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## ***In situ* detection of unexpected patterns of mutant p53 gene expression in non-small cell lung cancers**

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Many solid tumors, including non-small cell lung cancers (NSCLCs), are characterized by heterogeneous expression of p53 protein in the neoplastic cells. To analyse the molecular implications of this finding, we examined topographic distribution of p53 mutations using *in situ* polymerase chain reaction (PCR) in primary NSCLCs, showing distinct patterns of variable p53 overexpression by immunohistochemistry. Unique sets of primers for each mutation were designed, and optimal PCR conditions were determined by standard PCR using DNA from cloned mutants or cell lines established from these tumors. All tumor cell nuclei, regardless of the status of p53 overexpression, demonstrated homogeneous distribution of mutant p53 with specific primers, indicating that only subgroups of the mutated cells overexpressed p53 protein. *In situ* reverse transcription (RT)–PCR was applied to detect mutant mRNA in the individual tumor cells using specific primers. We found that in each case the distribution of mutant p53 mRNA coincided with that of immunohistochemical overexpression of p53 protein. Our results suggest that the regulation of mutant p53 expression, but not the genotype, is heterogeneous in the neoplastic cells. The topographic genomapping of p53 in NSCLC using *in situ* PCR provides a novel approach to view molecular mechanisms of lung carcinogenesis. *Oncogene* (2001) 20, 2579–2586.

**Keywords:** p53; lung cancer; immunohistochemistry; *in situ* PCR; *in situ* RT–PCR

### **Introduction**

Alterations in the tumor suppressor gene p53 are frequent events in non-small cell lung cancers (NSCLC), and may have prognostic importance (Takahashi *et al.*, 1989; Mitsudomi *et al.*, 1992; Horio *et al.*, 1993). The main mechanism of action involves a

point mutation in one allele and complete loss of the second allele (Greenblatt *et al.*, 1994).

Mutant p53 has a longer half-life than wild p53 (Oren *et al.*, 1981; Reihnsaus *et al.*, 1990) and we have previously established a correlation with the overexpression of p53 protein by immunohistochemistry and the presence of mutations in lung cancer cell lines (Bodner *et al.*, 1992). In lung tumors, we noted marked variability in the immunohistochemical staining patterns of p53 protein with two major patterns (Ebina *et al.*, 1994). One revealed focal positivity throughout the tumor at random locations giving a spotty appearance, and another consisted of positive staining along the periphery of the tumor. Interestingly, there was no correlation between the expression of p53 and proliferating cell nuclear antigen (PCNA) in the tumors, suggesting that the variability was perhaps not due to cell cycle dependent regulation (Prives, 1998).

There are two likely explanations for the heterogeneous distribution patterns of p53 immunoreactivity in tumors: (i) polyclonal expansion of the tumor cells with different p53 status, or (ii) differences in the regulation of mutant p53 expression through transcriptional activity or stability in tumor cells while they may all carry the same mutation. To begin to elucidate the underlying mechanisms it was necessary to assess the mRNA levels of mutant and wild-type p53 in individual cells. Thus we developed a novel method to detect specific p53 mutations in tissue sections by performing *in situ* polymerase chain reaction (PCR) on a glass slide, selectively amplifying the DNA fragments of interest (Hasse *et al.*, 1990; Martínez *et al.*, 1995). The selection of primers was based on the two base-pair mismatch method (Cha *et al.*, 1992). We also applied *in situ* reverse transcription (RT)–PCR to detect mutant mRNAs of p53. The reaction of *in situ* RT–PCR using mutant specific primers would be able to reveal both mutant cDNA fragments amplified within the cytoplasm and mutant DNA fragments amplified within the nucleus in a single tumor cell at the same time.

These techniques allowed us to visualize the same p53 mutation in all tumor cells, while the levels of mutant mRNA were variable. Moreover, there was a correlation between the expression of mutant mRNA and immunohistochemical staining for p53. Our findings support the concept that heterogeneous distribution patterns of p53 immunoreactivity in NSCLCs resulted

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from regulation of mutant p53 expression rather than genotypic differences in tumor cells.

**Results and Discussion**

*Diffuse pattern of p53 overexpression and a point mutation*

The specimen (Case 1) was a right lower lobe lung mass from a 55-year-old 30 pack year smoker. Histologically it was a poorly differentiated NSCLC which by immunohistochemistry revealed low level of diffusely distributed nuclear staining indicating p53 overexpression in most of the tumor cells. No Mdm2 expression was detected. A point mutation at codon 47 of p53 (CCG to TCG) was identified by DNA sequencing of the microdissected material (Figure 1a). This coincided with the mutation of the cell line NCI-H1373 which was established from the pericardial fluid of the same patient 18 months after the resection of the primary lung cancer. We selected an optimal PCR condition for each tumor with its individually designed primers including mutant specific (Muts, Figure 2a), wild-type specific (WTs), common (Com) primers, and omission of primers (0).

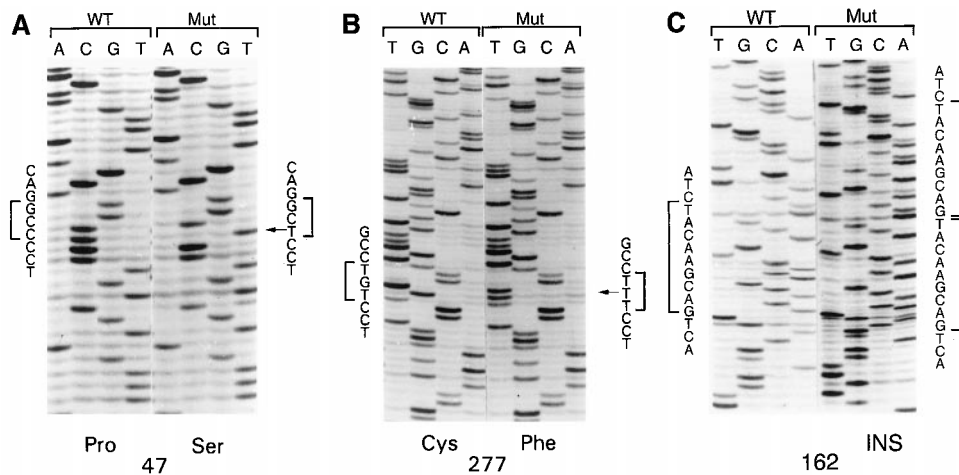
*In situ* PCR of the NCI-H1373 cell pellet, using Muts primers, demonstrated specific nuclear staining in over 90% cells (Figure 3a), whereas less than 1% faintly positive cells were seen in NCI-H460, which has a wild-type p53 gene, by these primers even after 35 cycles of PCR. As expected, when Wts primers were used, NCI-H1373 cells were negative (Figure 3b), while NCI-H460 were intensely positive. In the Case 1 tumor specimen, all tumor cells were amplified by *in situ* PCR with the Muts primers (Figure 3d). The amplicons obtained with Muts primers were confirmed by Southern blotting (88 base-pairs, Figure 3c). No tumor cells were positive with WTs primers although non-malignant cells, such as alveolar cells or macrophages in the normal appearing

lung tissue surrounding the tumor were positive with this set of primers (Figure 3e). As a positive control, *in situ* PCR using common (Com) primers revealed that all nuclei of both tumor and nonneoplastic cells were reactive (Figure 3f). When primers were omitted, as a negative control, most of the nuclei on the section remained negative, with the exception of rare cells conceivably undergoing apoptosis or DNA repair which were positive (Figure 3g). Scattered cells among unremarkable bronchiolar epithelial cells which showed p53 overexpression by immunohistochemistry (Figure 3h), were also positive with Muts primers (Figure 3i). These cells were too few for us to resolve if they represented tumor cells from the near-by focus of tumor or potentially premalignant airway epithelial cells.

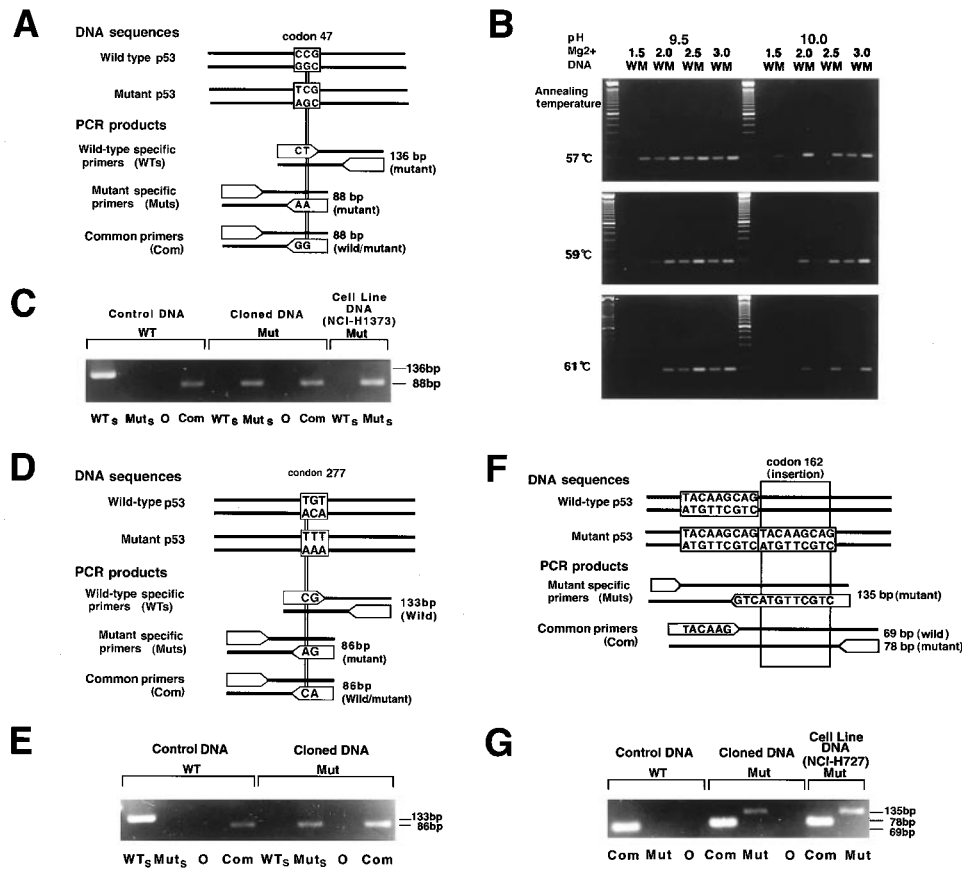
The reactions by the four sets of individually designed primers for each mutation do not only yield specificity, but potentially address the question whether a single p53-mutant cell reveals a loss of heterozygosity (LOH). The Case 1 tumor specimen indicated the presence of only mutant p53, because no amplification was observed with wild-type specific primers in tumors. The ability to document LOH at the cellular level is another potentially important application.

*Peripheral distribution of the p53 overexpressing tumor cells and a point mutation*

Case 2 tumor specimen was obtained from a right lower lobe tumor mass of a 66-year-old patient with severe chronic pulmonary emphysema. Histologically, it was a moderately differentiated papillary adenocarcinoma which demonstrated focal p53 overexpression by immunohistochemistry, composed of strongly stained nuclei distributed over the peripheral areas of the tumor, while most of the tumor cells were positive for proliferating cell nuclear antigen (PCNA) (Figure 4a). No Mdm2 expression was found in tumor cells. DNA sequencing revealed a point mutation of p53 at codon 277, TGT to TTT (Figure 1b). While the tumor cells



**Figure 1** p53 mutations in the three non-small cell lung cancer tumors examined in this study. (a) Case 1, a squamous cell carcinoma, had one point mutation at codon 47 (proline to serine). (b) Case 2, an adenocarcinoma, had a point mutation at codon 277 (cystein to phenylalanine). (c) Case 3, a carcinoid, had a nine base-pair insertion in exon 5. WT; wild-type p53. Mut; mutant p53



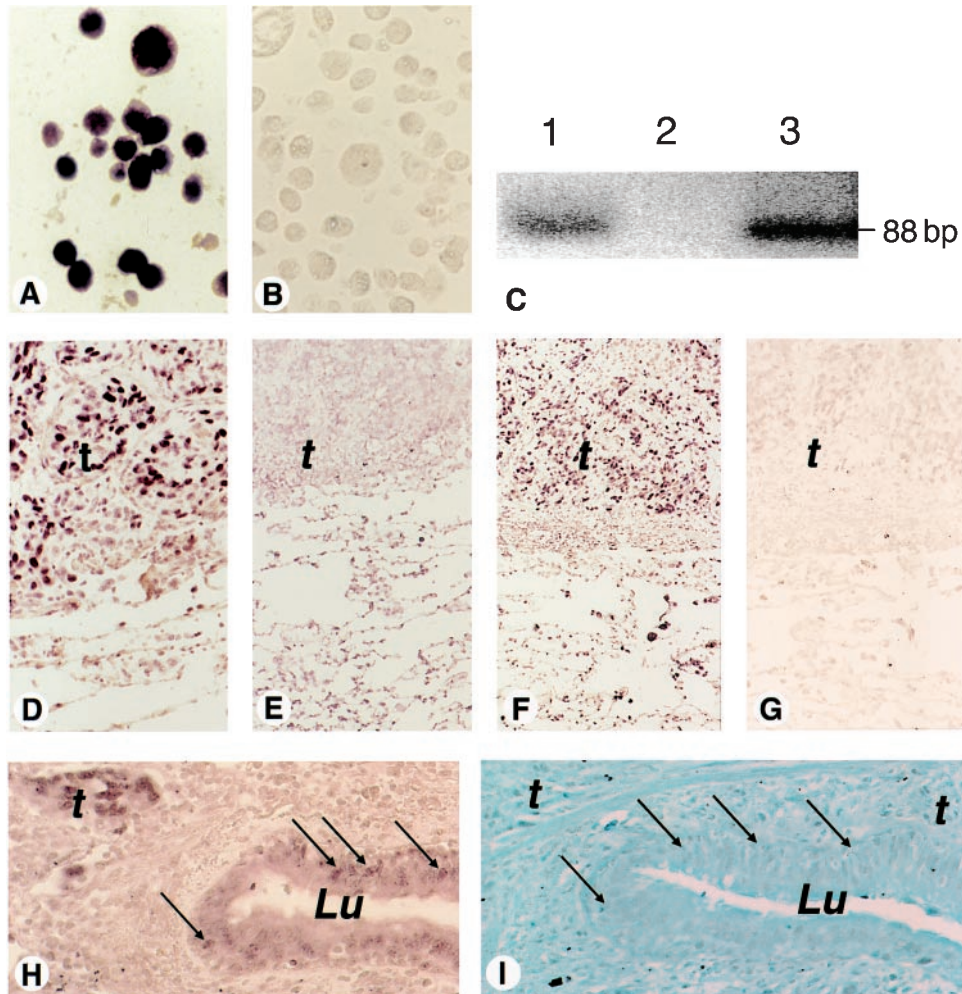
**Figure 2** Schema of the specific primers for each mutation-site and standard PCR reactions by the primer sets. The primers for wild-type specific (WTs), mutant specific (Muts), and common DNA fragments (Com) of each tumor, illustrated in schematic drawings, and the standard PCR reactions by these primer sets are shown in **a–c** (Case 1), **d** and **e** (Case 2), **f** and **g** (Case 3). Control DNA is human genomic DNA with wild-type p53. Mutant DNAs extracted from the tumor tissues were cloned and amplified using these primer sets. The cell lines, NCI-H1373 and NCI-H727, both of which were established from the tumor tissues of Cases 1 and 3, respectively, were also used as mutation controls. **(b)** The test reactions for selecting optimal PCR conditions (2.5 mM Mg<sup>2+</sup>, pH 10.0, with an annealing temperature of 61°C) for Muts primers in Case 1 are shown. W; wild-type p53 DNA and M; mutant p53 DNA. The PCR reactions without primers were always compared as negative control (0 in **c**, **e**, and **g**)

overexpressing p53 protein by immunohistochemistry in Case 2 were seen only in the periphery of the tumor, by *in situ* PCR all tumor cell nuclei were intensely stained after PCR amplification with Muts primers (Figures 2d and 4b), suggesting that all tumor cells carried the mutation. With WTs primers (Figure 2d), on the contrary, strong reaction was seen only in the nuclei of the normal cells surrounding the tumor (Figure 4c).

Moreover, using *in situ* RT-PCR, cDNA fragments which were reverse transcribed from the mutant p53 mRNA were amplified specifically with the Muts cDNA primers (sense in exon 7; 5'-GGCCATCCT-CACCATCA-3' and antisense in exon 8; 5'-TGCGCCGGTCTCTCCCAGGGAAC-3') resulting in cytoplasmic staining indicating the presence of this specific p53 point mutation at the mRNA level (Figure 4d). The signal was detected exclusively in the periphery of the tumor coinciding with the distribution of p53 protein-positive nuclei by immunohistochemistry. Interestingly, our *in situ* RT-PCR without DNase treatment clearly revealed both mutant DNA in the nuclei and mutant cDNA in the cytoplasm of the

appropriate cells (Figure 4d). The tumor cells without overexpression of p53 revealed mutant p53 without detectable mutant p53 mRNA by *in situ* RT-PCR using Muts primers (Figure 4e).

Our current data support calling tumors positive for p53 when there is only focal overexpression of p53 staining, and, moreover, these results clearly demonstrate how immunohistochemical studies of small biopsies may yield misleading conclusions, as staining for p53 may be negative while molecular analysis including *in situ* PCR would reveal the possible genetic change in the negative cells. The heterogenous staining pattern of these tumors would have been predicted to reflect a heterogenous pattern of gene mutation. This is clearly not the case and strongly suggests an interaction potentially with a variety of transcription factors which may control p53 expression (Kirch *et al.*, 1999; Schreiber *et al.*, 1999). The homogenous distribution of mutations of the p53 gene in NSCLCs does suggest that the alteration occurred before the clonal expansion of these tumors, which we have recently reported in adenocarcinoma of the lung (Kanazawa *et al.*, 2000).

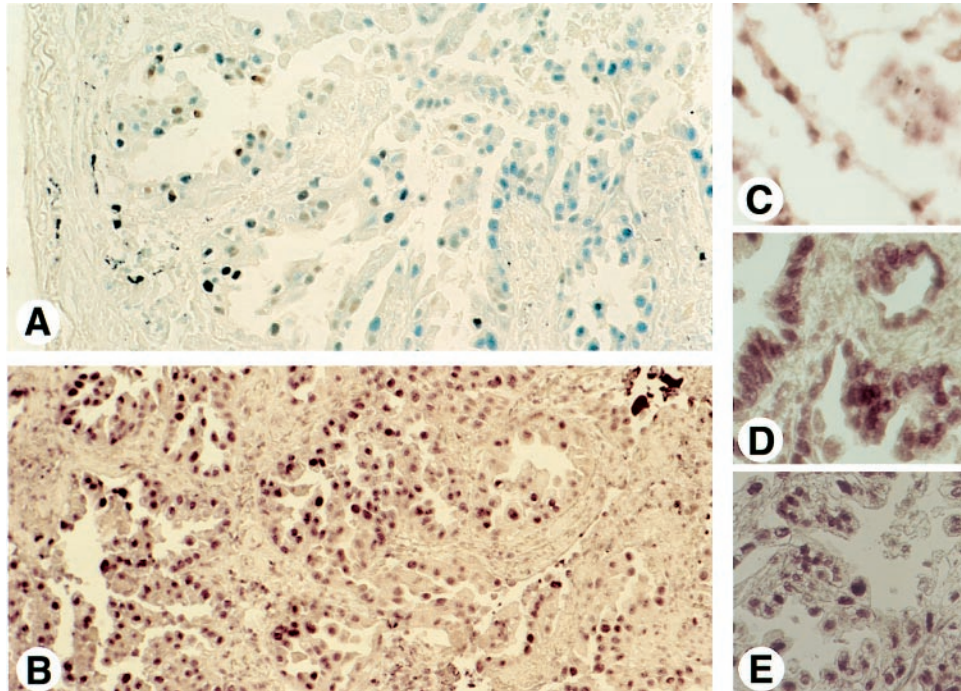


**Figure 3** Photomicrographs of the squamous cell carcinoma (Case 1) examined by *in situ* PCR. In the corresponding cell-line NCI-H1373, specific reactions are (a) positive with Muts primers, and (b) negative with Wts primers (originally  $\times 800$ ). Using sections derived from the tumor (d–g) (originally  $\times 80$ ), Muts primers amplified only mutant p53 found in the tumor cells (d), which was confirmed by Southern blot using mutant DNA fragments as a probe (c, lane 1: DNA fragments from tumor sections by *in situ* PCR with Muts primers; lane 2: wild-type fragments by standard PCR as negative control; and lane 3: mutant DNA 88 bp fragments by standard PCR as positive control). Wts primers amplified only wild-type p53 in the non-malignant cells (e, bottom). Common primers reacted with both wild-type and mutant p53 (f), while no amplification was seen without primers (g). Several nuclei in the lining of bronchiolar epithelium were found to have p53 mutation by *in situ* PCR (h, arrows,  $\times 200$ ). Same cells also revealed p53 overexpression by immunohistochemistry (i, arrows; immunoperoxidase stain,  $\times 200$ ). t; tumor

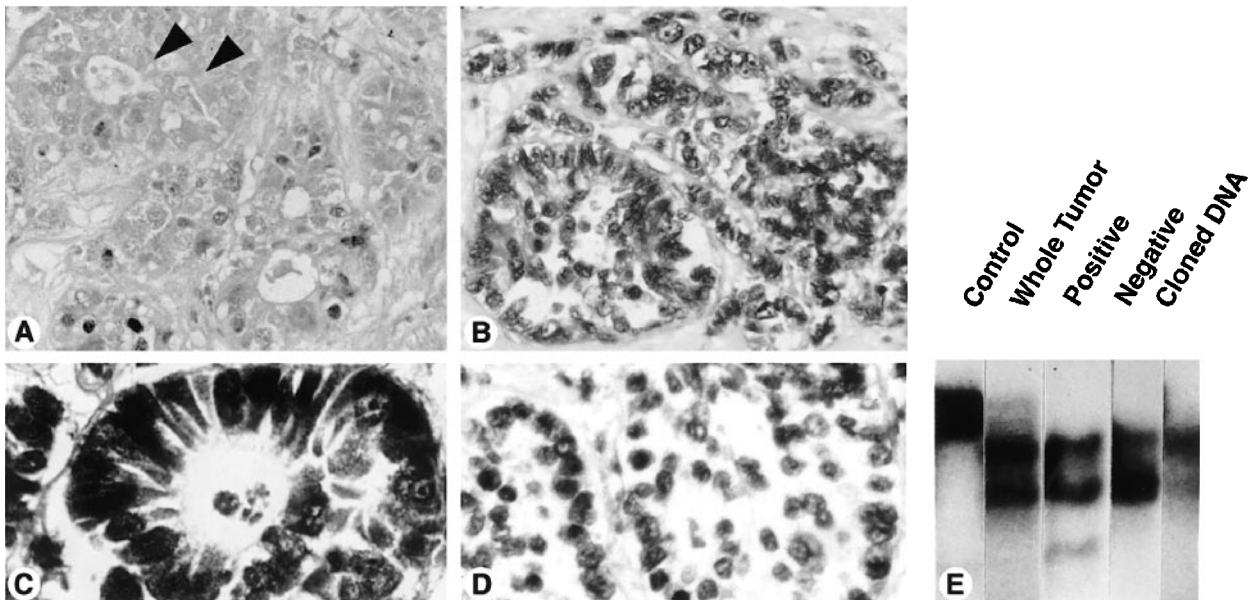
#### Scattered foci of p53 overexpression and a nine base-pair duplication

Case 3 specimen was obtained from a 65-year-old, 60 pack-year smoker who underwent a surgical resection for a right upper lobe lung mass which was found to extend to the pleura. Histologically the tumor was an atypical carcinoid which revealed scattered clusters of cells with p53 positive nuclei by immunohistochemistry (Figure 5a). The tumor was negative by Mdm2 staining. Our molecular analysis of the primary tumor identified a nine base-pair duplication in exon 5 of the p53 gene (Figure 1c). This abnormality was also present in the cell line NCI-H727 derived from this primary (Mitsudomi *et al.*, 1992). In this case, because designing Wts primers was impossible, only two sets of primers were synthesized.

The Case 3 tumor, which demonstrated p53 protein overexpression in a limited number of cells, was found to have the same mutation (nine base-pair duplication) throughout all the tumor cells, as indicated by amplification with Muts primers (Figure 2f) during *in situ* PCR (Figure 5b). This observation was confirmed by PCR–SSCP, which revealed similar shifted bands in both the p53 positive and the negative immunoreactive areas which were microdissected for this analysis (Figure 5e). In non-malignant lung tissue distant from the primary tumor, foci of Type II cell hyperplasia revealed no p53 overexpression by immunohistochemistry, no reaction with mutant specific (Muts) primers and no shifted band by PCR–SSCP, further confirming the reliability of the mutant specific reaction (data not shown). By *in situ* RT–PCR, a variable pattern was observed consisting of dark



**Figure 4** Photomicrographs of the adenocarcinoma (Case 2). (a) Double immunostaining for p53 and PCNA showed that most tumor cells are positive for PCNA (stained in blue), while p53 overexpression (in dark and light brown) is seen only in the periphery of the tumor (left side; immunoperoxidase stain,  $\times 200$ ). (b) By *in situ* PCR with MutS primers, all tumor-cell nuclei were positive ( $\times 200$ ). (c) With Wts primers reaction was limited to normal cells while large tumor cells were negative ( $\times 400$ ). (d) The cytoplasm of the p53 immunoreactive tumor cells was intensely positive by *in situ* RT-PCR for amplifying mutant cDNA fragments ( $\times 400$ ). Note that tumor cell nuclei were also staining because this reaction was done without digestion by DNase. (e) In contrast, tumor cells which were negative by immunostaining revealed a minimal reactivity by *in situ* RT-PCR ( $\times 400$ )



**Figure 5** Photomicrographs of the carcinoid (Case 3). (a) Focal reactivity for p53 was observed by immunohistochemistry. Note strong nuclear staining in the center, while other groups of tumor cells remained negative (arrow heads; immunoperoxidase stain,  $\times 400$ ). (b) All tumor cells reveal intense positivity following amplification using mutant specific primers while the surrounding non-malignant stromal cells are negative ( $\times 400$ ). (c) By *in situ* RT-PCR, without DNA digestion, the tumor cells in the positive foci by immunohistochemistry were significantly darker than (d) the tumor cells in the negative foci ( $\times 400$ ). (e) By PCR-SSCP analysis, DNA from the whole tumor (second lane from left), demonstrated a shift indicating the presence of a mutation, which is identical to the shifts in the DNA extracted from immunohistochemically positive (third lane from the left) as well as negative area (fourth lane from the left) and confirmed by the shifted band of cloned (mutant) DNA

cytoplasmic reactivity corresponding to the areas of p53 protein overexpression areas (Figure 5c,d).

Recent studies from multiple laboratories have demonstrated posttranslational mechanisms by which the wild-type p53 protein half life is extended including through Mdm2 interactions and possible phosphorylation (Prives, 1998). This leads to a disproportionate amount of p53 protein when compared to mRNA. Thus, it could be anticipated that one should find tumors with p53 immunoreactivity, low amounts of mRNA and a wild-type gene. Indeed, the pattern of p53 immunoreactivity could be heterogenous as the abnormalities such as hypoxia, or genotoxic stress may be present only in certain areas of the tumor. However, we examined tumors which displayed mutant p53 which by the virtue of its mutation has an extended protein half life. Our approach using RT-PCR may also help resolve discrepancies between the immunohistochemical studies and molecular analyses of p53 in human lung cancers using whole tumor DNA or mRNA (Brambilla *et al.*, 1993).

In summary, we describe an assay to examine the consequences of p53 mutations for gene expression in lung carcinogenesis at the cellular level. The somewhat unexpected finding of a correlation of the mutant p53 mRNA and protein expression in tumor cells underscores the importance of novel complementary techniques that may extend beyond microdissection (Park *et al.*, 1999). Potential applications of *in situ* methods include genetic analyses in many clinical settings employing existing, archival material (Nuovo *et al.*, 1999).

## Materials and methods

### *Tumors and cell lines*

Surgically resected specimens of the primary NSCLC tumors (Cases 1–3) and corresponding lung cancer cell lines NCI-H1373 and NCI-H727 were part of a prospective clinical trial of the patients with any stage or histologic subtype of pathologically confirmed NSCLC with an Eastern Cooperative Oncology Group performance status of three or better (at least partially ambulatory) who were entered into the study at the NCI-Navy Medical Oncology Branch as previously described (Shaw *et al.*, 1996). In addition, we used a well characterized NCI-H460 lung cancer cell line (NCI-Navy Medical Oncology Branch, Bethesda, MD, USA) as a control for wild-type p53 (Takahashi *et al.*, 1989). All material used in the current study was derived from investigations which were performed after approval by local institutional review boards.

Tumor specimens were fixed with 10% formalin and embedded in paraffin according to routine procedures. To simulate the conditions of tumor specimens, pellets of freshly grown cell lines were fixed for 2 h in 4% paraformaldehyde, washed in PBS, resuspended in 1 ml of 2% NuSieve low melting-point agarose (NuSieve<sup>®</sup>, FMC BioProducts, Rockland, ME, USA), allowed to solidify and embedded in paraffin (Takahashi *et al.*, 1989). Sections from the paraffin-embedded cell pellets were used to test the specificity of *in situ* PCR reactions.

### *Immunohistochemistry*

Following antigen retrieval, p53 immunostaining was performed using the mouse monoclonal antibody DO-7 against human p53 (Dako Corporation, Carpinteria, CA, USA) at 4.8 µg/ml, and avidin-biotinylated peroxidase method with Vectastain kit (Vector Laboratories, Burlingame, CA, USA) as previously described (Ebina *et al.*, 1994). Double staining for p53 and PCNA was also performed using an antibody against p53 in 9.5 µg/ml and PCNA, PC10 (monoclonal mouse antibody; Dako Corporation) in the concentration of 19.3 µg/ml, according to our previous study (Ebina *et al.*, 1994). For Mdm2 detection we used a mouse monoclonal antibody against the human protein from Oncogene Science (Cambridge, MA, USA) at dilution 10 µg/ml. Optimal concentrations for each antibody were determined by pilot studies using known positive control sections (Hall *et al.*, 1997).

### *Microdissection and DNA extraction*

From serially cut, dewaxed and dried sections, tumor foci corresponding to p53 positive or negative areas by immunohistochemistry, were microdissected using micropipet tips and collected into separate tubes. Specimens were digested with 0.5 ml of protein lysis buffer (50 mM Tris, 1 mM EDTA, 0.5% Tween 20, pH 8.5) containing 0.4 µg/ml proteinase K, for 3 h at 55°C. The reaction was stopped by heating at 94°C for 7 min. In addition, genomic DNA was extracted from freshly grown cell lines to be used in the standard PCR.

### *PCR/SSCP analysis and DNA sequencing*

A Perkin-Elmer 9600 Thermocycler (Perkin-Elmer Cetus, Norwalk, CT, USA) was used to amplify the DNA samples. The DNA extracted from the various tumor areas was used for PCR/single-strand conformation polymorphism (SSCP) analysis spanning p53 exons 4 through 9 (Mitsudomi *et al.*, 1992; Hall *et al.*, 1997). The exons showing shifted bands were cloned into a pCRII vector (Invitrogen, San Diego, CA, USA), and purified through Qiagen Mini Columns (Qiagen Inc., Chatsworth, CA, USA). DNA sequencing was performed using a dsDNA cycle sequencing system (Gibco-BRL, Gaithersburg, MD, USA) and primers end-labeled with  $\gamma$ -<sup>32</sup>P-ATP (Amersham Corp., Arlington Heights, IL, USA) (Figure 1).

### *Design of the primers and testing them in standard PCR*

Following the double mismatch approach (Cha *et al.*, 1992), we designed three to six pairs of mutant specific primers (Muts) for the cases with single base-pair mutations in p53, resulting in one base-pair mismatch with mutant p53 and two base-pair mismatches with wild-type. Likewise, specific primers for wild-type p53 (Wts) were synthesized which had one base-pair mismatch with wild-type and two base-pair mismatches with the mutant p53. Common primers (Com) had no mismatch with wild-type, but one base-pair mismatch with mutant p53, and, consequently, amplified both wild and mutant p53.

In the first case (Figure 1a) the sense primer of Muts was set within intron 3 (11999–12020; 5'-TGCTCTTTTCACC-CATCTACAG-3') while the antisense primer (5'-GAAC-CATTGTTCAATATCGTCCAA-3') was set to detect the mutation (TCG) in codon 47. They had one base-pair mismatch with mutant p53 and two base-pair mismatch with

wild-type p53. The wild-type specific (WTs) primers set within exon 4 (sense; 5'-GATGATTTGATGCTGCTCCT-3', antisense; 5'-CAGGGGCCGCGGTGTAGGAG-3'), which had one base-pair mismatch with wild-type p53 and two base-pair mismatch with mutant p53 were designed for detecting exclusively the wild-type (CCG) sequence (Figure 2a). Common (Com) primers set in intron 3 (sense; 5'-TGCTCTTTTACCCATCTACAG-3') and in exon 4 (antisense; 5'-GAACCATTGTTCAATATCGTCCGG-3'), were set as a positive control, with no mismatch with wild p53 and one base-pair mismatch with the mutant p53 (Figure 2a). The optimal PCR condition for each primer set was tested as shown in Figure 2b. The optimal PCR-mixture composition selected for these three different pairs of primers (WTs, Muts, and Com) was 2.5 mM Mg<sup>2+</sup>, pH 10.0, with an annealing temperature of 61°C. The sizes of the products were 136 base-pairs by Wts, and 88 base-pairs by Muts primers (Figure 2c).

In the second case (Figure 1b) the wild-type specific primers (Wts, sense in exon 8; 5'-GAGGTGCGTGTGGTGCCCG-3' and antisense in intron 9; 5'-CAGGGGCCGCGGTGTAGGAG-3') amplified their specific target sequences yielding a 133 base-pair product (Figure 2d). The sense primer of the mutant specific (Muts), and common (Com) primers was set in intron 7 (14424–14447, 5'-CTTGCTTCTCTTTTCTATCCTGA-3'), and antisense primers were set in exon 8 with the different sequence at the end of three prime (Muts; 5'-TGCGCCGGTCTCTCCCAGGGAAC-3' and 5'-TGCGCCGGTCTCTCCCAGGGACA-3') (Figure 2d). The optimal reaction required a PCR mixture containing 1.5 mM Mg<sup>2+</sup> at pH 10.0, and an annealing temperature of 60°C (Figure 2e).

As the third case was found to have a nine base-pair duplication in exon 5 (Figure 1c), only two sets of primers were designed: (i) mutant specific primers (Muts), for amplifying mutant p53 only and (ii) common primers (Com) which amplified both wild and mutant p53. As shown in Figure 2f, both sense and antisense primers of Muts were set within exon 5 (5'-TACTCCCCTGCCCTCAAC-3' and 5'-CTGTGACTGCTTGTACTG-3', respectively), which were also used to amplify mutant cDNA during *in situ* RT-PCR. The common primers (Com) were also set within exon 5 (sense; 5'-CGCGCCATGGCCATCTACAAG-3', antisense; 5'-CTCATGGTGGGGGCAGCGCT-3'), and the PCR products were 69 bp in wild-type p53 and 78 bp in this mutant p53 sequence with nine base-pair duplication. Standard PCR with these primers demonstrated their specificity by yielding 69 and 78 bp products for the Com and Muts primers, respectively (Figure 2g). Optimal PCR conditions were 1.5 mM Mg<sup>2+</sup>, pH 10.0 and an annealing temperature of 60°C.

All primers were synthesized in-house using a MilliGen 8700 DNA synthesizer (Millipore, Marlborough, MA, USA). As indicated, the specificity of the designed primers was tested by standard PCR, using human genomic DNA, DNA extracted from cell lines, or cloned DNA fragments. The MgCl<sub>2</sub> concentration and pH of the PCR mixture was selected using PCR Optimizer Kit (Invitrogen, San Diego, CA, USA). The appropriate annealing temperature was selected from a range between 51 and 63°C and settled for

each set of primers to avoid the possible effect by different annealing temperature (Figure 2b). The other conditions were kept identical for all reactions in the three tumors: denaturing at 94°C for 15 s, annealing time for 30 s and extension at 72°C for 30 s for 30 cycles, with an initial denaturing step at 94°C for 1 m. In order to perform 'hot start' in our PCR reaction (Nuovo, 1994), we applied the neutralizing antibody technique (Martínez *et al.*, 1995) using TaqStart antibody (Clontech Laboratories, Palo Alto, CA, USA).

#### In situ PCR

Direct *in situ* PCR was performed as previously described with minor modifications (Martínez *et al.*, 1995). Briefly, dried dewaxed sections on DNase/RNase free glass slides were incubated with protein lysis buffer (0.1 M Tris, 50 mM EDTA, pH 8) containing 20 µg/ml Proteinase K for 20 min at 37°C. The reaction was stopped with PBS containing 0.1 M glycine for 5 min. After thoroughly washing with PBS, 75 µl of the PCR optimal solution of, containing digoxigenin-11-[2'-deoxy-uridine-5']-triphosphate (DIG-11-dUTP; Boehringer Mannheim, Indianapolis, IN, USA) was added and the PCR reaction was carried out for 20–35 cycles in an Omni Slide System thermocycler (Hybaid, Hollobrook, NJ, USA). After washing twice in 0.1×SSC for 20 min each at 45°C, the incorporated PCR fragments were visualized by the digoxigenin detection kit (Boehringer Mannheim).

#### In situ RT-PCR

After Proteinase K (10 µg/ml) digestion at 37°C for 15 min, reverse transcription was performed using the SuperScript Preamplification System (Gibco-BRL) following the manufacturer's specifications. In brief, 70 µl DEPC-treated water containing 2.5 µg oligo(dT)<sub>12–18</sub> was added to each section, covered with parafilm, and incubated for 10 min at 70°C. After 1 min on ice, another solution (30 µl) including reverse transcriptase (100 U/section) was added to the sections, covered with parafilm, and the slides were incubated at 42°C for 50 min, followed by 15 min at 70°C. After thorough washes, direct *in situ* PCR was performed after this reverse transcription reaction using mutation specific primers as described before.

Controls included substituting the primers by H<sub>2</sub>O in the PCR reaction and omission of the reverse transcription in the case of mRNA.

#### Southern blotting analysis

After *in situ* PCR using mutant specific primers without DIG-11-dUTP, whole sections were scraped from the slide into tubes, and amplified DNA fragments were extracted by ethanol precipitation and loaded onto a 2% agarose gel. The separated fragments were transferred to nitrocellulose membranes, Nytran Plus (Schleicher & Schnell, Keene, NH, USA), hybridized with the probes, corresponding to the mutant DNAs labeled with α-<sup>32</sup>P-dCTP using Prime It (Stratagene, La Jolla, CA, USA).

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