Rapid and Sensitive p53 Alteration Analysis in Biopsies from Lung Cancer Patients Using a Functional Assay and A Universal Oligonucleotide Array: A Prospective Study

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ABSTRACT

Purpose: **Molecular profiling of alterations associated with lung cancer holds the promise to define clinical parameters such as response to treatment or survival. Because <5% of small cell lung cancers and <30% of non-small cell lung cancers are surgically resectable, molecular analysis will perforce rely on routinely available clinical samples such as biopsies. Identifying tumor mutations in such samples will require a sensitive and robust technology to overcome signal from excess amounts of normal DNA.**

Experimental Design: **p53 mutation status was assessed from the DNA and RNA of biopsies collected prospectively from 83 patients with lung cancer. Biopsies were obtained either by conventional bronchoscopy or computed tomography-guided percutaneous biopsy. Matched surgical specimens were available for 22 patients. Three assays were used: direct sequencing; a functional assay in yeast; and a newly developed PCR/ligase detection reaction/Universal DNA array assay.**

Results: **Using the functional assay, p53 mutation was found in 62% of biopsies and 64% of surgical specimens with a concordance of 80%. The sensitivity of the functional assay was determined to be 5%. Direct sequencing confirmed mutations in 92% of surgical specimens but in only 78% of biopsies. The DNA array confirmed 100% of mutations in both biopsies and surgical specimens. Using this newly developed DNA array, we demonstrate the feasibility of directly identifying p53 mutations in clinical samples containing <5% of tumor cells.**

Conclusions: **The versatility and sensitivity of this new array assay should allow additional development of mutation profiling arrays that could be applied to biological samples with a low tumor cell content such as bronchial aspirates, bronchoalveolar lavage fluid, or serum.**

INTRODUCTION

Over the past 20 years, lung cancer has remained the leading cause of cancer-related deaths in the world, and the overall 5-year survival has remained unchanged over this time at an abysmal 15% (1, 2). At present, clinical prognostic indicators such as Tumor-Node-Metastasis staging classification or performance status remain the main parameters used for treatment decisions. A major obstacle to curative treatment of lung cancer is the early onset of extrapulmonary dissemination. Small cell lung cancers are almost never accessible to surgical resection, whereas only 20–30% of non-small cell lung cancer patients presenting with apparently localized disease receive either surgery as sole treatment or multimodality treatment, including chemotherapy and/or radiotherapy with surgery (3).

Lung cancer is the clinical expression of a disease representing the end point of a series of specific somatic genetic and epigenetic changes that precede the invasive tumor by many years (4). These changes include loss of heterozygosity at chromosomes 3p, 9p, 17p, microsatellite instability, p16, and other tumor suppressor gene promoter methylation, K-*ras*, and/or *p53* mutations. The use of these changes as a clonal marker to detect rare tumor cells in body fluids such as sputum, bronchoalveolar lavage, bronchial aspirates, biopsy, and serum would be very promising for the early diagnosis of lung cancers. However, to date, the potential prognostic, predictive, and therapeutic value of detecting these alterations has been disappointing, partly due to the lack of power of a single alteration and partly due to heterogeneity between the various assays. Furthermore, many of the studies performed to date have been retrospective, using either frozen tissue or paraffin-embedded samples from surgical specimens. The use of these surgical specimens to screen for new molecular markers in either retrospective or prospective studies may be unintentionally biased because it tends to focus

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on only a subset of patients because: (*a*) most lung cancers are unresectable; (*b*) patients with resectable tumors have a better prognosis; and (*c*) patients with resectable cancer generally receive neoadjuvant chemotherapy before surgery.

To meet the challenge of molecular profiling of tumors, there is an urgent need to develop routine molecular diagnostic procedures to manage small or heterogeneous samples such as biopsies, bronchial aspirates, bronchoalveolar lavage, or sputum. It is equally urgent to develop sensitive assays able to overcome the small size and low percentage of tumor cell content of these samples. Biopsies are a suitable material because they are routinely performed in every patient suspected to have a lung tumor.

Among the various potential markers, accurate detection of p53 mutations could be clinically meaningful because this protein plays a key role in drug-induced apoptosis. Consequently, p53 mutational status could influence tumor response to chemotherapy. Furthermore, p53 mutations are frequent and occur early in lung cancer, making them attractive as markers for early detection of tumor cells. The discordance in the literature concerning the clinical relevance of p53 mutational status may be partly caused by different methods of analysis (5). We have recently established that the analysis of the central region of the gene (exons 5–8) misses ~13% of mutations, with half of these mutations corresponding to null mutations (5). The correlation between *p53* gene mutation and p53 protein accumulation in tumor cells is also only 70% based on studies analyzing the entire *p53* gene. This indicates that immunohistochemical analysis is not sufficiently sensitive. Moreover, recent studies have emphasized the concept that p53 mutants may present a heterogeneous behavior. Only a specific subset of p53 mutations could be of clinical value, and this subset could be different depending on the type of cancer or the treatment regimen used (6–11).

We have developed a prospective program to establish routine DNA and RNA extraction of biopsy specimen at the time of diagnosis. In this prospective study, we analyzed the p53 gene status using two sensitive methodologies: the yeast functional assay originally developed by Dr. Richard Iggo (12) and the PCR/ligase detection reaction (LDR)/Universal array developed by Dr. Francis Barany (13–15). We demonstrate that the yeast assay is more sensitive than direct sequencing for detection of p53 mutations in clinical specimens contaminated by a high proportion of stromal cells and can be used for routine analysis. Use of the PCR/LDR/Universal array also achieves a throughput and sensitivity that cannot be achieved by other currently available technologies.

MATERIALS AND METHODS

Patients. A cohort of 210 consecutive patients was prospectively evaluated for newly suspected lung cancer over a 20-month period (June 2000 to February 2002) in our chest surgery department. Fiber optic bronchoscopy was performed in all patients. Nonsurgical biopsies were used as the diagnostic procedure in 170 patients. Diagnostic material was obtained either by biopsy of an endobronchial lesion visualized during bronchoscopy or by computed tomography (CT)-guided percutaneous biopsy when bronchoscopy was not contributive. During bronchoscopy, four biopsies were taken and fixed in alcohol, formalin, and acetic acid for diagnosis, and two additional biopsies were taken and snap-frozen in individual cryotubes in liquid nitrogen at the time of endoscopy when the procedure was well tolerated (without respiratory intolerance, excessive cough, or bronchial bleeding). For CT-guided percutaneous biopsy, only one sample was taken and fixed in alcohol, formalin, and acetic acid, and a second biopsy was taken and snap-frozen at the time of CT scan, if well tolerated by the patient. No additional biopsy was performed for the purpose of this study, and all alcohol, formalin, and acetic acid-fixed and snap-frozen-paired biopsies were archived in the Tenon Hospital pathology department. Among the 134 patients from whom snap-frozen biopsies were obtained, the diagnosis of lung cancer could not be performed on alcohol, formalin, and acetic acid-fixed specimens in 28 cases, and the snap-frozen-paired biopsies were used to avoid another diagnostic procedure for the patient. Finally, frozen tissues from 106 patients (86 obtained by bronchoscopy and 26 obtained by CT-guided percutaneous biopsy) were the subject of the present study.

This procedure did not increase the number of biopsies for investigative purposes and only used specimens already acquired for routine diagnosis, as recommended by the French governmental Agence Nationale d'Accréditation et d'Evaluation en Santé in its "Recommendations for tumor cryopreserved cell and tissue libraries for molecular analyses."[5] As recommended, patients were informed that a part of the pathological specimens could be used for molecular analysis provided that a definitive pathological diagnosis was obtained on formalin-fixed samples.

Pathological Procedure. Snap-frozen biopsies, 1–3 μl in diameter and stored at −80°C, were cut in a cryostat chilled to −30°C. To avoid cross-contamination between tissues, the razor was moved 0.5 cm after each section was cut. In this way, a cryostat razor was used to cut 10–12 different specimens. After use, the razor was washed with distilled water, ethanol dried, and exposed for 30 min to a UV bank before starting a new series of sections. A first 5-μm slide was processed with Toluidine blue stain to assess the tumor cell content (Supplementary Figs. 1–7). If the slide contained at least 10% tumor cells, 10–20 adjacent 10-μm frozen sections were cut and immediately placed in a cryotube immersed in liquid nitrogen. Another slide was stained to check that the block still contained tumor cells. If the first frozen section slide did not contain tumor, a second or third section was cut deeper into the tissue block, and frozen slides were only prepared for molecular analysis if this microscopic examination showed the presence of tumor. If three consecutive Toluidine blue stain-stained slides were negative, the sample was not used, and the second frozen sample was accessed for similar processing. Among the 106 biopsies processed, 20 were eliminated because the biopsy was histologically negative for tumor cells, one was eliminated because it corresponded to a lung metastasis from a primary breast cancer, and 2 were eliminated because the tissue was too necrotic. A total of 83 samples was therefore processed for molecular analysis (Table 1). For 22 patients from whom biopsies

[5] Internet address: http://www.anaes.fr/ANAES/SiteWeb.nsf/wRubriquesID/APEH-3ZMHJP.

Table 1 Patient characteristics

Characteristics	Total patients (%)	p53 mutation
Age at diagnosis (yrs)		
<60	35 (42.2)	19
>60	48 (57.8)	32
(mean ± SD; range)	(60.8 ± 11.5; 19–82)	
Gender (M/F)	67 (80.7)/16 (19.3)	41/10
Histology		
Non-small cell lung cancer	65 (77)	35
Adenocarcinoma	21[a] (25)	10[a]
Squamous cell carcinoma	33 (39)	21
Large cell carcinoma	10 (12)	4
Typical carcinoid	1 (1.2)	0
Small cell lung cancer	19 (23)[a] (16.9)	17[a]
Smoking (mean ± SD, range)	(49.6 ± 27; 0–137)	
>30	69 (83.1)	43
<30	10 (12)	6
0	4 (4.9)	2
Disease extent		
Non-small cell lung cancer	65	35
IIIB/IV	32	17
I/II/IIIA	33[a]	19[a]
Small cell lung cancer	19	17
Localized	5[a]	4[a]
Disseminated	14	13
Total no. of patients	83	51

[a] 1ADC + small cell lung cancer (mixed).

were available, surgical specimens were also available leading to a total of 105 samples. The pathologist obtained the samples within 40–60 min after devascularization of the lobectomy or pneumonectomy. Histological control and sectioning were performed as described above. The pathologist (M. Antoine) classified these specimens semiquantitatively: + if it contained 0–25% of tumor cells; ++ if it contained 25–50%; +++ if it contained 50–75%; and ++++ if it contained 75–100%. The WHO international histological classification was used to assess the final pathological diagnosis. Specimens from 83 subjects were therefore studied in the present article.

Nucleic Acid Extraction and Processing. DNA and RNA extraction was performed simultaneously using the DNA/RNA minikit (Qiagen 14123). Genetic material from surgical specimens was resuspended in either TE [10 mM Tris (pH 8.0), 1 mM EDTA] (DNA) or water (RNA) in a final volume of 20 and 25 μl, respectively. Genetic material from biopsies was resuspended in a final volume of 10 μl. The yield of RNA and DNA allowed multiple independent PCR amplifications for either direct sequencing or functional p53 assay.

Reverse Transcriptase-PCR and PCR Analysis. Reverse transcription of RNA was performed using 2 μl of RNA. The RNA was incubated for 5 min at 65°C before adding 18 units of random primers (Invitrogen), 100 units of the Superscript II reverse transcriptase (Invitrogen), 10 mM DTT, 40 units of the RNase inhibitor, RNaseOUT, and 1.25 mM deoxynucleoside triphosphate. The reaction was incubated for 1 h at 45°C in a final volume of 20 μl. After inactivation at 72°C for 3 min, 2 μl of the cDNA preparation were used for PCR in a final volume of 20 μl [1.25 units of error-free PfU polymerase (Stratagene), 0.5 μM of each primer, 50 μM deoxynucleoside triphosphate, and 10% DMSO]. The amplification conditions were as follows: 5 min at 94°C, then 30 cycles of 30 s at 94°C, 30 s at 62°C, 2 min at 74°C, followed by 10 min at 74°C (final extension step). Five μl of the product were used for agarose gel analysis. For the yeast assay, the 5′- and 3′-region of p53 cDNA was amplified separately. For the 5′-region, we used phosphorothioate-modified primers P3 (ATTTGATGCTGTCCCCG-GACGATATTGAAsC, where s represents a phosphorothioate linkage) and P17 (GCCGCCCATGCAGGAACTGTTACA-CAsT). For the 3′-part, we used P16 (GCGATGGTCTGGC-CCCTCCTCAGCATCTTsA) and P4 (ACCCTTTTTGGACT-TCAGGTGGCTGGAGTsG). The size of these two reverse transcriptase-PCR products was 611 and 569 bp, respectively. For genomic DNA analysis, PCR was performed in a final volume of 25 μl [0.625 units of TaqGold polymerase (Applied BS), 0.2 μM of each primers, 200 μM of each deoxynucleoside triphosphate, 4 mM $MgCl_2$]. The amplification conditions were as follows: 10 min at 95°C, then 30 s at 95°C, 30 s at 60°C, 60 s at 72°C (35 cycles), and 10 min at 72°C (final extension step). Primers for amplification of genomic DNA have already been described previously (16). Five μl of the product were used for agarose gel analysis. DNAs were sequenced using the Big Dye Read reaction terminator kit (PE Biosystems) and an ABI 3100 genetic analyzer according to the manufacturer's instructions.

Yeast Assay. Transcriptional activation is the critical biochemical function of p53, which underlies its tumor suppressor activity. Mutant p53 proteins fail to activate transcription. A yeast strain (yIG397), defective for adenine synthesis because of a mutation in its endogenous ADE2 gene but containing a second copy of the ADE2 open reading frame controlled by a p53 response promoter, has been developed. Because ADE2-mutant strains grown on low-adenine plates turn red, yIG397 colonies containing mutant p53 are red, whereas colonies containing wild-type p53 are white. For the assay, the yeast strain was cotransformed with reverse transcriptase-PCR-amplified p53 and a linearized expression vector. p53 cDNA is therefore cloned in the vector *in vivo* by homologous recombination. To minimize mutations introduced during PCR, we used PfU DNA polymerase (Stratagene), a high-fidelity polymerase. In the original assay described by Flaman *et al.* (12), only one reverse transcriptase-PCR product was amplified and transformed in the recipient yeast. The cutoff for mutation was established as >15% red colonies, indicating the presence of a p53 mutation (12). Although >70% of red colonies are usually obtained for tumors with a high tumor DNA content, ambiguous results may be observed for tumors with a lower tumor cell content or with highly heterogeneous tumor cells. We and other authors (17–19) have also observed that the background of red colonies (false positive) can be heterogeneous from one sample to another, leading to difficulties defining a precise cutoff value. This heterogeneity was reproducible from one sample to another, suggesting that each sample of genetic material could have an inherent behavior that could be due either to the quality of the starting material, contaminating compounds affecting the processivity of the enzyme or both. Bearing this problem in mind, Waridel *et al.* (20) developed a split functional analysis of separated alleles in yeast (FASAY), where the p53 cDNA is amplified into two overlapping PCR fragments that are independently transformed in the recipient yeast with the appropriate vector. The first fragment (P3-P17) corresponds to residues 52–236, whereas the second fragment (P4-P16) corresponds to

residues 195–364. Because there is only one mutation/p53 cDNA, the main advantage of this improvement is that one PCR fragment for each sample will lead to background colonies, whereas the other fragment will lead to red colonies if a mutation is present.

Recovery of p53 Plasmids from Yeast and DNA Sequencing. For each sample yielding >15% of red colonies, the pooled plasmid DNA from 10 red yeast colonies was extracted and sequenced to make a final decision concerning mutations. The plasmid DNA was sequenced using the Big Dye Read reaction terminator kit (PE Biosystems) and an ABI 3100 genetic analyzer according to the manufacturer's instructions. For samples with <15% of red colonies, DNA from 10 red colonies was individually sequenced to distinguish true mutations from the background of PCR errors.

PCR-LDR Assay for p53 Mutations. PCR/LDR/Universal Array assays were generally performed as described in Favis *et al.*[6] and Gerry *et al.* (14).

p53 exons 5–8 were simultaneously amplified in single-tube reactions. Primer sequences, in 5′- to 3′-orientation, were as follows: exon 5 forward = CTGTTCACTTGTGCCCTGACTTTC; exon 5 reverse = CCAGCTGCTCACCATCGCTATC; exon 6 forward = CCTCTGATTCCTCACTGATTGCTCTTA; exon 6 reverse = GGCCACTGACAACCACCCTTAAC; exon 7 forward = GCCTCATCTTGGGCCTGTGTTATC; exon 7 reverse = GTGGATGGGTAGTAGTATGGAAGAAATC; exon 8 forward = GGACAGGTAGGACCTGATTTCCTTAC; and exon 8 reverse = CGCTTCTTGTCCTGCTTGCTTAC. To ensure amplification of all exons, PCR was performed by using primers containing a universal primer sequence at the 5′-ends. The initial PCR reaction was performed as previously described (13, 15) with the following modifications. The 25-μl PCR reaction mixture contained 3–5 μl of primary tumor DNA, all four deoxynucleoside triphosphates (400 μM of each one), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 0.625 units of AmpliTaq Gold (PE Applied Biosystems, Inc., Norwalk, CT), 2 pmol of gene-specific primers containing a 5′-universal sequence for exons 5, 6, and 8, and 4 pmol of a similar primer for exon 7. The reaction was preincubated for 10 min at 95°C. Amplification was performed for 15 cycles as follows: 94°C for 15 s and 65°C for 1 min. A second 25-μl aliquot of the reaction mixture, containing 25 pmol of universal primer, was then added. PCR was repeated for 25 cycles at an annealing temperature of 55°C for 1 min. Amplification was verified by examining the products on 3% agarose gel. Taq polymerase was inactivated by 3 cycles of freezing in dry ice.

After a multiplex PCR amplification of the regions of interest, each mutation was simultaneously detected using a thermostable ligase that joins pairs of adjacent oligonucleotides complementary to the sequences of interest. Ligation occurs only when there is perfect complementarity at the junction between the 5′-fluorescent-labeled upstream oligonucleotide, containing the discriminating base for the mutation on the 3′-end, and the adjacent downstream oligonucleotide, containing a complementary zip code sequence on the 3′-end. The complete set of LDR primers is described in Favis *et al.*[6] Ligation products are distinguished on the basis of differential labeling and capture of the zip code complement on its cognate zip code address on an universal array.

LDR reactions were carried out in a 20-μl mixture containing 20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM KCl, 10 mM DTT, 1 mM NAD^+, 25 nM (500 fmol) of the detecting primers, 2 μl of PCR product, and 25 fmol of Tth DNA ligase. Ligases were overproduced and purified as described previously (21, 22). LDR reactions were incubated for 5 min at 95°C and were then thermally cycled for 20 cycles of 30 s at 95°C and 4 min at 64°C. Quality control for LDR was performed using a synthetic template for each mutation to test the ability of the full mix of upper or lower ligation primers to produce the expected specific signal on the DNA microarray.

Preparation and hybridization were performed as previously described (13, 14), except that hybridization was carried out in the presence of 100 μg/ml sheared calf thymus DNA. Briefly, 20 μl of the LDR reaction were diluted with 20 μl of 2.0× hybridization buffer to produce a final buffer concentration of 300 mM 4-morpholineethanesulfonic acid (pH 6.0), 10 mM $MgCl_2$, and 0.1% SDS that was incubated for 5 min at 94°C before loading in the chips. The arrays were placed in humidified culture tubes and incubated for 1 h at 65°C and 20 rpm in a rotating hybridization oven. After hybridization, the arrays were washed in 300 mM bicine (pH 8.0), 10 mM $MgCl_2$, and 0.1% SDS for 10 min at 65°C. Arrays were reused three times and were stripped between uses by submerging for 1 min in a solution of boiling 100 mM bicine/0.1% SDS. Stripped arrays were rinsed in nanopure water, excess water was removed using forced air, and the arrays were stored in slide boxes at room temperature.

RESULTS

The clinical and histological characteristics of 83 patients with lung cancer are shown in Table 1. The distribution of the various histological types is in agreement with recent data concerning the distribution of lung cancer in France, indicating that no recruitment bias occurred during this prospective study (23).

Using total RNA extracted from either the biopsy or the tumor sample, reverse transcriptase-PCR amplification and FASAY analysis of all 105 samples (100%) were successful (Supplementary Fig. 1). FASAY analysis for the detection of p53 mutations has been extensively described, but most of these studies used a first generation assay with only one PCR product corresponding to residues 52–364. The cutoff value of red colonies for a positive result is usually arbitrarily defined between 10 and 20% (24–26). In the present study, we first used a 15% cutoff value, leading to the detection of p53 mutations in 44 of 83 biopsies and 14 of 22 tumors. Direct sequencing of pooled rescued plasmid DNA from yeast led to the identification of the p53 mutation in 100% of cases (Supplementary Figs. 1–7).

In the split methodology, the *p53* gene is cloned into two fragments. The basic idea is that the number of red colonies arising in the second fragment not containing the *p53* mutation will always correspond to background mutations. Two p53

[6] R. Favis, J. Huang, N. P. Gerry, A. Culliford, P. Paty, T. Soussi, and F. Barany. Harmonized microarray mutation scanning analysis of p53 mutation in undissected colorectal tumors, in press, Human Mutation, June 2004.

mutations are very rarely found in the same allele of the gene. We calculated the mean percentage of red colonies generated by the negative fragment of each tumor bearing a *p53* mutation. Samples with mutations in the overlapping segment of the two PCR products were carefully removed. Only samples with >15% of red colonies were taken into account in this analysis. This statistical analysis of the cutoff values was based on 39 samples of P3-P17 and 32 samples of P4-P16 fragments. The mean percentage of red colonies was 3.4 ± 2.6% for P3-P17 and 4.0 ± 2.4% for P4-P16. Similar mean values were obtained when the same analysis was performed on tumors negative for p53 mutations. Using cutoff values of 8.6 and 8.8% (mean ± 2 SDs), 7 biopsy specimens gave percentages of red colonies ranging between these cutoff values and our previous limit of 15% (Table 2). No new p53 mutations were detected among the surgical specimens. For these 7 specimens, sequencing of 10 individual red colonies led to the detection of p53 mutations (see "Materials and Methods"). The case of B32 is also noteworthy. Petri dishes transformed with the P3-P17 PCR product led to 7.2% of red colonies and 5.1% of pink colonies. These pink colonies have been shown to originate from leaky *p53* mutations that do not completely inactivate *p53* function (25, 26). Sequencing of 10 individual clones from pink colonies detected a single substitution at codon 180 of the p53 gene in a region known to lead to mutant p53 with a mild phenotype, whereas sequencing of individual clones from red colonies led to the identification of multiple mutations arising from PCR amplification. This particular example clearly shows that the split FASAY is a very sensitive method to detect mutant p53 in a highly heterogeneous tumor sample.

Therefore, using the new cutoff value defined above, 52 of 84 biopsies (62%) and 14 of 22 tumors (64%) were positive on the FASAY (Tables 2, 3, and 4).

The spectrum of missense mutations was as follows: 11 (G:C→A:T) transitions, 6 of which occurred at a CpG dinucleotide; 19 (G:C→T:A), 6 (T:A→C:G), 3 (A:T→T:A), and 5 (G:C→C:G) transversions. Nine frameshift mutations and 1 splice mutation were also revealed (Table 2). The high frequency of GC→TA transversions, which are usually only found in lung cancer patients, is associated with tobacco smoking (27). Five mutations were found in the 157–159 region, a hot spot region that has been shown to be the specific target of the tobacco carcinogen benzo(*a*)pyrene (28). The concordance between the pattern of p53 mutations described in this article and published literature based on more conventional procedures indicates that the functional assay used in the present study did not induce any specific selection bias for p53 mutations. This pattern of mutational events is not unexpected because the majority of patients in the present series were smokers (Tables 2 and 3).

In the series of 22 matched samples of biopsies with surgical specimens, 7 samples were wild-type in both samples, 11 had the same mutations, and 4 were discordant (Table 3).

To validate this FASAY analysis, direct sequencing was performed using either DNA or cDNA as starting material. The identity of the p53 mutation was confirmed in 28 of the 39 biopsies (71%) and 12 of the 13 (92%) surgical samples, whereas no mutation was detected in the remaining samples (Tables 2, 3, and 4). It is noteworthy that cDNA sequencing was more sensitive on 3 samples, confirming previous observations that mutant p53 RNA may be more stable or may be expressed at a higher level in tumor cells (29). Failure of sequencing is certainly caused by the low tumor cell content in the sample and the lack of sensitivity of automatic sequencing.

We have recently developed a microarray-based assay to detect p53 mutations that uses a thermostable ligase enzyme to discriminate between wild-type and mutant templates, resulting in separation of mutation detection and array hybridization (13–15).[6] This assay was used to efficiently detect *p53* mutations in surgical specimens from patients with colorectal cancer, but its sensitivity in nonsurgical samples such as biopsies has not been previously tested. Nine surgical specimens and 27 biopsies with p53 mutations detected by the FASAY were available for analysis by the array (Table 5 and Fig. 1*F*). The array confirmed mutations in all of the 27 biopsies (100%; Table 5), 7 (27%) of which were not confirmed by direct genomic DNA sequencing (Tables 2 and 3). Two mutations not detected by direct sequencing were also detected by the array. All *p53* mutations were detected by the array for the 8 surgical samples. For patient C6 in whom biopsy and surgical specimens were both available, histological examination of the specimen and FASAY analysis indicated a higher tumor cell content for the surgical specimen (70 *versus* 30%). Although FASAY easily detected a mutation at codon 249 in both samples, direct sequencing of the biopsy failed to detect the mutation, whereas the DNA chips clearly identified this event (Fig. 2, *A–F*). This feature can be applied to the majority of the samples analyzed in this study and emphasizes the high sensitivity of this array technology for biopsy specimens.

DISCUSSION

Lung carcinomas are typically late-stage and biologically aggressive, which accounts for their poor prognosis (4). The potential of new imaging and molecular techniques to significantly improve the detection of localized lung cancer provides an unprecedented opportunity to understand the biology, improve diagnosis, enhance treatment, and reduce mortality (30). Furthermore, recently developed proteomic and expression array technologies have intensified the search for new biomarkers that could be helpful in defining response to therapy or prognosis.

Only 30% of patients with non-small cell lung cancer and <5% of patients with small cell lung cancer are treated surgically, implying that the biological sample most frequently available for routine management at the time of diagnosis is biopsy. The size and heterogeneity of biopsies raise problems for current molecular diagnosis techniques. There is therefore an urgent need to develop sensitive assays for the detection of lung tumor-specific molecular alterations in routinely available specimens such as biopsies, bronchoalveolar lavage, or sputum. In the present prospective study, we demonstrate the feasibility of routine management and analysis of lung biopsy specimens for p53 mutation. This includes biopsies obtained using conventional bronchoscopy as well as CT-guided percutaneous biopsy. To our knowledge, this is the first time that material obtained by CT-guided percutaneous biopsy has been processed for molecular analysis despite the smaller sample size compared with biopsies obtained by conventional procedures. This is important in view of the increasing worldwide rate of adenocarcinoma in

Table 2 Analysis of lung biopsies for p53 mutations by functional analysis of separated alleles in yeast, direct sequencing, and DNA chips

			Functional analysis of separated alleles in yeast[b]		Mutation (FASAY analysis)				
Sample	Histology	% tumor cells[a]	% red clones 5′	% red clones 3′	Codon	Mutational event	DNA analysis[c]	RNA analysis[d]	Chips[e]
B1[f]	SCLC	100/75	10.8	87.8	249	AGG→AGT	(+)	ND	(+)
B2	SCC	50/75	97.1/96.9	68/71.7	205	TAT→TGT	(+)	ND	(+)
B3	LCC	75/75	64.3	2	132	AAG→AAC	(+)	(+)	NA
B4	SCLC	75/75	65.7	3.1	192	CAG→TAG	(+)	ND	NA
B5	SCLC	10/10	86.7	2.4	157	GTC→TTC	(+)	ND	(+)
B6	SCC	75/75	21.8	5.7	144	CAG→TAG	(+)	ND	(+)
B7	SCLC	75/100	0/4.4	44.2/18.4	del	Del part exon 8 and intron 8	(+)[g]	(+)[g]	NA
B8	SCLC	25/15	32.1	38.5	220	TAT→TGT	(+)	ND	(+)
B9	SCC	50/50	1.3	60.2	239	AAC→GAC	ND	(+)	NA
CTB10	SCC	15/0	2.1	91.8	267	CGG→CCG	(+)	ND	NA
B11	NSCLC	10/0	22.5	2.2	110	CGT→CTT	(−)	(−)	NA
B12	SCLC	10/50	13.9	63	249	AGG→TGG	(+)	ND	(+)
B13	ADC	75/50	81.9	33.6	195	ATC→AAC	(+)	ND	NA
B14	SCC	25/E	83	2.5	175	CGC→CAC	(+)	(+)	(+)
B15	SCLC	100/75	53.9	5.4	100	CAG→TAG	(+)	ND	NA
B16	ADC	0/5	76.6	5.3	157	GTC→TTC	(−)	Weak	(+)[h]
B17	SCLC	75/100	58.6	82.1	220	TAT→TGT	(+)	ND	(+)
B18	LCC	75/75	1.8	97.1	300	DEL C1	(+)	ND	NA
B19	SCLC	75/75	1.5	64	273	CGT→CTT	(+)	ND	(+)
B20	ADC	10/10	2.5/2.3	9.3/9.4	306	CGA→TGA	(−)	ND	(+)
B21	SCC	100/100	1.5	94.9	273	CGT→CTT	(+)	ND	(+)
B22	SCC	100/75	3.8/2.2/7.2	8.2/15.6/10.1	278–79	T insertion	ND	ND	NA
B23	ADC	75/75	82.6	1.8	179	CAT→CGT	(+)	ND	(+)
B24	SCC	75/75	69.2/64.8	2.5/1.6	110	CGT→CTT	(+)	ND	NA
B25	SCC	50/75	1.9	84.2	245	GGC→TGC	(+)	ND	(+)
B26	SCC	50/0	1.1	58.2	278	CCT→TCT	(−)	ND	(+)
B27	SCLC	75/50	2.1	64.6	286	DEL G1 (GGA)	ND	ND	NA
B28	SCLC	75/100	10.9–16.1[i]	4,1–80.8[i]	237	ATG→AAG	ND	ND	NA
B29[j]	ADC	10/E	2.8	31.3	273	CGT→CAT	(+)	ND	(+)
B30	ADC	75/100	5.2	41.7	298–99	17 bp insertion	ND	ND	NA
B31	ADC	75/75	10	5.9	134–35	TTTTGC→TTCACC	ND	ND	NA
B32	SCLC	10/10	7.2–5.1[i]	5.7	180	GAG→GAT	ND	ND	NA
B33	ADC	25/50	11.6	3.4	157	DEL C3 (GTC)	ND	ND	NA
B34	SCLC	75/75	9.5/7.2	49.6/49.5	307	DEL G1 (GCA)	ND	ND	NA
B35	SCLC	75/75	65.6	4.7	179	CAT→AAT	ND	ND	NA
B36[j]	ADC	50/15	13.4/15.4	6.8/8.7	175	CGC→CAC	(−)	ND	(+)
B37	ADC	10/15	6.1	30.5	237	ATG→ATT	(−)	ND	(+)
B38	SCLC	100/100	15.6	8	183	TCA→TGA	ND	ND	NA
B39	SCLC	100/100	47.3	0	192	CAG→TAG	ND	ND	NA
B40[j]	SCC	50/75	0.7/3.3	2.8/2.5	WT				
B41	ADC	10/10	8.1/6.7	4/5.2	WT				
B42	SCC	0/10	0.9/8.5	4.6/5.4	WT				
B43	SCC	15/15	3.8/7.9	3.2/6.1	WT				
B44[j]	ADC	15/25	0.9/3.3	1.9/2.5	WT				
CTB45	ADC	75/50	2.6/6.4	6.8/8.4	WT				
B46	SCC	0/0	4.6/1.3	5.8/4.2	WT				
B48	NSCLC	5/5	3.6/3	6.1/7.3	WT				
B49	NSCLC	10/0	3.6/4.3	6/7.9	WT				
CTB50	ADC	50/25	2.9/2.5	3.1/2.4	WT				
B51	NSCLC	15/10	6.7	2.1	WT				
B52	SCLC	0/0	3	3.1	WT				
B53	SCC	50/75	3.7/4.8	6.4/5.3	WT				
B54	SCLC	100/100	3.8/4.1	3.8/3.5	WT				
B55	ADC	10/15	1.4/2.4	2.7/3.9	WT				
B56	NSCLC	25/25	1.5/4.4	4.6/5.4	WT				
CTB57	NSCLC	10/0	4.7/1	2.3/5.2	WT				
CTB58	NSCLC	10/E	4.5/4.5	3.4/6.7	WT				
CTB59	SCC	10/E	0.8/8.1	7.3/2.9	WT				
B60	ADC	10/10	7.6/6.8	3/1.5	WT				
B61	SCC	10/10	2.3/0.7	2.9/0.8	WT				
B62	ADC	50/50	5/2.6	3.1/3.8	WT				

[a] The two values correspond to the top and bottom slides, respectively.

[b] Frequency of red clones is given for the 5′-part (P3-P17) and 3′-part of p53 (P4-P16). More than 1 assay was performed in several experiments, and all results are shown.

[c] Detection of p53 mutation by direct DNA sequencing of genomic DNA. +, the same mutation was detected in DNA; −, no mutation detected.

[d] Detection of p53 mutation by direct DNA sequencing of cDNA. +, the same mutation was detected in cDNA; −, no mutation detected.

[e] Chip analysis was always performed with genomic DNA, except for a few cases in which it was performed with cDNA.

[f] B, biopsy obtained by conventional bronchoscopy; SCLC, small cell lung cancer; ND, not done; SCC, squamous cell carcinoma; LCC, large cell carcinoma; NA, the mutation is not available on the chip; CTB, computed tomography-guided percutaneous biopsy; ADC, adenocarcinoma; E, block exhausted; WT, wild type; NSCLC, non small cell lung cancer; CT, carcinoid tumors.

[g] Mutation described previously (30).

[h] Signal obtained with cDNA amplified from the tumor. No signal was obtained with genomic DNA.

[i] Leaky mutations leading to both red clones (first number) and pink clones (second number).

[j] Nonsmoking patient.

Table 3 Analysis of biopsies and surgical specimens from matched patients for *p53* mutations by functional analysis of separated alleles in yeast, direct sequencing, and DNA chips

Sample[a]	Histology	% tumor cells[b]	Functional analysis of separated alleles in yeast[c]		Mutation (FASAY analysis)		DNA analysis[d]	RNA analysis[e]	Chips[f]
			% red clones 5′	% red clones 3′	Codon	Mutational event			
C1B	SCC[g]	10/20	6.7	15.8	275	TGT→TTT	(−)	ND	(+)
C1T	SCC	100/100	0	74.4	275	TGT→TTT	(−)	(+)	(+)
C2B[h]	SCC	0/0	6.6/7.2	18.6/10.9	WT		ND	ND	(−)
C2T	SCC + ADC	75/75	1.7/1.3	48.4/40.6	273	CGT→CTT	(−)	(+)	(+)[i]
C3B	SCC	100/100	12.8/11.2	4.3/1.4	71	DEL C1 (CCC)	(+)	ND	NA
C3T	SCC	100/100	19.3/26.9	1.7/2.1	71	DEL C1 (CCC)	(+)	ND	NA
C4B	SCC	75/100	2.3	92.1	242	TGC→TTC	(+)	ND	(+)
C4T	SCC	100/100	3.3	86.9	242	TGC→TTC	(+)	(+)	(+)
C5CTB	ADC	15/20	5.1/5.8	10.6/11.9	224	GAG→GTCTG	(+)	ND	NA
C5T	ADC	75/75	2.8/5	15.8/12.7	224	GAG→GTCTG	(+)	ND	NA
C6CTB	NSCLC	5/N	0.7	9.9	249	AGG→ATG	(−)	(−)	(+)
C6T	ADC	75/75	4.5	26.6	249	AGG→ATG	(−)	(+)	(+)
C7B	SCC	50/10	1.3	30.7	273	CGT→CAT	(−)	ND	(+)
C7T[j]	SCC	75/75	0.7	66.9	273	CGT→CAT	(+)	ND	(+)
C8B	SCC	100/100	1.7	78.9	273	CGT→GGT	(+)	ND	NA
C8T	SCC	100/100	1.4	82.9	273	CGT→GGT	(+)	ND	NA
C9CTB	NSCLC	75/75	52.2	2.3	159	GCC→CCC	(+)	(+)	(+)
C9T	ADC	100/100	51	2.5	159	GCC→CCC	(+)	(+)	(+)
C10B	SCC	5/0	18.3/43.2	7/10.3	175	CGC→CAC	(−)	ND	(+)[i]
C10T[j]	SCC	50/0	52.7/44.2	2.7/0	105	GGC→TGC	(−)	ND	NA
C11B	ADC + SCLC	100/100	3.5	60.3	248	CGG→CTG	(+)	ND	(+)
C11T[j,k]	SCLC	25/25	4.1	6.1	WT		ND	ND	(−)
C12B	SCC	75/50	44.5	98.8	218–221	DEL 9 PB	(+)	ND	NA
C12T[i]	SCC	75/75	30.5	67.1	218–221	DEL 9 PB	ND	ND	NA
C13CTB	ADC	20/10	21.1	4.6	193	CAT→CGT	ND	ND	(+)
C13T	ADC	75/75	44.3	3	193	CAT→CGT	(+)	ND	(+)
C14B[h]	SCC	0/5	6.2/6.5/9.1	8.5/9.7/4.9	WT		ND	ND	(−)
C14T[j]	SCC	75/100	6.4	71.4	248	CGG→CTG	(+)	ND	(+)
C15CTB	ADC	50/25	68.7	3.8	158	CGC→CTC	ND	ND	(+)
C15T[j]	ADC	100/100	77.4	1.5	158	CGC→CTC	(+)	ND	(+)
C16B	Ca	75/75	0.2	2.7	WT				
C16T	Ca	100/100	1.3	2.5	WT				
C17CTB	SCC	15/25	1.5	2.7	WT				
C17T	SCC	75/75	0.9	1.7	WT				
C18CTB	SCC	0/5	6.2	0.8	WT				
C18T	SCC	100/100	3.4	8	WT				
C19B	SCC	25/25	1.6	4.8	WT				
C19T	SCC	75/75	1.4	2.4	WT				
C20CTB	SCC	75/75	1.1	3.2	WT				
C20T	SCC	100/75	1.8	2.9	WT				
C21CTB	ADC	75/25	5.6	8.3	WT				
C21T	ADC	50/ND	8.6	3.7	WT				
C22CTB	ADC	15/5	1.6	2.9	WT				
C22T	ADC	50/75	0.3	1.3	WT				

[a] Matched biopsies (top lane, suffix B or CTB as defined in Table 2) and surgical specimens (bottom lane, suffix T). All patients were smokers.

[b] The two values correspond to the top and bottom slides, respectively.

[c] Frequency of red clones is given for the 5′-part (P3-P17) and 3′-part of p53 (P4-P16). More than 1 assay was performed in several experiments, and all results are shown.

[d] Detection of p53 mutation by direct DNA sequencing of genomic DNA. +, the same mutation was detected in DNA. −, no mutation detected.

[e] Detection of p53 mutation by direct DNA sequencing of cDNA. +, the same mutation was detected in cDNA. −, no mutation detected.

[f] Chip analysis was always performed with genomic DNA, except for a few cases in which it was performed with cDNA.

[g] SCC, squamous cell carcinoma; ND, not done; WT, wild type; ADC, adenocarcinoma; NA, the mutation is not available on the chip; NSCLC, non-small cell lung cancer; N, necrosis; SCLC, small cell lung cancer; Ca, carcinoid tumors; T, surgical specimen.

[h] The discrepancy between the surgical specimen and the biopsy could be due to the very low tumor cell content of the biopsy.

[i] Signal obtained with cDNA amplified from the tumor. No signal was obtained with genomic DNA.

[j] These patients received neoadjuvant chemotherapy.

[k] Histological examination of the biopsy detected a composite tumor consisting of SCLC and adenocarcinoma tissue. Histological examination of the surgical specimen after treatment showed only the SCLC component with WT p53, suggesting that the p53 mutation observed in the biopsy could arise from the adenocarcinoma component.

Table 4 Summary of *p53* mutation analysis

	Single biopsies	Matched biopsies/tumors	
		Biopsies	Tumors
FASAY[a]	39/62 (63%)	13/22 (60%)[b]	14/22 (63%)[b]
Sequencing[c]	22/28 (78%)	7/11 (63%)	12/13 (92%)
Chips[d]	18/18 (100%)	9/9 (100%)	9/9 (100%)

[a] Functional analysis of separated alleles in yeast (FASAY) represents the true frequency of p53 mutations in this series because no patient selection was performed for the analysis.

[b] Two patients were negative for the biopsies but positive for the tumor: 1 patient has a different mutation in the tumor and in the biopsy, and 1 patient with a mixed tumors (small cell lung cancer + non-small cell lung cancer) had a positive biopsy and a negative tumor (see text for more details).

[c] Only patients with positive FASAY are indicated. No p53 mutation was found in negative patient (see text for detail).

[d] Only patients with a p53 mutation and for whom the chips assay was available were tested.

Table 5 DNA chip analysis[a]

	SEQ+/ ARRAY+	SEQ−/ ARRAY+	SEQ ND/ ARRAY+	SEQ+/ ARRAY−
Tumors	9	0	0	0
Biopsies	17	8	2	0

[a] All samples analyzed by the array were shown to contain a p53 mutation after by functional analysis of separated alleles in yeast analysis. SEQ, detection of p53 mutation by direct sequencing; ARRAY, detection of p53 mutation by PCR/LDR array.

which CT-guided percutaneous biopsy is the method of choice for these peripheral tumors.

Although p53 mutations are common in lung cancer, the importance of these mutations for the patient's clinical outcome is still controversial (5), mainly because of the heterogeneous strategies used to assess p53 mutational status. Immunostaining lacks sensitivity because of false negatives from nonsense mutations, splicing mutations, and deletions that do not lead to p53 accumulation. In the present study, 10 mutations could not have been detected by immunostaining, and the splice mutation could not be detected by DNA sequencing (31). The majority of molecular analyses have also focused on the study of p53 exons 5 through 8. In a recent analysis of the p53 mutation database, we showed that this bias results in nondetection of ~13% of p53 mutations, and these false negatives may bias interpretation of the results during statistical analysis.

In the present study, we compared assays based on either DNA, direct sequencing or arrays, or RNA, the functional assay in yeast. Initially developed for the detection of germ-line mutations, the yeast assay has been widely used for the detection of somatic mutations in various types of tumors, including a few studies in lung cancer (32, 33). The yeast assay can be used to screen p53 from exons 4 to 10, which accounts for >95% of p53 mutations. In the present study, using the new split assay developed by Waridel *et al.* (20) and an experimentally defined cutoff value, we show that this assay may be sufficiently sensitive to detect p53 mutations in samples containing only 5% of tumor cells. Sequencing of rescued plasmids from red colonies allowed unambiguous identification of *p53* mutations in all cases, but direct sequencing of genomic DNA was only able to detect 72% of mutations in biopsy specimens. Until a more sensitive and specific methodology has been developed, we believe that the yeast assay should be considered as a reference method for the evaluation of p53 mutations in clinical specimens, especially specimens with a low tumor cell content. In addition to the advantages described above, the FASAY can easily distinguish true inactivating mutations from neutral mutations. Furthermore, the use of a short amplicon in reverse transcriptase-PCR also allows this assay to be performed on biological samples that could lead to extraction of partially degraded RNA (19).

Although sensitive, this assay has two major drawbacks: it has a low throughput and it does not provide any information about the precise p53 mutation, therefore, requiring sequencing of rescued plasmids. Although the first limitation could be circumvented by automation, the second limitation could be particularly inconvenient in view of the markedly heterogeneous behavior of various p53 mutants, leading to different clinical phenotypes. Several studies in breast cancer suggest that only specific p53 mutations are associated with *de novo* resistance to doxorubicin (9).

The PCR/LDR/Universal array assay provides both high throughput and allows direct identification of the mutational event, a feature that considerably reduces the cost of this assay. Furthermore, as demonstrated in the present work, it has a higher sensitivity than direct sequencing. One of the most useful aspects of the PCR/LDR/Universal array is its versatility because the same array can be used for the detection of mutations in multiple genes such as *p53*, *APC*, *K-ras*, or BRCA1 (13, 14). Our laboratories are also developing the PCR/LDR/Universal array to monitor gene promoter hypermethylation,[7] which is a frequent event in various types of cancer, including lung cancer (34, 35). Belinsky *et al.* (36) measured hypermethylation of the CpG islands in the sputum of lung cancer patients and demonstrated a high correlation with early stages of non-small cell lung cancer, which indicated that p16 CpG hypermethylation could be useful in predicting future lung cancer.

We envision the practical development of very sensitive PCR/LDR/Universal array assays, specifically programmed to a given type of cancer such as lung or colon cancer. By querying specific genes for each type of cancer (*e.g.*, gene mutations or hypermethylation), it would be possible to achieve a specificity of 90–95% for identification of tumor cells. Such universal array assays will be very useful to assess the tumor content of clinical specimens such as stool, serum, bronchoalveolar lavage fluid, and sputum—samples that are known to have a low tumor cell content. Using a new standardized extraction and conservation protocol, we have been able to extract RNA and DNA from bronchial secretions aspirated during fiber-optic bronchoscopy (bronchial aspirates) that are considered to contain tumor cells. FASAY and chips analysis were successfully performed with this material, indicating the feasibility of this type of analysis on heterogeneous specimens.[8]

Although the specificity of each gene queried is not high (current chips are programmed to detect only 50% of p53

[7] Y-W. Cheng and F. Barany, unpublished observations.

[8] C. Fouquet, M. Antoine, N. Rabbe, J. Cadranel, G. Zalcman, and T. Soussi, unpublished results.

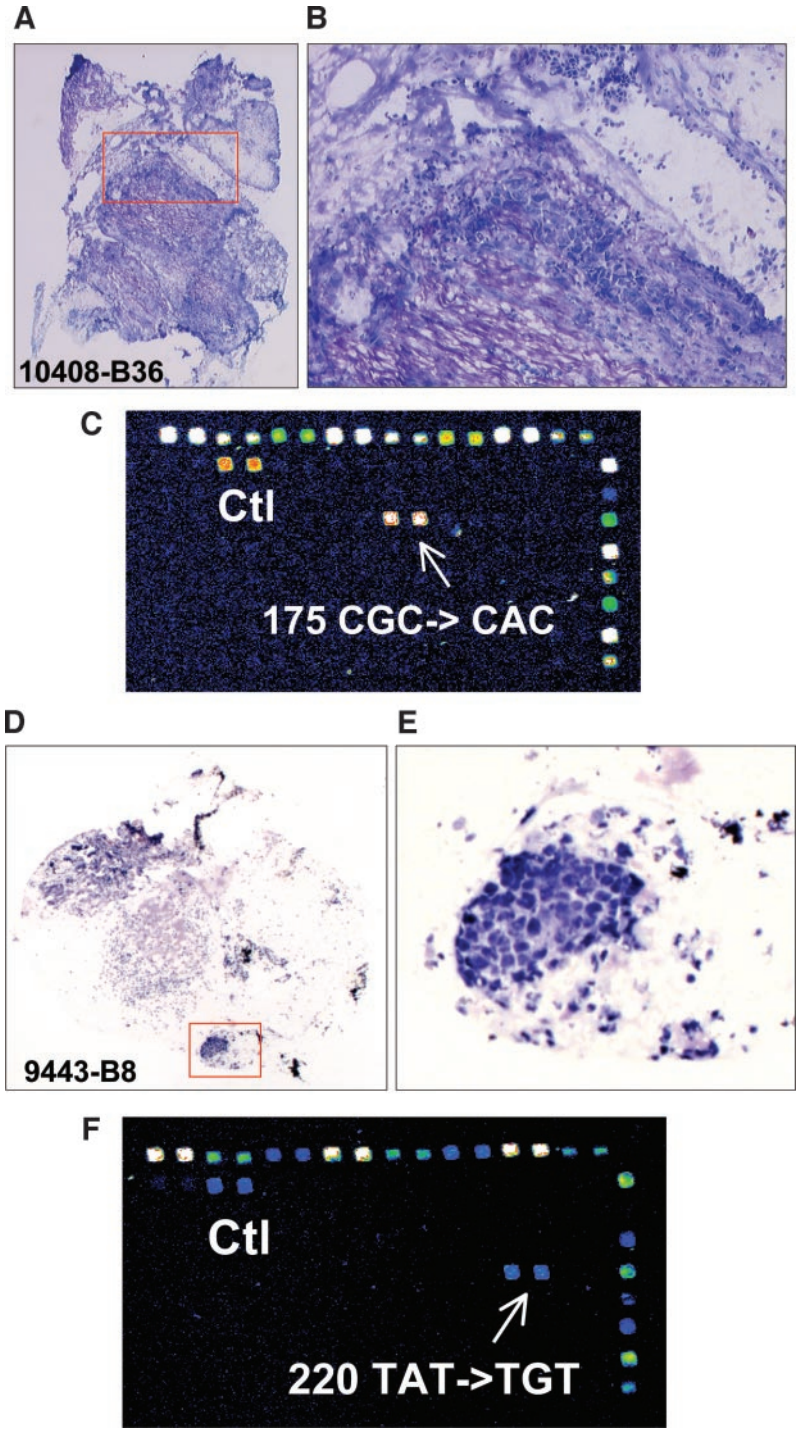

Fig. 1 Histology and array analysis of two bronchial biopsies. *A*, *B*, *D*, and *E*, Toluidine blue staining of an adenocarcinoma (*A* and *B*) and a small cell lung cancer (*D* and *E*). *A* and *D*, ×25; *B* and *D*, ×100. *C* and *F*, results of PCR/ligase detection reaction/Universal DNA microarray analysis of DNA. Addresses are double spotted onto a three-dimensional surface comprised of a loosely cross-linked polymer of acrylamide and acrylic acid. The three-dimensional surface combined with the zip code system allows hybridized arrays to be stripped of target and reused. Fiducials labeled with Cy3, Bodipy, and Alexa are spotted along the *top* and the *right side* of the array to provide orientation. Amplicon controls (Ctl) are seen in the next row; the Cy3 signal indicates that samples 10408 and 9443 present 175 G→A and 220 A→G mutations, respectively.

mutations in lung tumors), the probability of finding an index marker among the multiple genes queried is very high. The use of multiple fluorochromes could also improve the throughput of the assay.[9]

Many small and early lesions are now being detected in high-risk individuals by either low-dose CT scan screening programs or endoscopic fluorescence devices, but their true clinical significance remains uncertain. It is not possible to predict which of these lesions will really progress toward either overt cancer for dysplastic bronchial epithelial lesions or metastatic disease for early-stage cancers. It may be appropriate to target these premalignant changes or small stage I tumors for

[9] F. Barany, unpublished results.

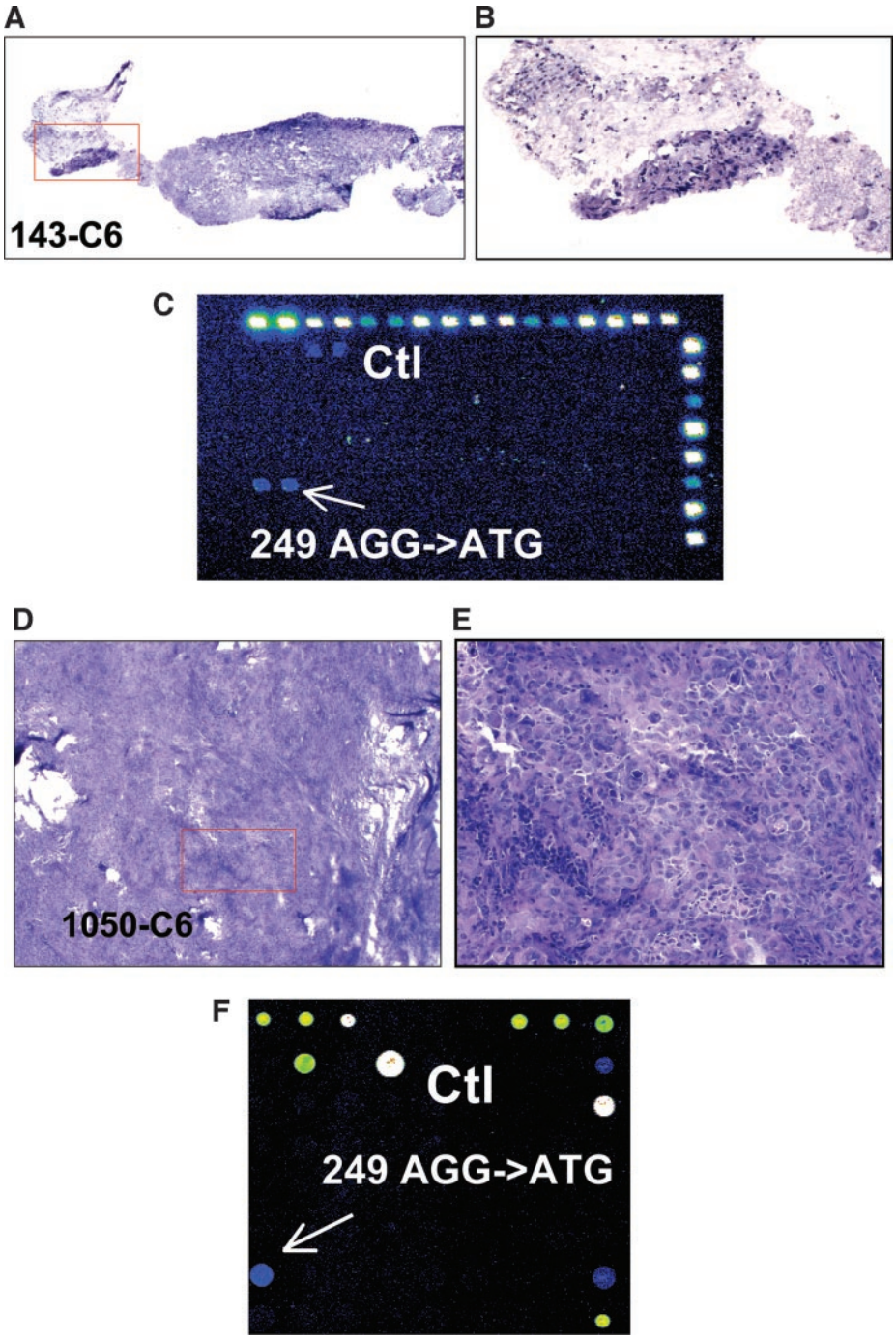

Fig. 2 Histology and array analysis of a matched biopsy and surgical specimen from the same patient. Toluidine blue staining of the biopsy (*A* and *B*) and surgical specimen (*D* and *E*) at two magnifications: *A* and *D* (×25); *B* and *D* (×100). *C* and *F*, results of PCR/ligase detection reaction/Universal DNA microarray analysis of DNA. Amplicon controls (Ctl) are seen in the *top row*; both samples display the same G→T mutation at codon 249. The arrangement of capture oligonucleotides in the array displayed in *F* is different because of a new spotting procedure.

early detection and intervention by fully profiling their molecular characteristics, including evaluation of response to specifically targeted intervention. High-throughput technologies such as genomics and proteomics are becoming widely available, and it will be crucial to apply these technologies to the detection of early lung carcinogenesis and outcome assessment. However, all of these technologies, including sample management and extraction of nucleic acids, must also be feasible as routine procedures in major clinical departments. The data presented here suggest that the PCR/LDR/Universal array assay, applied to samples containing a minority of tumor cells or DNA, recruited prospectively, meets these requirements.

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