[CANCER RESEARCH 51, 6194-6198, November 15, 1991]

Advances in Brief

Mutations in the *p53* Gene in Primary Human Breast Cancers[1]

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Abstract

Twenty-six primary breast tumors were examined for mutations in the *p53* tumor suppressor gene by an RNase protection assay and nucleotide sequence analysis of PCR-amplified *p53* complementary DNAs. Each method detected *p53* mutations in the same three tumors (12%). One tumor contained two mutations in the same allele. Single strand conformation polymorphism analysis of genomic DNA and complementary DNA proved more sensitive in the detection of mutations. Combining this technique with the other two a total of 12 mutations in the *p53* gene were demonstrated in 11 tumors (46%), and a polymorphism at codon 213 was detected in another tumor. Loss of heterozygosity on chromosome 17p was detected by Southern blot analysis in 30% of the tumor DNAs. Not all of the tumors containing a point mutation in *p53* also had loss of heterozygosity of the remaining allele, suggesting that loss of heterozygosity may represent a later event.

Introduction

There is now substantial evidence that genetic abnormalities affecting the *p53* gene are frequently associated with the pathogenesis of several neoplasias, particularly solid tumors such as breast, colon, and lung carcinomas (1). The mutations appear to cluster in highly conserved regions of the gene (2) and in some cases appear to inactivate the growth-regulatory functions of the protein (3, 4). This has been taken as evidence that *p53* is a tumor suppressor gene which when mutated, contributes to the malignant progression of the tumor. The frequency of *p53* mutations in primary breast carcinomas has been directly determined in only a limited number of cases (5–9). However, using immunohistochemical detection of abnormal *p53* protein in primary breast tumors (7, 10, 11) it has been inferred that about 50% of the tumors contain a *p53* mutation. Similarly, examination of primary breast tumor DNAs has revealed LOH[4] on the short arm of chromosome 17, the location of the *p53* gene, in approximately 50% of cases (6, 12–15). In the present study we have undertaken a comprehensive molecular analysis to directly determine the frequency of genetic abnormalities affecting the *p53* locus in 26 primary breast tumors as well as to determine how this is related to LOH on chromosome 17p.

Received 9/3/91; accepted 9/27/91.

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[4] The abbreviations used are : LOH, loss of heterozygosity; RFLP, restriction fragment length polymorphism; cDNA, complementary DNA; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism; ORF, open reading frame.

Materials and Methods

Sample Acquisition and Preparation. Infiltrating ductal carcinomas were obtained from 27 patients at the S. Giovanni Vecchio Hospital (Turin, Italy) who had not undergone treatment prior to surgery. Macroscopically normal mammary tissue was manually removed, and the tumor tissue was quickly frozen and embedded in OCT compound (Miles Scientific, Kankakee, IL) for intraoperative diagnosis. Areas of the specimen with a predominant neoplastic component were collected and stored at −70°C for further analysis. Lymphocytes from the same patients were isolated from 20 ml heparinized blood by density gradient centrifugation on LSM Ficoll medium (Organon Teknica Co., Durham, NC) according to the manufacturer's instructions.

DNA and RNA Preparation. High-molecular-weight DNA was prepared from frozen tissues and lymphocytes as described (15). After ethanol precipitation, the DNA samples were dissolved in 10 mM Tris/1 mM EDTA (pH 7.4) and stored at −20°C. Total RNA was extracted as described (16), redissolved in 10 mM Tris buffer, pH 7.5, and stored precipitated at −70°C.

DNA Probes and Analysis of Genomic DNA and RNA. The following probes were used: pYNZ22.1 (chromosome 17p13.3, D17S5, ATCC 57575; Ref. 17) was obtained from the American Type Culture Collection (Rockville, MD) and identifies a variable number tandem repeat RFLP in *Bam*HI- and *Pst*I-digested human genomic DNA; and pBHP53 (18), a genomic *p53* fragment, identifies a *Bam*HI RFLP within the *p53* gene. The *p53* locus was analyzed by two independent methods: (*a*) Southern blot analysis using the pBHP53 DNA probe and (*b*) PCR-based amplification of a 250-base pair genomic fragment containing the *Tha*I RFLP at codon 72, as described (19). The DNA probes were labeled with [^{32}P]dCTP (Amersham, Arlington Heights, IL) using random primers (Stratagene, La Jolla, CA). Genomic DNAs (10 μg each) were digested to completion with the restriction enzyme of choice (Gibco-BRL, Gaithersburg, MD), subjected to electrophoresis in 1% agarose gels, and transferred to Genatran[45] Nylon membranes (Plasco, Woburn, MA). Hybridization and autoradiography were carried out as previously described (15). Membranes were stripped at 65°C and rehybridized with control probes (pDF1.8-*Pst*I, chr. 1q; *Erb*-A2, chr. 3p) to confirm equivalent DNA loading. Northern blot analysis and RNase protection assays were performed on total RNA samples as previously described (20), using three overlapping probes which covered the entire coding region of *p53*.

SSCP Analysis of Genomic and cDNA. The PCR/SSCP method (21) was modified to screen for point mutations in the *p53* gene (22). Genomic DNA/PCR fragments (438 base pairs spanning exons 5 and 6, or 670 base pairs containing exons 7 and 8) and cDNA/PCR fragments spanning exons 4 to 7 (codons 116 to 242) or exons 7 to 9 (codons 242 to 327) were amplified using 100 ng of genomic DNA or 250 ng of random-primed cDNA as templates and 0.5 μl of [^{32}P]dCTP (Amersham) in 10-μl reaction volumes. To localize the possible point mutations to a specific exon, 1 μl of the PCR product was digested with *Aat*I (USB Corp., Cleveland, OH) for the exon 5/6 fragment or *Dra*I (Bethesda Research Laboratory, Gaithersburg, MD) for the exon 7/8 fragment, and *Alw*NI (New England Biolabs, Beverly, MA) for the cDNA/PCR product. The reaction was diluted 1:5 with loading buffer (95% formamide, 2 mM EDTA, pH 8.3). Two μl of each diluted sample were denatured (90°C for 5 min) and loaded onto a 6% nondenaturing acrylamide gel in 89 mM Tris-borate, 2 mM EDTA, pH 8.3, and electrophoresed for 5 h at 4°C at 25 W. The gels were subsequently dried and autoradiographed.

cDNA/PCR/Cloning/Sequencing. First-strand cDNAs were synthesized from 20 μg of total cellular RNA using random primers (Pharmacia, Piscataway, NJ). Subsequent PCR amplification of the products was performed using *p53*-specific oligonucleotide primers located just outside the ORF, as described (20), except that the primers contained a *Hind*III or *Xba*I site at their 5′ and 3′ ends, respectively. The primers used were: sense, 5′-AGTCAAGCTTGACGGTGACACGCTTCCCTGGATT-3′, and antisense, 5′-AGTCTCTAGATCAGTGGGGAAGAAGAAGTGGAGA-3′. The PCR products were cloned into the *Hind*III-*Xba*I sites of the plasmid pRC/CMV (Invitrogen, San Diego, CA). Plamid DNAs were prepared from pooled clones using the Miniprep Kit Plus (Pharmacia) and sequenced using *p53* ORF-specific primers and the Sequenase II kit (USB) with [^{35}S]dATP (Amersham).

Results

Detection of Mutations in *p53* Genomic DNA, RNA, and cDNA. RNase protection assays were performed on total RNA samples from tumors using probes spanning the entire *p53* ORF. An abnormal pattern of protected RNA species was observed in tumors 32, 39, and 87 (Table 1). These patterns suggested that the mutations map within the *p53* ORF (20). To confirm and extend these results the nucleotide sequence of the *p53* ORF was determined on pools of PCR-amplified *p53* cDNA clones for each of the 26 tumors (Table 1). This analysis showed that tumor 32 had two point mutations (codon 280 AGA → ACA, arg → thr; codon 285 GAG → AAG, glu → lys). Further sequence analysis of individual *p53* clones showed that both mutations were present in the same allele (data not shown). Two other tumors each contained a single mutation. Tumor 39 contained a single point mutation at codon 238 (TGT → TTT, cys → phe), while tumor 87 contained a deletion encompassing exon 4 (codons 33 to 126). This latter abnormality probably arose as a mRNA splicing error resulting from an intronic mutation similar to one described in a case of small cell lung cancer (23). The mutations detected in these three tumors by nucleotide sequence analysis corresponded exactly to the location of the abnormalities indicated by RNase protection assay. Moreover, there was no evidence for the expression of a normal sequence coexisting with the mutant *p53* sequence.

We were concerned about the apparent low frequency (12%) of mutations in the *p53* gene relative to the published higher frequencies of LOH in this region of chromosome 17p (50%) and immunohistochemical staining of *p53* protein in tumor sections (50%). It seemed possible that some mutations could have been missed due to the presence of contaminating normal stromal tissue or a heterogeneity of tumor cells containing the mutation in the tumor biopsy material. To address this possibility we surveyed 24 of the tumor DNAs and RNAs using the SSCP assay (21). SSCP assays were performed on PCR-amplified DNA fragments using tumor-derived genomic DNA (covering exons 5 through 8, amino acids 126–306) and cDNA (covering exons 4 through 9, codons 33–331) as templates. Genetic abnormalities were observed in genomic DNA samples from ten tumors (tumors 28, 32, 37, 53, 66, 75, 77, 80, 81, and 85; Fig. 1 and Table 1). However, in tumor 28 the alteration mapped to codon 213 which we have recently found represents a naturally occurring polymorphism. In tumor 87 the *p53* mutation detected by RNase protection assays and cDNA sequencing was a deletion of exon 4, an area not covered in the genomic SSCP assay. Thus a total of 11 of the 24 tumors (46%) had a mutation(s) in the *p53* gene. SSCP analysis of cDNA confirmed a majority of the mutations found in genomic DNA (Fig. 2).

RFLP Analysis of the *p53* and pYNZ22.1 Loci. To determine whether LOH on chromosome 17p correlates with mutation in the *p53* gene, genomic DNA samples from the breast tumor DNAs were compared to the constitutional (lymphocyte) genomic DNAs from the same patients for LOH at the *p53* and pYNZ22.1 loci (Fig. 3 and Table 1). Nineteen of 26 patients (73%) were constitutionally heterozygous for *p53* (analyzed by pBHP53, *Bam*HI RFLP and/or codon 72 RFLP), while six (30%) had LOH at the *p53* gene. Five of these six tumors also had a mutation(s) in the remaining *p53* allele (Table 1). How-

Table 1 *Status of the p53 gene in primary human breast tumors using various molecular techniques*

Tumor	17p13.3 pYNZ22.1	17p13.1 *p53*	RNase protection	cDNA clone sequence	cDNA mutation[a]	SSCP exons 5/6	SSCP exons 7/8
7	Heterozygous	Heterozygous	Normal	Normal		Not tested	Not tested
13	Heterozygous	Heterozygous	Normal	Normal		Normal	Normal
27	Heterozygous	Non inf.[b]	Normal	Normal		Normal	Normal
31	Deleted	Deleted	Normal	Normal		Normal	Normal
35	Non inf.	Heterozygous	Normal	Normal		Normal	Normal
36	Heterozygous	Non inf.	Normal	Normal		Normal	Normal
41	Non inf.	Heterozygous	Normal	Normal		Normal	Normal
45	Non inf.	Non inf.	Normal	Normal		Not tested	Not tested
48	Heterozygous	Non inf.	Normal	Normal		Normal	Normal
58	Heterozygous	Heterozygous	Normal	Normal		Normal	Normal
65	Non inf.	Heterozygous	Normal	Normal		Normal	Normal
78	Non inf.	Heterozygous	Normal	Normal		Normal	Normal
82	Heterozygous	Heterozygous	Normal	Normal		Normal	Normal
86	Deleted	Heterozygous	Normal	Normal		Normal	Normal
28	Heterozygous	Heterozygous	Normal	Normal		Codon 213 pol.[b]	Normal
32	Heterozygous	Deleted	Abnormal	Mutated	280 & 285	Normal	Mut. exon 8
37	Heterozygous	Not tested	Normal	Normal		Normal	Mut. exon 7
39	Deleted	Deleted	Abnormal	Mutated	238	Normal	Not tested
53	Heterozygous	Non inf.	Normal	Normal		Mut. exon 5	Normal
66	Heterozygous	Heterozygous	Normal	Normal		Mut. exon 5	Normal
75	Non inf.	Non inf.	Normal	Normal		Normal	Mut. exon 8
77	Deleted	Deleted	Normal	Normal		Mut. exon 5	Normal
80	Non inf.	Heterozygous	Normal	Normal		Normal	Mut. exon 7
81	Heterozygous	Heterozygous	Normal	Normal		Mut. exon 5	Normal
85	Deleted	Deleted	Normal	Normal		Mut. exon 6	Normal
87	Deleted	Deleted	Abnormal	Mutated	Del. 33–126[b]	Normal	Normal

[a] Number indicates codon position.
[b] Non inf., non-informative at the particular locus; del., deletion; pol., polymorphism.

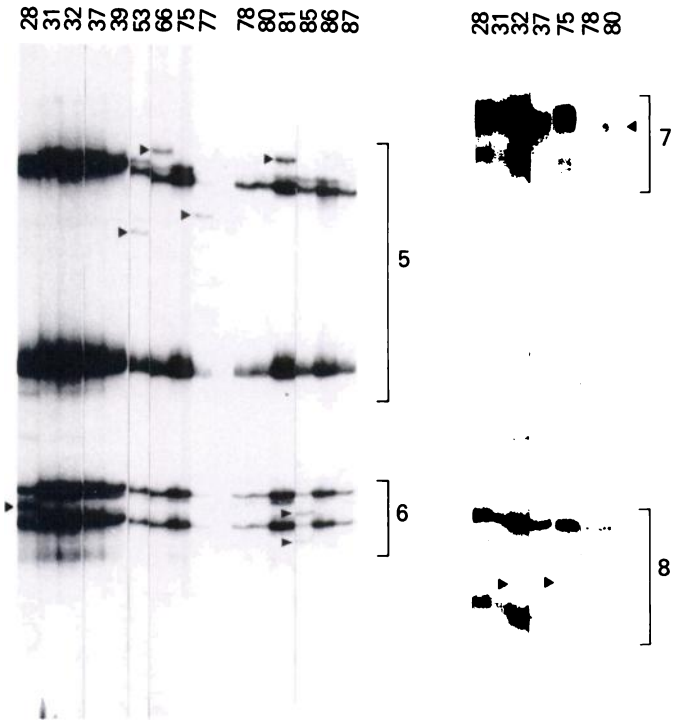

Fig. 1. SSCP analysis of PCR-amplified *p53* DNA from primary human breast tumor DNAs. The primers and conditions of the PCR are given in "Materials and Methods." The PCR products were digested with either *Aat*I (exons 5/6) or *Dra*I (exons 7/8) and then electrophoretically separated on a 6% nondenaturing acrylamide gel. *Brackets*, alleles for exons 5/6 and exons 7/8. The *numbers above each lane* correspond to specific breast tumors DNAs. *Arrows*, novel or mutant alleles.

ever, three of the tumors having a *p53* mutation retained heterozygosity at the *p53* locus (tumors 66, 80, and 81). At the pYNZ22.1 locus 19 of 25 patients tested were constitutionally heterozygous. Six (32%) of the informative patients showed a LOH in the tumor DNA. Four of these had mutations in *p53* while two, 31 and 81, did not (Table 1). In tumor 31, there are several possible explanations for the apparent absence of a *p53* mutation in the remaining allele: (*a*) a mutation occurred in a region of *p53* not covered by our analysis; (*b*) LOH has preceded the development of a mutation in the remaining *p53* allele; (*c*) the remaining *p53* allele in the tumor was transcriptionally silent and the cDNA was generated almost exclusively from RNA from normal cells in the tumor specimen; and (*d*) another unknown tumor suppressor gene, closely linked to *p53*, is altered. Consistent with the latter possibility tumor 86 showed LOH for pYNZ22.1 while retaining two normal *p53* alleles.

Discussion

A variety of genetic alterations have previously been observed in the *p53* gene in various malignant tissues (reviewed in Ref. 1). In general, these abnormalities appear to abrogate the normal function of the gene product as well as prolong its half-life in the cell. It is thought that the prolonged half-life of the altered protein is primarily responsible for its detection in 50% of primary human breast tumors by immunohistochemical techniques. In our study, 11 of 24 (46%) primary breast carcinomas contained mutations in the *p53* gene. Although this frequency of mutations is similar to that inferred from immunohistochemical techniques, it is clearly dependent on the methodology used to detect the mutation. Thus, in our tumor panel only three of the eleven mutations detected by SSCP analysis were detected by RNase protection assays or nucleotide sequence analysis of the PCR product of *p53* cDNA. We believe this reflects the variable contamination of the tumor biopsy material with normal stroma tissue as well as the probable heterogeneity of tumor cells which contain the mutation within some biopsies. In addition, the published rate of detection of mutations by the

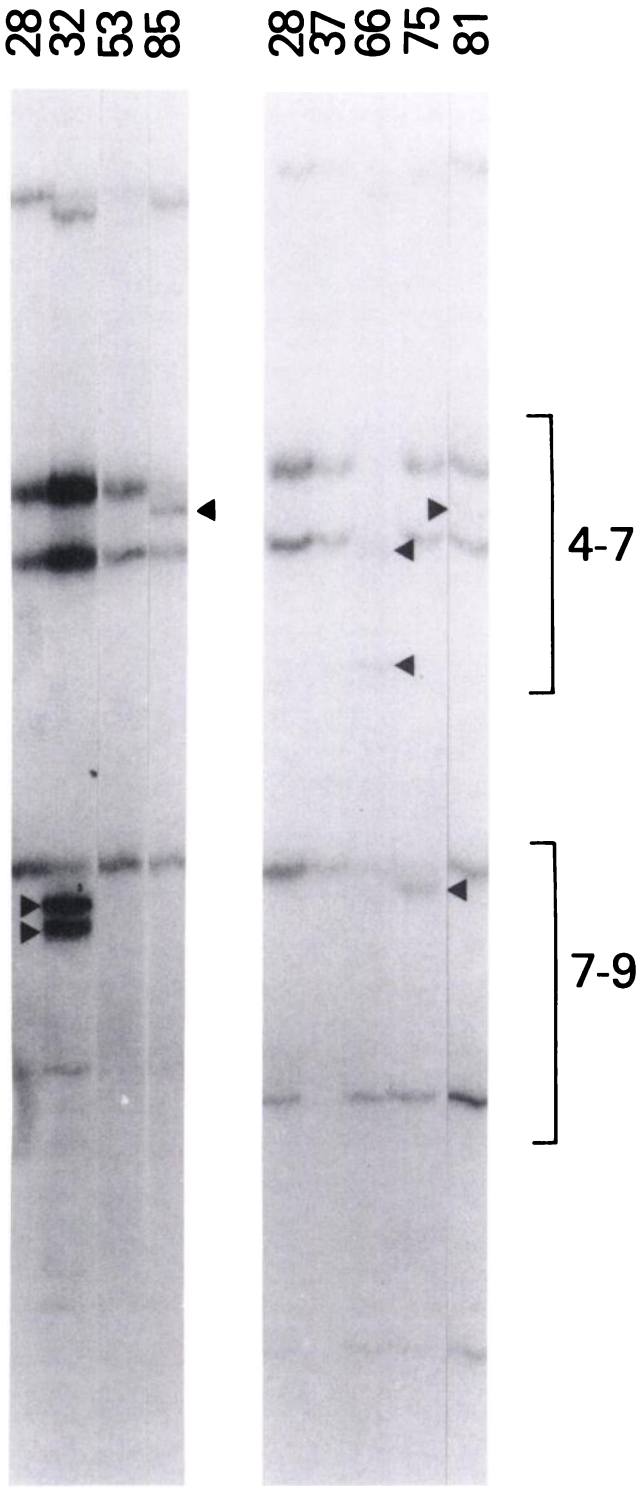

Fig. 2. SSCP analysis of PCR-amplified DNA from primary breast tumor-derived *p53* cDNA. The conditions for cDNA synthesis and PCR amplification are given in "Materials and Methods." The PCR products were digested with *Alw*NI and then separated on a 6% nondenaturing acrylamide gel. *Brackets*, alleles for exons 4–7 and exons 7–9. The *numbers above each lane* correspond to specific breast tumors. *Arrows*, novel or mutant alleles.

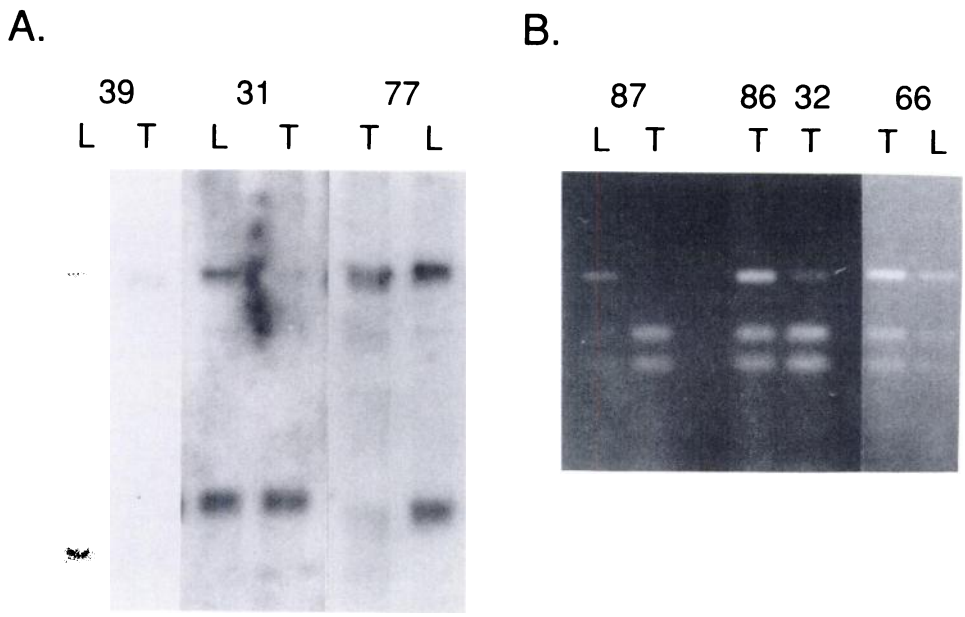

Fig. 3. Genetic abnormalities for markers on chromosome 17p in human breast cancer. The locus pYNZ22.1 (*A*) was analyzed by conventional Southern hybridization. Tumor DNAs 31, 39, and 77 represent examples of a LOH at the pYNZ22.1 locus. The *p53* locus (*B*) was analyzed by a novel PCR-based technique (19). Examples of LOH at the *p53* locus are tumor DNAs 32 and 87, while in tumor DNAs 86 and 66 there is a normal amount of each allele. *L* and *T*, lymphocyte and tumor DNA samples, respectively, from the same patient.

RNase protection assay is only 50% (20).

The site of the mutations observed may have important consequences for the biological activity of the protein (3, 4). For instance, three of the four mutations detected in the present study have previously been observed in other tumors. The deletion in tumor 87 was identical to that occurring in a lung tumor cell line (20). The point mutation at codon 285 in sample 32 has been observed in a breast cancer cell line, and codon 280 was also the site of mutation (nonidentical) in another breast cancer cell line (8). Of the 12 mutations detected in this study, one occurred in exon 4, four in exon 5, one in exon 6, and two in each of exons 7 and 8. Although in our panel exon 5 is affected most frequently by mutation, when our data is added to the published work (1, 5–9, 14) there does not appear to be a preferential selection for mutations occurring in one conserved domain over another in primary human breast tumors.

Inactivation of a tumor suppressor gene requires two independent mutagenic events (reviewed in Ref. 24). LOH on chromosome 17p has been frequently detected in sporadic primary human breast carcinomas (6, 12–15). However, with respect to the inactivation of *p53* there is little information available as to whether in sporadic breast cancer there is a preferred order of mutagenic events during tumor progression. In our study, there were nine breast tumors from patients which were informative for the genomic *p53* restriction site polymorphisms and contained point mutations in *p53*. Three of the tumors (tumors 66, 80, and 81) had not lost the remaining normal allele, whereas the others had LOH at *p53*. This suggests that the clonal outgrowth of cells containing a point mutation in *p53* precedes LOH in this region of chromosome 17 during tumor progression. An extreme example of this hypothesis occurs in the Li-Fraumeni hereditary cancer syndrome, in which breast cancer is a major component (25). In one affected patient with an osteosarcoma, there was a somatic LOH of the remaining *p53* allele. One possible exception in our study to this scenario was tumor 31, in which LOH occurred at the *p53* and YNZ22.1 loci but the remaining *p53* allele was normal. However, there may be another explanation for this tumor as well as tumor 86, in which both *p53* alleles were normal but LOH had occurred at YNZ22.1. These tumors may also contain an independent mutation in another tumor suppressor gene which has been postulated to be located on chromosome 17p, telomeric to *p53* (26, 27). Further studies are required to dissect the genetic lesions on chromosome 17p and document the stage of tumor progression at which *p53* point mutations and LOH begin to appear.

Taken together, our data show that the SSCP technique represents a highly sensitive approach to determining the frequency and relative location of *p53* mutations in primary human breast tumors. This is important since frozen biopsy materials are frequently contaminated with normal cells of different origins which can mask the presence of mutations detected through other molecular approaches. Using SSCP it should be possible to rapidly screen larger panels of primary breast tumor biopsy material to determine the various clinical parameters with which it is associated, including patient prognosis. In this regard it is pertinent that we (26) have found that LOH on chromosome 17p has a significant association with tumors having a high proliferative index.

Acknowledgments

The pBHP53 probe was a generous gift of Dr. Y. Nakamura.

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