Frequent Detection of ras and p53 Mutations in Brush Cytology Samples from Lung Cancer Patients by a Restriction Fragment Length Polymorphism-based “Enriched PCR” Technique

Moira Behn, Shen Qun, Wulf Pankow, Klaus Havemann, and Marcus Schuermann


ABSTRACT

RFLP-mediated PCR has been successfully applied as a reliable tool in the detection of ras mutations in many cancers and provides a basis for “mutant-enriched PCR” protocols. We have, therefore, modified this technique to the sensitive detection of K-ras codon 12 and also p53 “hot spot” mutations, which, frequently in lung cancer, affect codons at the positions 157, 175, 245, 248, 249, and 273. With a high sensitivity of 1 mutant allele in 10<sup>6</sup> normal alleles, our enrichment assay allows the detection of oncogene alleles when only a few tumor cells are present within a normal cell population. Brush cytology material obtained from the tumor site of 20 patients with endoscopically apparent bronchial carcinoma was compared to macroscopically normal mucosa taken from the contralateral bronchus (“control” cytology). We found K-ras codon 12 mutations in 5 cases (25%) and p53 mutations in 13 cases (65%) in the tumor-derived cell material but, with the exception of two cases, not in cell material taken from the control cytology. Seventy-five percent of the samples analyzed showed that at least one of the two oncogenes was affected. In several cases, two p53 lesions were detected concomitantly. The majority of the mutations could be reconfirmed by an alternative approach exploiting changes in the genomic RFLP pattern induced by these mutations and were also demonstrated in separate diagnostic biopsies taken. Thus, we conclude that the established enriched PCR protocol ensures a high sensitivity and preserved specificity for the diagnosis of oncogene lesions associated with lung cancer. Because conventional techniques normally yield a lower incidence of corresponding ras and p53 mutations, we think that both the high rate and the heterogeneity of p53 mutations found in some cases are, indeed, related to the increased sensitivity of this new enriched PCR technique.

INTRODUCTION

Early diagnosis of lung cancer is important because most cases are already inoperable at the time of diagnosis and, thus, bear a grave prognosis. The development of sensitive techniques for the detection of genetic lesions occurring early in the course of the disease may, therefore, improve the chance of successful local surgical resection. In lung cancer, potential diagnostic molecular markers include dominant oncogenes and tumor suppressor genes (for reviews see Refs. 1–4). Of these, the frequencies of ras and p53 oncogene mutations have been analyzed extensively. ras mutations are found in non-small cell lung cancer, predominantly in ADC, where the rate ranges between 15% and 50% of the cases according to the sensitivity of the assay used (5–11). The vast majority of the ras lesions analyzed affect the K-ras gene in codon 12 (>90%). Mutations of the p53 gene located on chromosome 17p are detectable in 50–70% of the cases and, contrary to K-ras mutations, are found in all histological types of lung cancer (12–16). These mutations comprise both allelic loss and/or point mutations. Interestingly, a large proportion of the point mutations are clustered within four small sections located between exons 4 and 9, a region encoding the central DNA-binding core region of the p53 protein (17). Of these, several codons are considerably more frequently affected than others, hence their designation as “hot spot” regions of the p53 gene (17). Although K-ras mutations seem to occur late in lung cancer tumorigenesis (18), somatic alterations of the p53 gene can be found at different tumor stages (19–21). p53 mutations may even occur at a precancerous stage because lesions are found in metaplastic and dysplastic states (20, 21).

A major problem in determining oncogene lesions in lung cancer occurs whenever tissue samples contain a large amount of genetically normal cells derived from the site of tissue sampling. This applies in particular to bronchoscopic biopsies, broncho-alveolar lavage, and brush cytology samples, which may harbor only a few cancer cells within a population of normal respiratory epithelium and alveolar macrophages. One of the methods of overcoming this problem has been the application of PCR technology in combination with RFLP analysis (22–24). Briefly, this method consists of a two-step nested PCR in which a mismatch primer is used in the second step to introduce a restriction site into PCR products derived from normal but not

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3 The abbreviations used are: ADC, adenocarcinoma; PCR-RFLP, RFLP-mediated PCR.
mutant alleles. The resulting PCR product is then digested by the corresponding restriction enzyme, leading to the cleavage of wild-type allelic products but not mutant ones. This second step then can then be repeated, thereby allowing the mutant alleles to serve as templates, and, thus, eventually results in the accumulation of restriction enzyme-harboring fragments can be distinguished easily from remaining wild-type alleles, given their different bp lengths. by mutation-harboring fragments can be distinguished easily from remaining wild-type alleles, given their different bp lengths. by standard phenol-chloroform extraction procedure and could be stored either as a dry pellet for up to 1 year or as a solution.

To address this problem, we have established an enrichment protocol for mutant alleles, based on the PCR-RFLP technique, that allows the sensitive detection of p53 lesions mutated in several hot spot positions, which are most frequently affected in lung cancer. We have used this protocol to determine the frequency of K-ras codon 12 and p53 hot spot mutations in brush cytology material taken from sites of visible tumor growth. Here, we report that circumscribed p53 lesions can be obtained in material obtained at bronchoscopy.

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**MATERIALS AND METHODS**

**Cell Lines and Tissue Source**

Cell lines NCI-H1573 (of ADC origin, no K-ras codon 12 mutation, homozygous p53 codon 248 mutation) and NCI-H2009 (ADC cell line, heterozygous K-ras codon 12, homozygous p53 codon 273 mutation) were obtained from Dr. A. Gazdar (Southwestern Medical Center, Dallas, TX; described in Ref. 26). Cell line LCLC-97TM1 (large cell carcinoma cell line, homozygous K-ras codon 12 mutation) was established in Marburg (described in Ref. 27).

**Bronchoscopy Samples**

Cytology and biopsy samples were collected during routine bronchoscopy at the Neukölln Hospital (Berlin, Germany) in 1992 and 1993. The study included only patients with clear signs of endoluminal tumor growth. The clinical data obtained at the time of bronchoscopy and the subsequent histology of the tumors found are listed in Table 1. In each case, brush cytology material was first taken from the nonaffected contralateral bronchial mucosa, followed by taking of brush cytology and biopsy material from the tumor site. To avoid cross-contamination, a sterile new brush was taken for each sample picking. The material derived from brush cytology was air-dried on microscope slides and spray-fixed; thereafter, biopsy material was fixed in formalin and embedded in paraffin.

**DNA Preparation**

For DNA analysis of brush cytology material, the cell debris was removed from the glass by scratching with a scalpel, transferred to Eppendorf tubes, and incubated in 500 µl of lysis buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, and 0.5% Tween 20] containing protease K (0.5 mg/ml) overnight at 56°C with regular shaking. The DNA was extracted by standard phenol-chloroform extraction procedure and could be stored either as a dry pellet for up to 1 year or as a solution in 50 µl of 10 mM Tris-HCl (pH 8.3) for 4–6 weeks at 4°C.

In the case of biopsy material, paraffin-embedded tissue samples were cut into 10–15-µm sections. Two to four sections of each paraffin block were incubated in 500 µl of lysis buffer for 4 h to dissolve DNA fragments and treated in the same way as brush cytology samples.
A Detection of K-ras mutations by "enriched-PCR" analysis

Primer pairs for pre-amplification
PCR product
K-ras 5' / K-ras 3' 296 bp

Primer pairs for PCR-RFLP
PCR product
digested PCR product
K-ras 5'/K-ras 3' 180 bp 139 + 41 bp

B Detection of p53 "hot spot" mutations by "enriched-PCR" analysis

Primer pairs for pre-amplification
PCR product
p53 codon

Primer pairs for PCR-RFLP
PCR product
digested PCR product
p53 codon

Primer pairs and digestion products for allele-specific restriction enzyme site analysis

Parameters for the "Enriched PCR" Analysis

Preamplification. For the first amplification step, 100–300 ng of either nontreated or predigested genomic DNA were amplified in a volume of 50 μl containing 60 mM Tris-SO₄ (pH 9.1), 18 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 200 μM each dNTP, 250 nM each primer, and 1 unit of ELONGase (Life Technologies, Inc.), a mix that consists of Taq and Pyrococcus spp. GB-D thermostable DNA polymerases. This enzyme mix was chosen to increase the proofreading activity, leading to higher fidelity. The reaction mixes were overlaid with 80 μl of mineral oil (Sigma Chemical Co., St. Louis, MO). The amplification parameters were 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, for a total of 30 cycles.

The oligonucleotide primers used for the preamplification

Fig. 1 Schematic representation of the primers used in the analysis. A, location of primers used in the analysis of K-ras. B, primers used in the detection of p53 lesions. Shown is the exon-intron structure of the genes (not to scale). □, exons. Names of the primer are indicated above or below primer positions (arrows). Lower, primer pairs used in the preamplification step (primers for first PCR) and in the subsequent PCR-RFLP steps (primers for PCR-RFLP), along with the sizes of the expected and of the digested PCR products.
PCR-based Oncogene “Hot Spot” Detection in Lung Cancer

...of human K-ras and p53 sequences were as follows (nucleotide substitutions are underlined; see also Fig. 1): K-ras exon 1, K-ras' (5'-GTTATACTCATTTTGATGACCACATGTTCTATT-3') and K-ras' (5'-ACCCTGAGAATTTGCAGAGAAGAC-3'). 53 exons 5 and 6, p53p3 (5'-CTTTCACCTGTGGTCCTCTTCCCTTCACTGACT-3') and p53p4 (5'-ACTGCTACACCGAGGACGTGACACCCAC-3'). p53 exon 7: p53p5 (5'-CCTCATCTGGTGCTATCTCCTAAGTGTTGCT-3') or p53p6 (5'-CCTCATCTGGTGCTATCTCCTAAGTGTTGCT-3') and p53p7 (5'-CCTCCACCGGTCTTGTGCTTGCATTCCCTC-3') and p53 exon 8, p53p8 (5'-CTTCTGGTCTCTCTTTCTATCGAGTAGTGGTA-3') and p53p9 (5'-GGTCCGGATGTAGCTGACGGGGTGAAAAAT-3').

Purification of PCR Products. DNA fragments resulting from the first amplification were purified by centrifugation through quick spin columns (QiAquick PCR Purification Kit; Qiagen) to ensure that all residual exonuclease activity from the first amplification step was eliminated and thus, prevent any interference with the intentional introduction of site-directed mismatch positions (as described below). The samples were then diluted 1:100–1:300, and 2 μl were subjected to the PCR-RFLP analysis.

PCR-RFLP Analysis. Two μl of an appropriate dilution from the purified products of first step PCR were amplified in a volume of 50 μl containing the following buffer components: 10 mm Trit-HCl (pH 8.3), 50 mm KCl, 1.5 mm MgCl₂, 0.2 mm each dNTP, 25 pmol of each primer, and 1.5 units of Taq DNA polymerase (Boehringer Mannheim). The samples were overlaid with 80 μl of mineral oil, and amplifications were performed for 24 cycles as above. The following oligonucleotide mismatch primers were used (nucleotide substitutions are underlined; see also Fig. 1): K-ras, primer sequences, K12Mva1 (5'-CTGGCTGAAAATGACTGAAATATAAAGCTGTGATGTTGAGCT-3') and K12as-1 (5'-CCTTATCTGTATGATCAAAGATTGTC-3'), p53, sense primer sequences, p53p4 (5'-GGTCTCTCTCCAGC-3') and p53p5T (5'-CCTCATCTGGTGCTATCTCCTAAGTGTTGCT-3') and p53p6 (5'-CTTCTGGTCTCTCTTTCTATCGAGTAGTGGTA-3') and p53p7 (5'-GGTCCGGATGTAGCTGACGGGGTGAAAAAT-3').

Endonuclease Digestion of PCR-RFLP Products. Five-μl aliquots of the PCR-RFLP were digested with 25 units of the following enzymes: K-ras codon 12, Mva1 (Boehringer Mannheim); p53 codon 157, AatII (New England Biolabs); p53 codon 215, BstUI (New England Biolabs); p53 codon 245, BglII (Life Technologies, Inc.); p53 codon 248, CspI (Stratagene); p53 codon 249, Bsu36I (Stratagene); and p53 codon 273, MluI (Boehringer Mannheim). The digestion was performed for 3 h in a total volume of 25 μl under conditions recommended by the supplier (PCR components were ignored). Twenty μl of the digestion products were electrophoresed through a 3% ethidium bromide-stained NuSieve Agarose gel (Bionzym; FMC Bioproducts, Rockland, ME).

Subsequent Enrichment Steps. The digestion products from above were diluted between 50-fold, and 2 μl were amplified using the same conditions for PCR amplification and digestion as described before. Thus, the complete enriched PCR protocol comprised 102 PCR cycles (30 cycles of preamplification and 72 cycles of PCR-RFLP).

Allele-specific Restriction Enzyme Site Analysis

Prior to the first PCR amplification step, 300–500 ng of genomic DNA were digested in a total volume of 30 μl containing 10 units of Hgal (New England Biolabs) for the analysis of p53 codon 157, AciI (New England Biolabs) for codon 245, Mspl (Boehringer Mannheim) for codon 248, and HaeIII (Boehringer Mannheim) for codon 249. The digestion was performed for 3 h in a total volume of 25 μl under conditions recommended by the supplier. The subsequent PCR amplification was performed over 24 cycles using ELONGase (Life Technologies, Inc.) using primer combinations as indicated in Fig. 1B. PCR products were then diluted as described before, and mutant...
alleles were allowed to enrich in two additional digestion and reamplification steps, as described above, in the presence of ELONGase instead of conventional Taq DNA polymerase. Thus, the protocol comprised a total of 72 PCR cycles.

Sequence Analysis

PCR products purified from agarose gels were sequenced using an ABI PRISM 377 sequencing automat (Applied Biosystems), AmpliTaq DNA polymerase, and an ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems), according to the manufacturer’s protocol.

RESULTS

Primer Design and Specificity. The PCR-RFLP protocol used in the analysis of K-ras codon 12 mutations was performed as described by Mitsudomi et al. (6) and Sugio et al. (18) using K-ras primers and mismatch primers, generating the restriction endonuclease cleavage site MvaI/BstNI, as indicated there. The strategy of this protocol is shown in Fig. 1A and was applied to DNA from a non-small cell lung cancer cell line known to harbor a homozygous K-ras mutation in codon 12 (LCLC-97TM1), yielding a clear MvaI-resistant fragment after one step of PCR-RFLP (Fig. 2, Lane 7). Conversely, PCR products derived from DNA of a cell line with a normal K-ras codon 12 status (NCI-H1573) were digestible with MvaI (Fig. 2, Lane 1). To test the sensitivity of this method further, we diluted DNA from a cell line LCLC-97TM1 into DNA of a cell line NCI-H1573 at various ratios. We then analyzed this mixture in three subsequent steps of PCR-RFLP, each time using DNA aliquots derived from the previous step to enrich for mutant alleles. In this way, we were able to improve the detection of mutant K-ras alleles up to a limit of 1 allele in 10⁸ normal alleles. Under these conditions, however, we also observed gradual accumulation of MvaI-resistant fragments in the cell line with no K-ras mutation (Fig. 2). This suggested that extended cycles of PCR amplification (more than 80 cycles) led to increasing nucleotide misincorporations by the conventional Taq polymerase used. To suppress these polymerase-borne errors, we used an enzyme mix containing Taq and a secondary thermostable DNA polymerase that possesses a 3’- to 5’-exonuclease or “proofreading” activity, thereby increasing the bp fidelity (28, 29). As shown in Fig. 2, this enabled us to reduce the background level of nonspecific fragment resistance, as long as the total number of PCR amplification steps did not exceed 70–80 cycles with conventional Taq polymerase. With this modification, we were able to increase the sensitivity of the assay approximately 50-fold, without loss of specificity. As an additional test for this hypothesis, we performed three steps of PCR-RFLP analysis screening for the same mutations in material that we obtained from archival brush cytology material (see below). Fig. 3 shows that, with conventional Taq polymerase alone, a substantial “background” of MvaI-resistant fragments becomes prevalent in the second enrichment step, whereas this phenomenon does not occur if proofread amplified DNA material is taken instead.

A similar PCR-RFLP protocol for the detection of p53 mutations was then designed. Because this oncogene bears mutations at multiple sites, we concentrated on six positions that are most frequently affected in lung cancer, namely, positions at amino acids 157, 175, 245, 248, 249, and 273. The selection of these hot spot sites was based on data compiled by Greenblatt et al. (17), with reference to their incidence in lung cancer. Fig. 1B shows the strategy used to determine mutations in these codons. In these cases, we chose artificial restriction enzyme sites,
which allowed the use of inexpensive but effective enzymes, capable of cutting within 1 h. In addition, the site introduced allowed the detection of all those mutations in the corresponding codon, which resulted in an amino acid substitution (usually mutations in the first or second position of the respective triplet). The protocol was applied to the analysis of cell lines NCI-H1573 and NCI-H2009, which harbor p53 mutations at positions 248 and 273 of the p53 gene, respectively (6). In a serial dilution assay with DNA from a nonaffected cell line, up to 1 allele in 10^4 normal alleles could be detected, which is comparable to the sensitivity seen in the analysis of K-ras mutations. Finally, the specificity of our modified PCR-RFLP protocol was tested by screening for K-ras and p53 mutations in WBCs obtained from 20 healthy donors. Three subsequent PCR-RFLP enrichment steps were performed for each of the tested p53 and K-ras positions. In no case were we able to detect a single point mutation (data not shown).

Analysis of Archival Brush Cytology Material. Having established an improved enriched PCR protocol, we next performed a study on cytological material derived from lung cancer patients during routine bronchoscopy (see “Materials and Methods”). This material was screened systematically for individual K-ras and p53 mutations in the aforementioned codons and compared to material derived from the contralateral bronchial mucosa in each of the patients. With the primer combinations given in Fig. 1, we were able to obtain, in each case, a PCR product of the expected length in the preamplification step. In the subsequent enrichment for mutant alleles by three consecutive steps of PCR-RFLP, we found a considerable number of digestion-resistant PCR products indicative of one or more site-specific K-ras or p53 mutations in the respective sample. To confirm the results and check for possible erroneous mutations occurring in the course of enrichment, all enriched PCR steps were repeated once again, starting from the same original tumor DNA samples. As an example, Fig. 4 shows the results obtained in the analysis for p53 codon 157, 248, and 249, whereas the “control” mucosa samples remained restriction endonuclease sensitive, even after two steps of enrichment for mutated alleles (data not shown).

Our survey of 20 brush cytology samples yielded a total of...
Table 2  Detection of K-ras and p53 mutations in brush cytology specimens from the tumor site

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>K-ras</th>
<th>p53</th>
<th>No. of mutations</th>
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<td>Codon 12</td>
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<tr>
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<td>1</td>
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Fig. 5  Verification of K-ras and p53 mutations by sequencing. Shown is the sequence around K-ras and p53 hot spot areas analyzed in four patients. Top, DNA derived from normal mucosa; middle, sequencing of DNA from cytology samples from the tumor site; and bottom, sequencing of DNA from corresponding biopsies of the same patients. Mutations found are indicated below the respective samples along with the resulting amino acid substitution.

five K-ras codon 12 mutations (summarized in Table 2), whereas no analogous lesion was found in the control mucosa sample from each patient. Similarly, we found a number of p53 mutations in samples derived from the tumor site. Mutations of codon 248 and 249 (five cases each) were seen most frequently, followed by mutations of codon 273 (four cases), codon 157 (two cases), and codon 175 (one case). No mutations were found in codon 245. In several cases, we found more than one p53 mutation (patients 1, 2, 3, and 16; see Table 2). Three brush cytology samples revealed both a K-ras and a p53 mutation (patients 3, 11, and 17). One cytology sample even harbored tumor cells collectively bearing three mutations (patient 3; see Table 2). Altogether, we found 5 lesions in the K-ras and 17 in the p53 gene. These could be attributed to 5 patients bearing.
K-ras mutations (25%) and 13 patients with p53 mutations (65%). Five of the 20 samples (25%) showed no mutation with respect to the seven positions examined in the two genes. Brush cytology samples taken from macroscopically normal control bronchial epithelium revealed only one digestion-resistant fragment, indicative of a mutation in codon 157 of the p53 gene. In the remaining cases, no alteration in the digestion pattern was observed, even after three subsequent PCR-RFLP steps (data not shown).

Finally, all PCR products obtained were sequenced (summarized in Table 2). We found that all K-ras or p53 fragments contained individual point mutations that occurred within the first codon following the primer binding site. All mutations detected also gave rise to an amino acid substitution. Conversely, the remaining sequence and PCR products obtained from the contralateral mucosa showed no deviation from the expected sequence.

**Correlation between Cytology and Biopsy Samples.**
To establish whether the mutations found in the cytology specimens were derived from cells representative of the tumor apparent in the bronchoscopy, we analyzed the biopsy samples taken during this diagnostic procedure for the histopathological classification of the tumor. In all cases, the histological examination had confirmed the presence of carcinoma cells in the biopsy (see Table 1). DNA extracted from the same paraffin-embedded material was subjected to three steps of PCR-RFLP enrichment, with the result that the majority of the mutations were detected within the first PCR-RFLP step and the remainder were detected within the first of the two subsequent enrichment steps. As performed previously, all mutations were confirmed by sequencing. Fig. 5 gives an example of four cases of K-ras and p53 mutations examined this way. In each case, we found an identical point mutation in the biopsy and cytology sample taken from the same tumor site. A summary of all results obtained is given in Table 3. In nearly all cases, the location and sequence of the individual oncogene lesion were identical in the brush cytology and the biopsy specimens (19/22). Two mutations found in one patient could not be retrieved in the original biopsy material. In one biopsy sample, we found an additional third p53 lesion located in codon 245. Conversely, the remaining sequence and PCR products obtained from the contralateral mucosa showed no deviation from the expected sequence.

**Relation of Oncogene Mutations to Tumor Origin.** Finally, to demonstrate that the lesions found are present in the starting material, we tested the same DNA for allelic-specific restriction site alterations, making use of naturally occurring restriction sites in most of the examined p53 codons. These are located in the p53 gene at codons 157, 245, 248, and 249. Mutations in codon 157 destroy a Hgal site present in the genomic DNA, codon 245 mutations destroy an AcI site, codon 248 mutations destroy an MspI site, and codon 249 mutations give rise to a HaeIII-resistant PCR product. The Hgal/HaeII site in codon 175 could not be used in this assay because the high number of recognition sites would cut the PCR product into too-short fragments. In one instance (HaeII site in codon 249), an additional HaeIII site in the resulting fragment was eliminated by the use of a mismatch primer, p53p5, changing an internally located G into T, as indicated in the primer sequence (see "Materials and Methods"). This had no measurable effect on the assay conditions. Due to the higher yield of DNA from biopsy samples, it was possible to demonstrate the successful cleavage of all DNA fragments before PCR amplification because, otherwise, false positive fragments would occur (data not shown). Our reanalysis of the four p53 codons mentioned above confirmed 8 of the 11 mutations found in the enriched PCR assay. Results obtained for codons 248 and 249 of the p53 gene are shown in Fig. 6. From this figure, it is also evident that three mutations were missed by this method (one in codon 248 and two in codon 249), which have been detected by our enriched PCR protocol (see Fig. 4). Subsequent sequence analysis confirmed that the nucleotide substitutions were identical in both cases (data not shown).

**DISCUSSION**
Here, we have adapted and modified existing enriched-PCR protocols based on the PCR-RFLP technique, which we applied to the sensitive detection of ras and p53 mutations in cytological material from patients with lung cancer. This was achieved by repetitive steps of PCR-RFLP, resulting in the accumulation of mutant alleles. Because this procedure risks producing a high rate of false-positive results due to increasing polymerase-borne error rates, we used a Taq polymerase with enhanced proofreading activity in the first amplification step. In this way, we were able to increase the sensitivity to 1 mutant allele in 104 normal alleles without loss of specificity. According to our results, this method can be applied to fresh or fixed tissue samples, as well as to air-dried archival material. Our enriched-PCR protocol, however, should be limited to two steps of enrichment for mutated fragments (equal to 3 PCR-RFLP steps or 72 PCR amplification cycles with conventional Taq polymerase). After three PCR-RFLP enrichment steps (corresponding to 96 PCR cycles; see Fig. 2), we observed a minor population of endonuclease-resistant fragments in all samples.

### Table 3
Comparison of mutational analysis in cytology and biopsy material

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<td>20</td>
<td>p53, V157</td>
<td>p53, V157</td>
</tr>
<tr>
<td>Total</td>
<td>5 K-ras; 17 p53</td>
<td>4 K-ras; 16 p53</td>
</tr>
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</table>
which may indicate that, during further enrichment steps, non-specific mutations accumulate.

Several precautions were taken before this assay was tested on tumor material. A number of cell lines with known mutations in the ras and p53 genes were tested, and the respective mutations could be retrieved, even if the samples were diluted several-fold (see Fig. 2). Serial testing of DNA isolated from WBCs of 20 healthy donors yielded no sign of digestion resistance in any of the seven codons tested, even after three PCR-RFLP steps. Moreover, with one exception, no mutations were detected in macroscopically normal contralateral mucosa. This one mutation occurred in codon 157 of the p53 gene. We found the same mutation in both cytology sample and biopsy material from the tumor site (p53, codon 157). The corresponding tumor was histologically classified as SCLC and originally grew at two different sites in the right lung. Therefore, it is possible that the finding of a mutation in the contralateral mucosa was due to tumor cell contamination, either during bronchoscopy or due to early intrapulmonary metastasis.

Additional evidence for the fidelity of our enriched PCR protocol stems from three facts: we reexamined the genomic material obtained from the brush cytology samples, which yielded the same results; we tested analogous DNA material from biopsy specimens taken on the same occasion but kept separately; and we tested the same DNA samples for alterations within naturally occurring allele-specific restriction sites. The latter alternative approach, however, could only be performed with respect to four codons of the p53 gene. Although, by this method, we could only reconfirm 8 of 11 cases, it largely excludes the possibility that these alterations were "de novo" artifacts, occurring repetitively as an intrinsic parameter of our enriched PCR protocol. Finally, the sequence analysis revealed that all mutations found were, indeed, missense mutations, leading to amino acid substitutions. Moreover, the predominant type of mutation found are G to T transversions (54.5%), as has been reported in several studies previously (5–7, 17, 30). From these data, we, therefore, conclude that at least the majority of the mutations found by either PCR approach are, indeed, indicative of the original tumor lesion.

Another aspect resulting from our study is an unexpectedly high frequency of K-ras and p53 mutations found in both the cytology and biopsy material from lung cancer patients. The frequency of lesions of the p53 gene was particularly high (65%), although only six hot spot positions of the gene were investigated. This frequency is significantly higher than previous estimations, which have been based on the analysis of histological sections that contain clonal tumor material (17). Although this high percentage is based on a relatively small number of cases, we think that our study may be indicative of a higher prevalence of such oncogene mutations in lung cancer patients than previously thought. This may be explained by the high sensitivity of our technique, which has a detection limit far beyond conventional techniques such as PCR-single-strand conformation polymorphism, direct sequencing of PCR products, or PCR followed by allele-specific oligonucleotide hybridization (24). A similar observation has been made by Mills et al. (10), who detected a 50% frequency of K-ras codon 12 mutations in lung cancer ADC using a similar PCR technique. They speculated that the high frequency of K-ras mutations found could be due to the enhanced sensitivity of this technique.

Interestingly, apart from the frequency, we also found a high percentage of multiple point mutations, several of which occurred within the p53 gene itself. This would not be expected in a unifocal and clonal tumor cell population. We, therefore, compared the number of mutations found in with the tumor-node-metastasis status at bronchoscopy (given in Table 1), as

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Fig. 6 Examples of allele-specific restriction site analysis of biopsy material from 20 patients screening for p53 codon 248 (top) and 249 mutations (bottom). Genomic DNA was digested by MspI or HaeIII prior to 30 cycles of PCR amplification using primer pair p53p5/p53p6. The resulting material was subjected to three sequential PCR-RFLP steps. For details see Figs. 1B and 3 and "Materials and Methods."
one might expect the number of oncogene lesions to increase with tumor size. The average T or N status, however, did not correlate with the individual number of mutations. Instead, we observed several cases with multiple mutations, which clinically presented with limited disease (cases 1, 2, and 17), whereas others at the stage of advanced disease showed no detectable K-ras or p53 mutation (cases 4, 6, and 10). An attractive alternative explanation is provided by the model of “field cancerization,” in which sites of multiple tumorigenesis are thought to occur. This would imply that the site of histological sampling either contains nontransformed respiratory epithelium bearing multiple genetic damage or areas of multifocal tumor formation. Evidence for field cancerization by chronic exposure to alcohol and tobacco at the molecular level was recently shown by Waridel et al. (33). These authors used a functional assay detecting transcriptionally inactive p53 mutants and showed that, in patients with multiple head and neck tumors, normal mucosa also contains a high frequency of p53 alterations, contrary to patients presenting with a single tumor. This finding may point to the possibility that multiple clones of mutant p53-containing cells expand independently in chronically damaged epithelium. Whether this situation holds true for lung cancer patients needs to be shown in further studies using analogous sensitive techniques.

Finally, it is interesting to note that we also found an unusual distribution of K-ras mutations with respect to tumor histology. Our own relatively small collection of bronchoscopically accessible tumors contained only three samples classified as ADC, one of which carried a K-ras mutation. We found instead four K-ras lesions in other types of tumors, of which three were histologically classified as squamous cell carcinomas and one remained unclassified. This finding differs from previous published data, which suggests that K-ras mutations are only rarely found in tumors other than ADC (6, 7, 34). Several factors may contribute to this bias: the small nonrepresentative collection of samples may lead to an overestimation of incidental findings and/or the high sensitivity of the PCR-RFLP technique may, as discussed above, detect mutations in surrounding epithelium or multifocal tumor cell populations arising in the tumor area.

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Frequent detection of ras and p53 mutations in brush cytology samples from lung cancer patients by a restriction fragment length polymorphism-based "enriched PCR" technique.

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