exons V through VIII because they seem to represent the hot spots residing in the highly conserved region of the gene. Of 36 patients, we detected 15 (41.6%) with *p53* mutations on tumor samples. DGGE analysis on DNA from tissue showed nine mutations (25%) on exon V (Fig. 1*A*) with five distributed on fragment Va and four on fragment Vb; five mutations (13.8%) on exon VI and six mutations (16.6%) on exon VIII (Fig. 1*B*). No mutation was detected on exon VII. In two patients, we detected simultaneous mutations on both exon VI and VIII; in two patients, we detected concomitant mutations on exons V and VIII; and in one patient, we detected triple mutation on exons V, VI, and VIII. DGGE analysis of DNA from BAL samples revealed seven (19.4%) mutations distributed on *p53* exons with a 46% concordance with respect to tumor tissue. They were distributed as follows: 4 (22%) of 18 with squamous cell carcinoma, 2 (15%) of 13 patients with adenocarcinoma, and 1 (25%) of 4 patients with large cell carcinoma. *p53* mutations were more frequent in squamous cell carcinoma (9/18, 50%) than in adenocarcinoma (3/13, 23%; *P* = 0.12), confirming previous results (24). We found a trend toward accumulation of *p53* mutations in centrally located tumors (*P* = 0.07), of *p53* exon VIII mutations in well-differentiated tumors (*P* = 0.07) and of *p53* exon V mutations in smokers (*P* = 0.08). There was a significant correlation between *p53* exon V mutations and squamous cell histotype (*P* = 0.04) or central location (*P* = 0.006). We found a strong correlation between *p53* mutations and smoking habit (*P* = 0.0001).

Exon V BAL analysis showed three mutations, all on exon Va, with 60% concordance with tumor sample. Exon VI BAL analysis showed presence of mutations in two of five mutated tumors, with a 40% concordance. Exon VII analysis showed 100% concordance, with no mutation being detected either in tumor or in BAL. Exon VIII mutations were found in two BALs of six mutated primary tumors with a 33.3% concordance. Overall concordance between tumor and BAL samples was equal to 46%. It is noteworthy that when an altered migration pattern was present simultaneously in the primary tumor and in the paired BAL, an identical bandshift on the gel was always observed. Three time consecutively repeated experiments confirmed these results (Fig. 1, *A* and *B*).

In Table 2, comparative analysis of tumor and BAL samples is summarized.

K-*ras* Exon I DGGE Mutational Analysis. Of 36 patients, we detected 7 (19.4%) with K-*ras* exon I mutations on tumor samples. DGGE analysis of DNA from BAL samples revealed three (8.3%) mutations distributed on K-*ras* exon I with a 42% overall concordance with respect to tumor tissue. The mutated BAL sample always showed in repeated experiments the same bandshift on the gel in comparison with the correspondent primary tumor. BAL mutations of K-*ras* were distributed in relation to histotype as follows: 1 (7.6%) of 13 patients with adenocarcinoma, 1 (5.5%) of 18 patients with squamous cell carcinoma, and 1 (25%) of 4 patients with large cell carcinoma. There was a tendency for K-*ras* mutated cases to be more frequent in adenocarcinoma (4/13, 30%) than in squamous cell carcinoma (2/18, 11%; *P* = 0.18), as reported by other authors (25).

p53 and K-*ras* Comparative Analysis

Comparing *p53* and K-*ras* mutations, 17 samples (47.2%) had neither *ras* nor *p53* mutations (*ras*−/*p53*−), 4 (11.1%) had only *ras* mutations (*ras*+/*p53*), 12 (33%) had only *p53* mutations (*ras*−/*p53*+), and 3 (8.3%) had both *ras* and *p53* mutations (*ras*+/*p53*+). The frequency of *ras* mutations in the *p53* mutation group (3/15, 20%) was not significantly different from that observed in a *p53* wt group (4/21, 19%), which suggested that *ras* mutations and *p53* mutations occurred independently of each other (*P* = 0.94). There was no association between K-*ras* or *p53* mutation and other clinical parameters.

Tumor location strongly influenced the ability to detect molecular alterations in the BAL fluid. Tumor specific gene mutations were more often detected in BAL fluid from patients with central tumors than parenchymal or peripheral tumors (*p53*: 85.7% *versus* 14.3%, *P* = 0.0004; *ras*+/*p53*−: 75% *versus* 25%, *P* = 0.001). The detection rate for *p53* mutations in BAL fluid was greater in samples obtained from patients with squamous cell carcinomas (22%) than in samples obtained from patients with adenocarcinomas (15%).

Decision diagnostic criteria showed that molecular screening by DGGE of *p53*-amplified DNA from BAL had 46.6%

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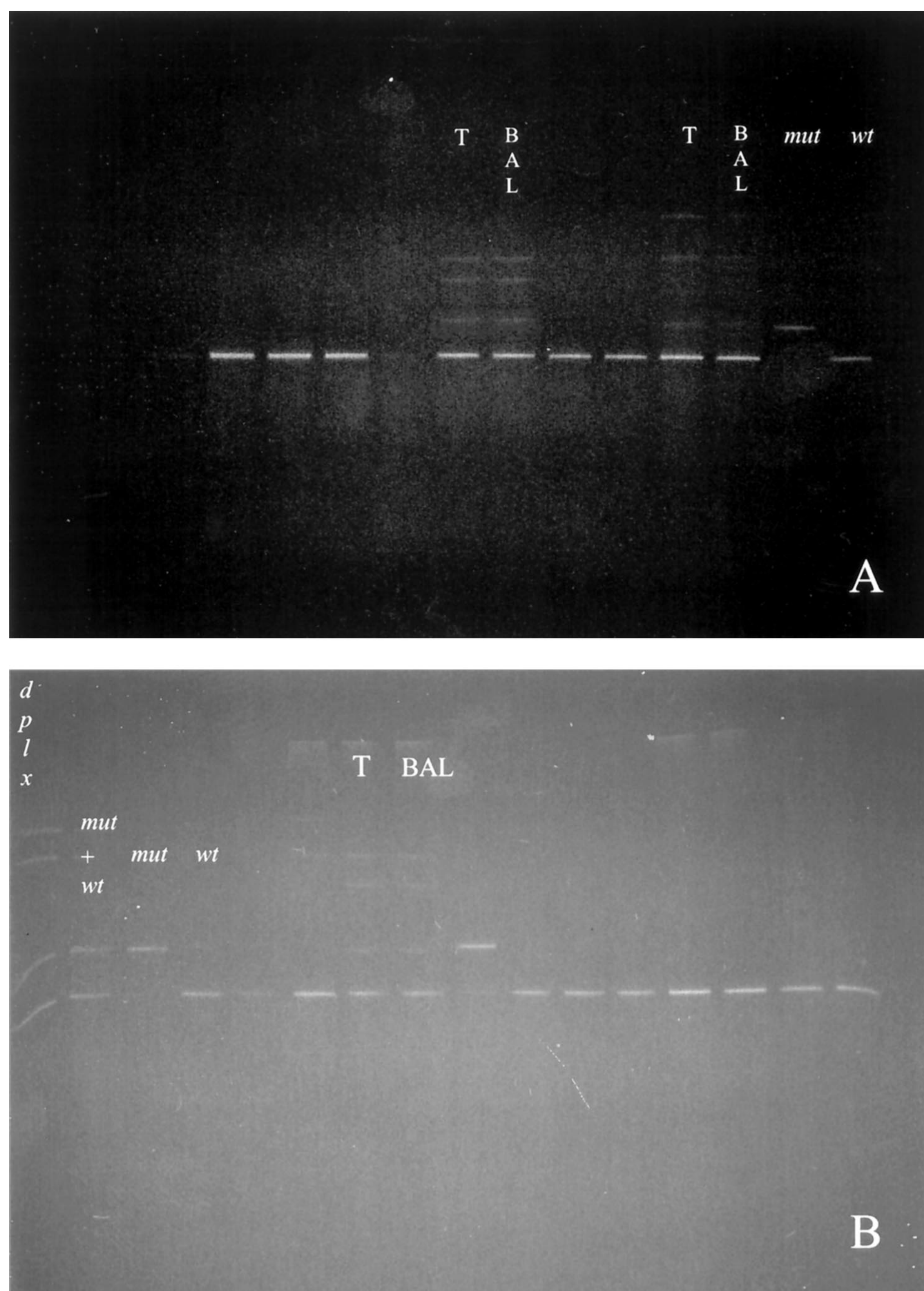

Fig. 1 *A,* exon Va PCR-DGGE analysis of lung biopsies and paired BALs specimens. *T,* tumor; *wt,* wt MCF-7 cell line DNA; *mut,* codon 141 mutated DNA. *B,* exon VIII PCR-DGGE analysis of lung biopsies and paired BALs specimens. *T,* tumor; *wt,* wt MCF-7 cell line DNA; *mut,* mutated NCI-H510 cell line DNA; *dplx,* heteroduplexes.

sensitivity, 100% specificity, 77.7% accuracy, 100% prediction of positive result, and 72.4% prediction of negative result. For K-*ras* BAL mutation detection, we obtained 43% sensitivity, 100% specificity, 88.8% test accuracy, 100% positive predictive value, and 80.5% negative predictive value.

Because of the contamination of the affected tissue with surrounding healthy, blood or connective tissue, the amount of mutated allele can be decreased and varies between the different DNA samples. Our BAL samples required a minimum of 40% neoplastic cells for mutation detection by DGGE. To better test sensitivity of the method, a mixture of each wt DNA (negative control) containing a decreasing concentration of each mutated DNA (positive control; 50%, 40%, 30%, 20%, 10%, 5%, 3%) was performed. The mutation could be identified at 10% DNA mutation content by DGGE.

DISCUSSION

Lung cancer is characterized by multiple genetic changes, which include the activation of proto-oncogenes and the inactivation of tumor suppressor genes. *FHIT,* K-*ras* and *p53* gene alterations are the most common molecular events identified in

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Table 2 Comparative analysis of K-*ras* and *p53* mutations in tumor and BAL samples.

Patient	K-*ras*		*p53*		V		VI		VIII		K-*ras*-*p53*
	T[a]	B	T	B	T	B	T	B	T	B	
1	−	−	+	−	−	−	+	−	−	−	+
2	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	−	−
4	+	−	+	+	+	+	−	−	+	−	+
5	−	−	+	−	+	−	−	−	−	−	+
6	−	−	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	−	−	−	−
8	+	−	−	−	−	−	−	−	−	−	+
9	+	+	−	−	−	−	−	−	−	−	+
10	−	−	+	−	+	−	−	−	+	−	+
11	−	−	+	−	−	−	−	−	+	−	+
12	−	−	−	−	−	−	−	−	−	−	−
13	−	−	−	−	−	−	−	−	−	−	−
14	−	−	+	+	−	−	+	+	+	−	+
15	−	−	−	−	−	−	−	−	−	−	−
16	−	−	−	−	−	−	−	−	−	−	−
17	−	−	−	−	−	−	−	−	−	−	−
18	−	−	+	+	+	+	−	−	−	−	+
19	−	−	−	−	−	−	−	−	−	−	−
20	−	−	−	−	−	−	−	−	−	−	−
21	−	−	+	+	+	+	−	−	−	−	+
22	+	+	+	+	−	−	+	+	−	−	+
23	−	−	−	−	−	−	−	−	−	−	−
24	−	−	−	−	−	−	−	−	−	−	−
25	−	−	+	−	−	−	+	−	−	−	+
26	−	−	−	−	−	−	−	−	−	−	−
27	+	+	+	+	+	−	−	−	−	−	+
28	−	−	+	−	−	−	−	−	+	+	+
29	−	−	+	+	+	−	+	−	+	−	+
30	−	−	−	−	−	−	−	−	−	−	−
31	−	−	−	−	−	−	−	−	−	−	−
32	+	−	−	−	−	−	−	−	−	−	+
33	+	−	−	−	−	−	−	−	−	−	+
34	−	−	+	−	+	−	−	−	−	−	+
35	−	−	−	−	−	−	−	−	−	−	−
36	−	−	+	−	+	−	−	−	−	−	+

[a] T, tumor; B, corresponding BAL; V, *p53* exon V; VI, *p53* exon VI; VIII, *p53* exon VIII; a, *p53* exon Va.

NSCLC, suggesting a key role of these genes in tumor lung carcinogenesis. Furthermore, it has been reported that *p53* or K-*ras* mutations as well as 3p deletions in patients with NSCLC were found to be associated not only with the genesis and progression of lung cancer but also with shortened survival as predictors of poor prognosis, although this notion remains controversial (26–30). It has been demonstrated that genomic damage has an independent prognostic value of poor clinical evolution in NSCLC (31).

Enthusiasm for the use of cytological analysis of sputum or chest X-rays as techniques for early detection of lung cancer was reduced by several studies that showed that screening and subsequent resection did not reduce lung cancer mortality, even in early stage because of microscopic metastatic disease (32).

Apparent limitations of the present diagnostic and follow-up examinations in monitoring patients with high risk lung cancer necessitate the development of noninvasive tests for risk assessment and early diagnosis of NSCLC. Examination and characterization of exfoliated cells in BAL from lung cancer patient can be a useful tool in screening high risk populations, detecting epithelial abnormalities for the presence of malignancy, and assessing response to treatment (33, 34). Cytology has several shortcomings, the most significant of which are high rates of atypia in patients with benign conditions, such as inflammatory disease, and false-negative results in patients with low grade tumors. The limitation of sputum examination have lead to the development of invasive procedure for lung cancer diagnosis, including the BAL technique, which involves the infusion and reaspiration of a sterile saline solution in distal segments of the lung via a fiberoptic bronchoscope. Although the number of epithelial cells in BAL fluid is small, the determination of the cellular components of BAL fluid can aid cancer diagnosis, especially for peripheral tumors. Because of the high sensitivity of the molecular approach, our strategy could prove to be a valuable support to more traditional methods (35). To evaluate the possibility that tumor cells containing K-*ras* and *p53* gene mutations were shed into the bronchoalveolar fluid, we attempted to identify these mutations in DNA amplified from BAL. The PCR-DGGE strategy was used for primary tumors and correspondent BAL samples in an attempt to find a sensitive and specific assay for detection of non-small cell carcinoma. Sensitivity of PCR-DGGE in detection of known mutations has

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been assessed in different reports (36–38) indicating that optimized procedure is highly sensitive and can detect >90% of mutations. Data reported elsewhere illustrated that DGGE is a method with an even higher sensitivity for identifying mut alleles in a mixture of mut and wt alleles compared to direct sequencing. Therefore, DGGE can detect point mutations that might be missed by direct sequencing when mut levels are low, as in tumors (37).

Among the 36 patients with NSCLC, the percentage of *p53* mutation-positive BAL fluid was higher for patients with squamous cell carcinoma than for patients with adenocarcinoma, confirming previous observations that *p53* mutations are most common in squamous cell carcinoma. Tumor location strongly influenced the ability to detect molecular alterations in the BAL fluid. In fact, *p53* mutations in BAL fluid were more frequent in central (predominantly squamous) tumors ($P = 0.0004$). K-*ras* BAL mutations were also more frequent in central tumors, but without a statistical significance because of the high diagnostic value of BAL in peripheral (adenocarcinoma) lung cancer and the higher frequency of K-*ras* mutations in these tumors. For *p53* and K-*ras* DGGE BAL mutation-screening, we calculated, respectively, a 46.6% and a 43% concordance. Similar results has been showed in a previous report (3). Authors examined the frequency of tumor-specific oncogene mutations (*p53*, K-*ras*, p16, and microsatellite instability) in 50 consecutive, prospectively collected BAL from patients with resectable NSCLC. They reported an overall 53% concordance between tumor and paired BAL samples.

When a different melting pattern compared with the wt was present either in the primary tumor or in the corresponding BAL, it always showed an identical bandshift on the acrylamide gel, strongly suggesting that the method was able to detect the same molecular change both in tumor and paired BAL. Moreover, using five different amplification products, we were able to screen the portions ("hot spots") of the *p53* gene within which >80% of mutations have been detected. Unfortunately, we could not distinguish *p53* mutations from *p53* genetic polymorphism, although it have been described mainly outside of the studied regions (codons 21, 31, 47, and 72) remaining the polymorphism at codon 213, the only one which we were not able to differentiate from a somatic mutation (39). Direct sequencing is labor-intensive. Although point mutations could escape the detection in some cases (*e.g.,* domains outside of the regions covered by the five fragments), PCR-DGGE remains a simple, nonisotopic technique for clinical specimen screening (20, 21).

These results have different potential clinical applications. Patients at high risk for primary NSCLC (such as heavy smokers or patients exposed to occupational carcinogens) could be screened for the presence of neoplastic cells in the BAL by analysis of K-*ras* and *p53* mutations. Such screening would be more useful if these mutations are found at an early stage that precedes more advanced and surgically incurable disease. Furthermore, DGGE mutational analysis of BAL sample could be useful in follow-up of patients with known NSCLC treated by lung-sparing surgical strategies. Such patients could have their tumors analyzed for *p53* mutations and, if found, BAL samples could be periodically retested by DGGE for the presence of the mutations. Other tumors in which cells are exfoliated (such as those of the bladder, colon, or cervix) could undergo similar studies.

Findings reported in this study showed that molecular analysis by testing BAL fluid for early diagnosis of a primary lung tumor is an outstanding field of interest. DGGE is a feasible method, but an improvement of sensitivity on fluid sample is required.

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Detection by Denaturant Gradient Gel Electrophoresis of Tumor-specific Mutations in Biopsies and Relative Bronchoalveolar Lavage Fluid from Resectable Non-Small Cell Lung Cancer

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