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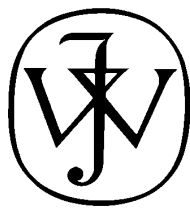
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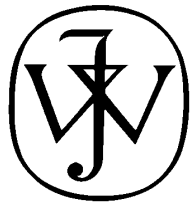
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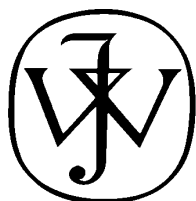
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## DETECTING LUNG CANCER IN PLASMA WITH THE USE OF MULTIPLE GENETIC MARKERS

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AQ: 1

**Recent studies have demonstrated the possibility to detect genetic changes in plasma DNA of cancer patients. The goal of this study was to validate a panel of molecular markers for lung cancer detection in plasma DNA. Three markers, p53, FHIT and microsatellite alterations at loci on chromosome 3p, were used to detect mutations in tumor and plasma DNA of 64 stage I–III non small cell lung cancer patients. p53 mutations were studied by direct sequencing of exons 5 through 8 in tumor DNA and by plaque hybridization assay and sequencing in plasma DNA. Allelic losses were evaluated by fluorescent PCR in tumor and plasma DNA. p53 genomic mutations were detected in 26 (40.6%) of 64 tumor DNA samples and the identical mutation was identified in plasma of 19 (73.1%) of them. Microsatellite alterations at FHIT and 3p loci were observed in 40 (62.5%) tumors and in 23 (35.9%) plasma samples. Of the 40 patients showing microsatellite alterations in tumors, 19 (47.5%) displayed the same change in plasma DNA. At least 1 of the 3 genetic markers (p53, FHIT and 3p) was altered in plasma of 51.6% of all patients and 60.7% of stage I patients. Moreover, genetic markers in plasma identified 29 of 45 (64.4%) of all stages and 15 of 22 (68.2%) of stage I patients whose tumors had an alteration. These results provide the proof of principle that plasma DNA alterations are tumor-specific in most cases and support blood testing as a noninvasive strategy for early detection.**  
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**Key words:** lung cancer; early detection; genetic markers; p53; FHIT

Lung cancer is one of the leading causes of cancer mortality in the world, especially in developed countries.<sup>1</sup> A major problem in lung cancer is the lack of clinically efficient noninvasive methods for early detection and screening of asymptomatic high-risk individuals.

In the past, the most common screening techniques, such as chest radiography and sputum cytology, were unable to reduce the mortality.<sup>2</sup> Recent results achieved by spiral CT have opened new prospects for significant reduction of lung cancer mortality but proper selection of high-risk population and differential diagnosis are critical elements.<sup>3</sup> In fact, even though for stage I lung cancer patients surgical resection can achieve a 60–70% 5-year survival, over 70% of cases are detected in stage II–IV patients where survival is poor.<sup>4</sup> Thus, the development of novel molecular methodologies is needed to facilitate early detection of lung cancer. Lung cancer is associated with a variety of genetic alterations, including p53<sup>5</sup> and K-ras mutations,<sup>6</sup> inactivation of the fragile histidine triad (FHIT) gene,<sup>7,8</sup> allelic imbalances at multiple chromosomal loci<sup>9,10</sup> and aberrant promoter methylation of several genes, mainly p16<sup>INK4a</sup>.<sup>11</sup> Most of these changes have also been described in premalignant lesions and early phases of lung carcinogenesis.<sup>12</sup> The use of sensitive molecular techniques has enabled the detection in the plasma of lung cancer patients of the same genetic alterations observed in their tumors.<sup>13–15</sup> In addition, several studies have demonstrated the presence of significantly higher concentrations of circulating DNA in the plasma/serum in patients with different types of cancer,<sup>16–19</sup> including primary or recurrent lung cancer.<sup>20</sup> Thus, quantification of plasma DNA and characterization of specific molecular changes could represent useful bi-

omarkers of lung cancer. In an attempt to validate a grid of molecular genetic markers detectable in plasma DNA of lung cancer patients, we analyzed a series of 64 patients with stage I–III non small cell lung cancer (NSCLC), focusing our attention on 3 very common alterations: p53, FHIT and allelic imbalances affecting other 4 loci on 3p. The ultimate goal of the study was the validation of molecular approaches that might be useful for an effective early detection and monitoring of NSCLC.

### MATERIAL AND METHODS

#### Pathologic and immunohistochemical methods

Clinical and pathologic data are illustrated in Table I. There were 38 squamous cell carcinomas, 19 adenocarcinomas, 5 large cell carcinomas, and 2 non small cell carcinomas not further defined. Stage I accounted for 45%, stage II for 27% and stage III for 28% of the tumors. For immunohistochemical analysis, formalin-fixed and paraffin-embedded samples obtained at surgery were investigated for p53 and Fhit markers according to previously refined methods.<sup>21</sup> All cases were evaluated blindly without knowledge of the patients' identity, pathologic diagnosis, clinical outcome or plasma DNA status. The percentage of p53 and Fhit-immunoreactive tumor cells was evaluated by scoring a minimum of 1,000 tumor cells in representative fields of immunostaining.

TI

#### Samples collection and DNA isolation

A consecutive series of 64 NSCLC patients admitted from 1995 to 1997 at the Royal Brompton Hospital, London, was analyzed (Table I). All patients gave informed consent to have their banked specimens analyzed in future molecular studies. All tumor specimens were surgically resected and immediately stored at –140°C. Peripheral blood samples were obtained from the patients on the day of admission and collected in lithium heparin. Plasma was immediately separated from the cellular fraction by centrifuging twice at 2,500 rpm for 10 min at 4°C. The resulting supernatant (plasma) and 2 ml of whole blood were frozen at –80°C. DNA was extracted from tissues, plasma and blood cells samples by using QIAamp DNA Mini Kit (Qiagen, Chatsworth, CA) accord-

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TABLE I – PATIENTS FEATURES (n = 64)

	n	%
Age		
Median	64	
> 60	42	66
Female	18	28
Smokers	62	97
Mean ciga/day	24	
> 20 ciga/day	28	44
Type		
Squamous	38	59
Adeno	19	30
Other	7	11
Stage		
I	29	45
II	17	27
III	18	28
Surgery		
Pneumonectomy	18	28
Lobectomy	40	63
Segmentectomy	2	3
Mediastinal biopsy	4	6

ing to the tissue protocol and blood and body fluids protocol; 1,000  $\mu$ l of plasma were purified by 5 passages on the same column (Qiagen) and the resulting DNA was eluted in 50  $\mu$ l of sterile bidistilled water and stored at  $-20^{\circ}\text{C}$ . Tumor and whole blood DNA concentrations were estimated by spectrophotometry.

#### *p53 mutations detection by polymerase chain reaction and direct sequencing*

A 1,672 bp fragment of the TP53 gene, exon 5 through exon 8, was amplified from 64 available primary tumor DNAs by PCR (GeneAmp PCR system 9700, PE, Applied Biosystem, Foster City, CA); 100 ng of DNA purified from primary tumor were used for PCR amplification, performed using the following primers (from MWG oligo) located in the introns flanking exon 5 and exon 8: 312 exo5 (sense), 5'-TTCAACTCTGTCTCCTTCCT-3'; 8.3 exo8 (antisense), 5'-AAGTGAATCTGAGGCATAAC-3'. The resulting 1,672 bp fragment of the TP53 gene was then used as a template to sequence separately the different exons, 5, 6, 7 and 8. PCR amplification was performed as follows: 2.5  $\mu$ l of  $10 \times$  buffer II gold PE, 2.5  $\mu$ l of 2.5 mM  $\text{MgCl}_2$ , 1  $\mu$ l of 2.5 mM dNTP mix, 2  $\mu$ l of each primer (312 and 8.3) 20  $\mu\text{M}$ , 0.3  $\mu$ l of 5 units/ $\mu$ l Amplitaq Gold (Applied Biosystem) and 37.7  $\mu$ l of sterile water. The final volume was 50  $\mu$ l. Samples were then processed through an incubation of 10 min at  $95^{\circ}\text{C}$  and 40 cycles consisting of 40 sec at  $94^{\circ}\text{C}$ , 40 sec at  $62^{\circ}\text{C}$  annealing temperature and 2 min at  $72^{\circ}\text{C}$ , for the first 10 cycles, and 40 sec at  $94^{\circ}\text{C}$ , 40 sec at  $60^{\circ}\text{C}$ , 2 min at  $72^{\circ}\text{C}$  for the last 30 cycles with a final extension at  $72^{\circ}\text{C}$  for 5 min. The PCR products were purified (Qiaquick PCR purification Kit, Qiagen) and exons 5–8 were sequenced individually by cycle sequencing using appropriate primers located in the introns.

#### *Plaque hybridization assay*

The search for p53 mutations in plasma was performed in 26 patients (27 mutations) found to have p53 mutation in their primary tumor with oligonucleotide plaque hybridization assay. The exon containing the p53 mutation found in the primary tumor was amplified from plasma DNA by PCR with the appropriate programs and primers for each exon; 5  $\mu$ l of plasma DNA were used for the amplification in a final volume of 50  $\mu$ l. The fragment was then purified and cloned in a pGEM-Teasy vector (Promega, Madison, WI). After ligation, carried out at RT for 1 hr, the transformation in bacterial cells was performed o/n at  $37^{\circ}\text{C}$  and between 1,000 and 3,000 clones were transferred to nylon membranes (Stratagene, La Jolla, CA).

The membranes were then hybridized with  $\text{P}^{32}$  end-labeled oligonucleotide probes specific for the p53 mutation identified in the primary tumor as previously described. As a positive control,

the primary tumor was used; as a negative control, a plasma DNA sample without mutation was used. Hybridizing plaques indicated the presence of mutation of p53. The positive plaques were picked up and sequenced to confirm the mutation. The ratio of mutant to total white colonies ranged from 1/50 to 1/700. In the negative control, no positive plaques were found. For some plasma samples, where the mutation was not confirmed by sequence even in the presence of conspicuous hybridized plaques, other techniques such as restriction endonuclease analysis (REA) and mutation allele-specific amplification (MASA) were used.

#### *Restriction endonuclease analysis*

REA was performed only in plasma samples that had a selective mutation, arginine-to-serine substitution,<sup>22</sup> identified as a hotspot mutation in lung cancer, located in exon 7 of the p53 gene. The plasma samples were analyzed by REA as follow: 5  $\mu$ l of purified plasma DNA were used to amplify exon 7 of p53 in a final volume of 50  $\mu$ l with the use of previously described conditions with specific primers located in the introns. After incubation at  $95^{\circ}\text{C}$  for 10 min, the PCR consisted of different steps: 30 sec at  $95^{\circ}\text{C}$ , 30 sec at  $63^{\circ}\text{C}$ , 1 min at  $72^{\circ}\text{C}$  for 10 cycles, 30 sec at  $95^{\circ}\text{C}$ , 30 sec at  $62^{\circ}\text{C}$ , 1 min at  $72^{\circ}\text{C}$  for 30 cycles, followed by a final extension at  $72^{\circ}\text{C}$ ; 10  $\mu$ l of PCR product were digested with *HaeIII* (New England Biolabs, Beverly, MA) and the amplification products were visualized on 3% agarose gel with ethidium bromide. Two major fragments of 92 and 66 bp and several small fragments from wild-type sequence were clearly identified on the gel, because the restriction endonuclease cleaved a GG/CC sequence between codon 249 and 250 of exon 7, whereas the presence of an un-cleaved band of 158 bp was indicative of the mutation in codon 249, since the enzyme was not able to cut the GG/CC sequence that is destroyed by the mutation (AGG-to-AGT). The complete cleavage was ensured by the absence of the 254 bp fragment. To enhance the sensitivity of our test, the digested product was subjected to an additional PCR reaction to amplify the mutant PCR product selectively; 5  $\mu$ l of digested products were used for a second amplification using the following nested primers located in exon 7: Nes1 (sense), 5'-AGGCGCACTGGCCTCCTT-3', and Nes2 (antisense), 5'-TGTGCAGGGTGGCAAGTGGC-3'. The final volume was 50  $\mu$ l. The samples were processed through 30 cycles consisting, after incubation at  $95^{\circ}\text{C}$  for 10 min, of 30 sec at  $94^{\circ}\text{C}$ , 30 sec at  $60^{\circ}\text{C}$  and 30 sec at  $72^{\circ}\text{C}$ , followed by a final extension at  $72^{\circ}\text{C}$  for 5 min. In this case, a fragment of 182 bp was obtained; 15  $\mu$ l of PCR product were then digested with restriction endonuclease *HaeIII* for 1 hr at  $37^{\circ}\text{C}$  in a final volume of 20  $\mu$ l. Two fragments, of 92 and 66 bp, representative of wild-type sequence and one undigested enriched 158 bp fragment, indicative of the mutation arginine-to-serine in codon 249 of exon 7, were clearly identified on the gel. In the positive control (a tumor sample carrying the mutation), the 158 bp fragment was clearly detected, whereas the negative control (a sample from plasma DNA of a healthy donor) did not show the 158 bp fragment.

The sensitivity of this test was calculated by serially diluting a control DNA containing the mutation at codon 249 from 1:10 to 1:10,000. The presence of the un-cleaved 158 bp fragment was still visible at ratio of 1:10,000. To confirm the presence of the mutation, these fragments were separated on a 3% agarose gel stained with ethidium bromide, transferred to a nylon membrane (Hybond-N<sup>+</sup> Amersham Pharmacia Biotech, Piscataway, NJ) and hybridized with  $\text{P}^{32}$  end-labeled oligonucleotide probes containing the p53 mutation at codon 249. The presence of a hybridized 158 bp band in plasma confirmed the mutation.

#### *Mutation allele-specific amplification*

One case carrying a deletion of a conspicuous number of bases (27 bp) was analyzed by MASA. The amplification was performed with primers centered upstream and downstream the deletion. The reaction mixture contained 5  $\mu$ l of DNA, 5  $\mu$ l of  $10 \times$  buffer, 2.5 mM  $\text{MgCl}_2$ , 1  $\mu$ l dNTP mix, 2  $\mu$ l of each primer 20  $\mu\text{M}$ , 0.3  $\mu$ l of 5 units/ml Amplitaq Gold and 37.7  $\mu$ l of sterile water. The final



volume was 50 µl and the samples were processed through 30–35 cycles at an appropriate annealing temperature for each primer. As a positive control, the corresponding tumor DNA carrying the deletion was used; as a negative control, DNA from lymphocytes of a healthy donor was used. The PCR products were then visualized on a 3% agarose gel stained with ethidium bromide. The presence of an amplified fragment confirmed the deletion in plasma DNA.

*Microsatellite analysis*

The analysis of microsatellite instability and loss of heterozygosity was performed by studying microsatellite alterations at loci at 3p14.2 (D3S1300, FHIT locus), 3p21 (D3S1289), 3p23 (D3S1266), 3p24.2 (D3S2338), 3p25-26 (D3S1304) that are hot-spots of deletions in lung cancer. The sequences of nucleotide markers for microsatellite analysis are available through the Genome Database.

A total of 30 ng of tumor and lymphocyte DNA were used for the analysis; 2–30 ng of purified DNA was used for PCR amplification of plasma by using primer pairs synthesized with FAM, HEX or NED fluorescent labels (PE Applied Biosystems ABI Prism Linkage Mapping Set). PCR protocol was as follows: Buffer II gold PE (10 ×) 1.5 µl, MgCl<sub>2</sub> (2.5 mM) 1.5 µl, dNTP mix (2.5 mM) 0.2 µl, labeled primer mix (10 µM) 1 µl, AmpliTaq Gold (5 U/µl) 0.12 µl, sterile water 9.8 µl. Final volume of the reaction was 15 µl. Samples were processed in a GeneAmp PCR system 9700 thermal cycler through 45 cycles, each cycle consisting of 10" at 96°C, 30" at 55°C annealing temperature, 3' at 70°C. Pools of the fluorescent PCR products for each clinical specimen were separated electrophoretically on a 5% polyacrylamide gel and analyzed by laser fluorescence using ABI Prism DNA Sequencer (377 PE Applied Biosystem) equipped with GeneScan TM 2.1 software. Loss of heterozygosity (LOH) and the presence of allele shifts indicating genomic instability are recorded in the various samples and compared with the profiles obtained in DNA from normal peripheral lymphocytes. LOH was scored when a reduction of at least 30% of allele intensity in the experimental sample was seen. All the DNA samples with microsatellite alterations were amplified at least twice to rule out PCR artifacts or sample contamination. In the presence of allelic imbalance in plasma, increasing amounts of plasma DNA were used in the PCR reaction in order to exclude unreliable allelotyping.

*Statistical analysis*

Qualitative data are presented as frequencies and/or percentages and compared using chi-square test or Fisher's exact test. Exact 95% confidence intervals for proportions were calculated using the binomial distribution. An association was considered statistically significant if the corresponding *p*-value was ≤ 0.05.

RESULTS

*p53 mutations, FHIT and 3p LOH analysis in tumor and plasma samples*

Table II records the frequency of molecular changes in tumor and plasma. The frequency and type of p53 genomic mutations in

TABLE II – FREQUENCY OF p53 MUTATIONS, FHIT AND 3p LOH IN TUMOR AND PLASMA SAMPLES FROM NSCLC PATIENTS

	Tumor	Plasma	Plasma <sup>a</sup>
p53 mutations	26/64 (40.6%)		19/26 (73.1%)
FHIT LOH <sup>b</sup>	22/56 (39.3%)	9/56 (16.1%)	7/22 (32%)
3p LOH <sup>c</sup>	40/64 (62.5%)	23/64 (35.9%)	19/40 (47.5%)
Any change	45/64 (70.3%)	33/64 (51.6%)	29/45 (64.4%)

<sup>a</sup>Fraction of plasma samples with alterations from patients showing a corresponding change in the tumor.– <sup>b</sup>In specimens unable to determine presence or absence (noninformative).– <sup>c</sup>Number of cases containing at least 1 microsatellite alteration in the panel of 5 markers analyzed.

tumor samples detected by exon 5–8 amplification and direct sequencing are reported in Table III. Twenty-six out of 64 (40.6%; 95% confidence interval = 28–54%) samples studied showed the presence of genomic mutations in the coding sequence of the p53 gene. Two different mutations in exons 5 and 6 were found in one tumor sample. Twenty-three mutations were missense and 4 were null (Table III). The analysis of microsatellite alterations at 5 loci on 3p showed allelic imbalances affecting at least 1 locus on 3p in 40 of 64 tumors (62.5%; CI = 49–74%). The frequency of alterations at any single locus was D3S1300 (3p14.2), 22/56 (39.3%); D3S1289 (3p21), 15/54 (27%); D3S1266 (3p23), 20/50 (40%); D3S2338 (3p24.2), 22/54 (44%); D3S1304 (3p25-26), 24/53 (45%).

The search for p53 mutations in plasma was done in 26 patients (27 mutations) displaying p53 mutation in the tumor sample by using direct sequencing analysis, plaque hybridization assay (Fig. 1) and sequencing, REA (Fig. 2) and MASA. In 19 of 26 (73.1%; CI = 52–88%) plasma DNA analyzed, we detected, by using one or more of the assays described above, the same p53 mutation identified in the corresponding tumor.

Plasma samples from 64 patients who were informative (*i.e.*, heterozygous) for at least one locus on 3p were evaluated for the presence of microsatellite changes. Twenty-three of 64 plasma samples (35.9%; CI = 24–49%) showed alterations at one or more 3p loci (Table II). Of the 40 patients having microsatellite changes in the tumors, 19 (47.5%; CI = 31–64%) had a corresponding alteration in plasma DNA. In 4 plasma samples, a microsatellite alteration was observed in the absence of a corresponding tumor alteration. Nevertheless, the presence of FHIT and 3p LOH alterations in plasma samples was significantly associated with FHIT and 3p LOH alterations in tumor specimens (*p* = 0.02 in both cases, using Fisher's exact test).

When plasma samples were grouped according to alterations found in any genetic markers (FHIT, 3p, p53), tumor-specific changes were detected in 33 of 64 (51.6%; CI = 39–64%) of all patients and in 17 of 29 (58.6%; CI = 39–76%) of stage I patients. Moreover, genetic markers in plasma identified 29 of 45 (64.4%; CI = 49–78%) of all stages and 15 of 22 (68.2%; CI = 45–86%) of stage I patients whose tumors had a detectable alteration at any of the 3 markers. The frequency of molecular alterations in p53, FHIT or 3pLOH, whether in tumor or in plasma, was not statistically different when compared by stage, histology or age of the patients (data not shown).

TABLE III – CHARACTERISTICS OF p53 MUTATIONS

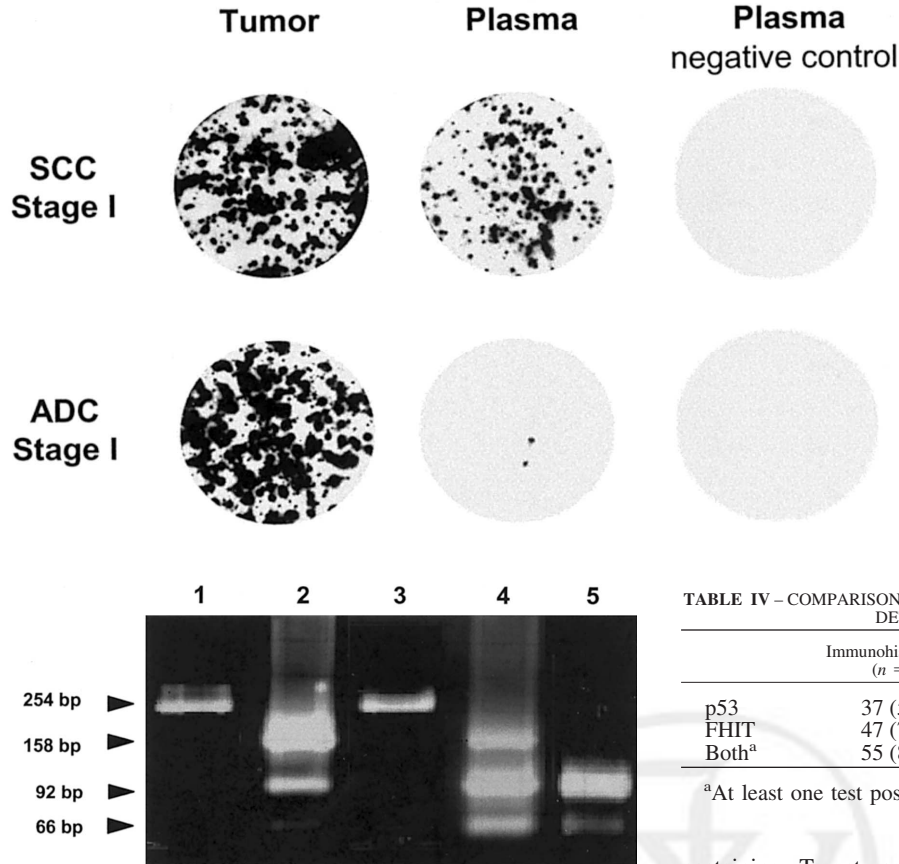
Number of cases with mutation	Exon	Mutation	Codon	Amino acid change
1	5	G→A	135	Cys→Tyr
1	5	Insertion C	151	Frameshift
1	5	G→T	154	Gly→Val
2	5	G→T	157	Val→Phe
2	5	C→T	159	Ala→Val
1	5	Deletion 27 bp	159	Frameshift
1	5	Insertion CC	161	Frameshift
1	5	A→T	168	Cys→Tyr
1	6	Deletion 5 bp	187	Frameshift
1	6	T→A	216	Val→Glu
1	6	T→G	218	Val→Gly
1	6	A→G	220	Tyr→Cys
2	7	T→G	234	Tyr→Cys
1	7	G→T	237	Met→Ile
1	7	G→T	248	Arg→Leu
3	7	G→T	249	Arg→Ser
1	7	A→T	249	Arg→Trp
1	8	G→A	273	Arg→His
1	8	G→A	275	Cys→Tyr
1	8	A→T	280	Arg→Stop
1	8	C→T	282	Arg→Trp
1	8	G→T	294	Glu→Asp

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T3

F2



**FIGURE 1** – Analysis of p53 mutations by plaque hybridization assay in plasma DNA from lung cancer patients showing variable number of mutant p53 alleles in stage I tumors. Nylon membranes were hybridized with mutant-specific oligonucleotides. Representative hybridizing clones were selected for sequencing. As a negative control, plasma DNA from individuals without p53 mutation was used in each experiment.

**FIGURE 2** – Detection of plasma Ser-249 p53 mutation in an NSCLC patient with REA. Lane 1, amplification of exon 7 from tumor sample by PCR; lane 2, fragment of exon 7 from tumor sample digested with *Hae*III. The 2 fragments of 92 and 66 bp originate from wild-type, because the restriction endonuclease cleaves a GG/CC sequence between codon 249 and 250 of exon 7; the presence of the 158 bp fragment is indicative of the mutation in codon 249, since the enzyme is not able to cut the GG/CC sequence that is destroyed by mutation. Lane 3, amplification of exon 7 from plasma sample by PCR; lane 4, fragment of exon 7 from plasma sample digested with *Hae*III. The 2 fragments of 92 and 66 bp are indicative of wild-type sequence, whereas the 158 bp fragment is indicative of mutation. Lane 5, control subject. The presence of only the 2 fragments of 92 and 66 bp is indicative of wild-type sequence.

**TABLE IV** – COMPARISON OF IMMUNOHISTOCHEMICAL AND MOLECULAR DETERMINATIONS IN TUMOR

	Immunohistochemical (n = 64)	Molecular (n = 56)	Both <sup>a</sup> (n = 56)
p53	37 (58%)	6 (41%) <sup>b</sup>	43 (67%) <sup>b</sup>
FHIT	47 (73%)	22 (39%)	43 (77%)
Both <sup>a</sup>	55 (86%)	31 (55%)	51 (91%)

<sup>a</sup>At least one test positive.– <sup>b</sup>Assay done in 64 specimens.

*Immunohistochemical analysis of p53 and fhit proteins*

Positive p53 immunostaining (> 10% reactive cells) was detected in 37 of 64 (57.8%; CI = 45–70%) samples. Twenty of 26 (76.9%; CI = 56–91%) cases showing p53 genomic mutation in exons 5–8 displayed positive p53 immunostaining compared to 17 of 38 (44.7%; CI = 29–62%) tumors without p53 mutation (*p* = 0.02, Fisher’s exact test). In 3 samples, in spite of the presence of missense mutations in exon 6 (1 case) and exon 8 (2 cases), lack of p53 immunoreactivity was observed. Overall, only 20 (54%; CI = 37–70%) of the 37 p53-overexpressing tumors that were also analyzed for genomic mutations showed underlying p53 gene mutations in exons 5–8, suggesting alternative mechanism for p53 protein stabilization in tumor samples.

Since in 39% of the tumor samples allelic imbalances at the D3S1300 locus located in intron 5 of the FHIT gene were observed, we analyzed Fhit protein expression by immunohistochemistry in tumor sections. Complete absence of Fhit protein immunostaining was recorded in 47 of 64 (73.4%; CI = 61–84%) tumors. Of the 17 Fhit protein-positive cases, 2 showed reduced immunoreactivity, with only 10% of cells showing weak immu-

nostaining. Twenty-one of 22 (95.5%; CI = 77–99%) tumors showing FHIT LOH also displayed complete absence of Fhit protein expression compared to 21 of 34 (61.8%; CI = 43–78%) tumors without FHIT LOH (*p* = 0.005, Fisher’s exact test).

No statistically significant association between p53 and Fhit protein expression was observed in tumor specimens, whereas tumors showing either FHIT LOH or 3p LOH displayed a significantly higher frequency of p53 genomic mutations. In fact, in samples with 3pLOH, 21 of 40 exhibited p53 mutations (52.5%; CI = 36–68%), whereas in samples without 3p LOH, 5 of 24 showed p53 mutations (20.8%; CI = 7–42%; *p* = 0.018, Fisher’s test). Similarly, in samples with FHIT LOH, 12 of 22 exhibited p53 mutations (54.5%; CI = 32–76%) and only 9 of 34 samples without FHIT LOH showed p53 mutations (26.5%; CI = 13–44%; *p* = 0.034, chi-square test).

The combined frequency of p53, FHIT and 3p microsatellite alterations in tumors detected by both immunohistochemical and molecular assays was 93.7% (60 of 64 patients; CI = 85–98%), whereas by molecular assays it was only 70.3% (45 of 64 patients). As illustrated in Table IV, immunohistochemical essays revealed a higher prevalence of p53 and of Fhit alterations in tumor compared to mutational analysis, whether considered individually or combined, and in agreement with high values previously reported.<sup>23</sup>

DISCUSSION

Several studies have provided the evidence that tumor-like DNA is present in plasma of cancer patients and can be detected with sensitive techniques.<sup>13,22,24</sup> Our results confirm and extend at other 3p loci the data previously reported<sup>14</sup> regarding the presence of microsatellite changes at FHIT locus in plasma DNA of lung cancer patients. Moreover, we report here for the first time a p53 mutational analysis in plasma samples performed with sensitive molecular assays.

The choice of the 3 genetic targets, p53, FHIT and 3p loci, was motivated by the consensus on these changes as the most frequent

and earliest alterations in lung carcinogenesis, thus fulfilling the criteria for optimal biomarkers. Two other major molecular changes, p16<sup>INK4A</sup> promoter hypermethylation and K-ras mutations, were already analyzed by our group in a different series of lung tumor and plasma samples.<sup>15</sup> While p16<sup>INK4A</sup> methylation resulted as a promising marker, being detectable in 54% of plasma samples, K-ras mutations were not identified in any of the plasma sample analyzed, precluding the use of K-ras as a molecular marker in plasma-based molecular alteration detection for NSCLC.

Combining 5 markers located on chromosome 3p (including FHIT), we observed that 62.5% of patients showed allelic imbalances in tumor samples and 35.9% in plasma samples, whereas 47.5% of the cases showing tumor alteration displayed a corresponding change in plasma DNA. The theoretical sensitivity of the microsatellite assay ranges from 1:100 to 1:200.<sup>25</sup>

p53 mutations in tumors, analyzed by PCR and direct sequencing, identified 40.6% of the patients. However, the frequency of p53 mutations in plasma samples using this technique was very low (less than 1%) due to the coexistence of tumor and normal DNA in plasma that hampers the detection of single-base mutations. The lack of p53 mutations in normal DNA indicates that the mutations identified in plasma samples by more sensitive techniques, plaque assay and, in specific cases, REA and MASA are neither germline nor polymorphisms. Moreover, the p53 mutations detected in our cases were already reported in tumors of different types, including lung (<http://www.iarc.fr/p53/>).<sup>26</sup> Plaque hybridization assay is a technique with high sensitivity (1:1,000–1:10,000) that has been successfully used to identify p53 mutations in cytologic samples.<sup>27</sup> In the latter study, 39% of lung cancer patients analyzed carried in their bronchoalveolar lavage the same mutation identified in the tumor sample, whereas only 14% of the patients showed microsatellite changes. In our study, the sensitivity of p53 mutation detection in plasma with the use of plaque assay, REA and MASA raised from < 1% to 73%.

With the use of sensitive techniques, the efficiency of p53 mutation detection in plasma seems thus higher than microsatellite alterations assay and the combination of the 3 markers (p53, FHIT, 3p) is able to detect alterations in plasma DNA of 51.6% of all patients and 60.7% of stage I patients. Moreover, genetic markers in plasma identified 64.4% of all stages and 68.2% of stage I patients whose tumors had a detectable alteration in 1 or more of these 3 markers supporting a possible use of molecular assays in plasma for detection of early stage lung cancer.

The genetic analyses used in this study showed that alterations in the plasma matched exactly those found in the primary tumors of the corresponding patients in 29 of 45 (64.4%) patients. In 4 patients, microsatellite alterations were detected in plasma only and even though these changes were confirmed in separate experiments, they may nevertheless represent artifactual imbalances due to the low amount of DNA in the PCR reaction. However, these discordant findings could also reflect intratumoral clonal heterogeneity and biased tissue sampling as previously reported.<sup>28</sup> Nevertheless, since 35.5% (16 of 45) of the patients with alterations in tumors did not have a detectable mutation in 1 of the 3 markers in their plasma DNA, there is a clear need to improve sensitivity of using mutations in plasma DNA to test for lung cancer. On the other hand, the proportion of tumors detected with the 3 genetic markers used in this study is 70% and nearly 30% of the tumor

specimens analyzed resulted negative for the presence of any type of the molecular changes. To be able to detect a higher prevalence of genetic changes in tumors, alterations in other genes/markers would have to be assessed.

A major limitation of studying p53 gene mutations by plaque assay, REA or MASA resides in the fact that these molecular assays require the previous knowledge of the mutation present in the tumor. The distribution of p53 mutations in our patients included many different types of mutations scattered all along the DNA binding domain of the p53 protein, from exon 5 to exon 8, although a prevalence of mutations affecting exon 5 ( $n = 10$ ) compared to the other exons ( $n = 16$ ) was observed. As already reported in lung cancer from smokers,<sup>5</sup> we found a predominance of G-to-T transversions that were present in 12 of 27 (44%) of our patients. A frequent p53 mutation identified as a hotspot in hepatocellular carcinoma in a population exposed to aflatoxin B is an arginine-to-serine substitution at codon 249 (Ser-249).<sup>29</sup> Ser-249 mutation is nevertheless reported in 200 of the 10,385 p53 mutation described in human cancer (<http://www.iarc.fr/p53/>),<sup>26</sup> and 25% of these Ser-249 mutations were found in lung cancer patients. Previous studies have also shown that benzo(a)pyrene-diol-epoxide induces guanine adducts at specific hotspots of the p53 gene, codons 157, 248, 249, 273, in normal human bronchial epithelial cells.<sup>30,31</sup> In the present study, we detected low frequency of mutations in codon 157 (2 cases) and a higher frequency in codons 248–249 (5 cases). Overall, only 7 (24%) of the 26 p53 mutations detected in our patients reside in the reported hotspots, suggesting that a thorough mutational screening is needed in order to use p53 as an informative marker in biologic fluids. However, the p53 mutational analysis could be automated by using a high-throughput format in order to reduce time and costs. Since plaque assay is based on the discrimination of p53 mutation on a single molecule basis through oligonucleotide hybridization, new nanotechnology tools such as DNA microarrays could be applicable for screening purposes, allowing the achievement of good sensitivity with this approach.

In conclusion, the sensitivity of molecular assays either in tumors or in plasma still remains the limiting step for a routine use of molecular markers in clinical practice. Nevertheless, the establishment of a panel of tumor-specific molecular markers detecting 100% of lung tumors undoubtedly represents the ultimate target, although for the time being it appears a difficult achievement. Specificity of molecular assays also needs testing in healthy individuals and we have previously reported that, using the same methods described in this study, microsatellite alterations in plasma DNA were not found in a control group of 43 individuals (including 8 smokers), indicating a very low occurrence of false positive results. Furthermore, recently we could also find good sensitivity and specificity of a real-time quantitative PCR assay in plasma DNA in a case-control study of cancer patients and asymptomatic chronic smokers (data not shown). It is likely that the combination of quantitative and qualitative molecular assays on plasma DNA, developed by high-throughput platforms, will improve the noninvasive approach to lung cancer detection.

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