Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 5006–5010, June 1991
Medical Sciences

Genetic basis for p53 overexpression in human breast cancer

(protein expression/gene mutation/allelic deletion)

ANDREW M. DAVIDOFF*, PETER A. HUMPHREY†, J. DIRK IGLEHART*, AND JEFFREY R. MARKS*‡

Departments of *Surgery and †Pathology, Box 3873, Duke University Medical Center, Durham, NC 27710

Communicated by Judah Folkman, March 8, 1991 (received for review August 30, 1990)

ABSTRACT **Overexpression of an activated form of the p53 protein may be involved in neoplastic transformation. We found widespread overexpression of p53 by immunohistochemical staining in 11 (22%) of 49 primary invasive human breast cancers. Northern blot analysis showed that this overexpression was not due to an increase in the steady-state level of p53 mRNA. The p53 gene was directly sequenced in 7 of these tumors with elevated levels of the protein and, in each case, a mutation that altered the coding sequence for p53 was found in a highly conserved region of the gene. Whereas 4 of these tumors contained only a mutant p53 allele, the other 3 tumors exhibited coding sequences from both a mutant and a wild-type allele. p53 mutations have previously been correlated with allelic loss of part of chromosome 17p that contains the p53 locus. Examination of all 49 breast tumors revealed a 61% frequency of deletion at or near the p53 locus. However, the presence of allelic deletion did not correlate with overexpression of the protein. Six tumors that were deleted but did not express high levels of the protein were sequenced and all retained a wild-type p53 allele. In this series of human breast cancers, overexpression of the p53 protein, not allelic loss on chromosome 17p, was always associated with mutation of the p53 gene.**

The nuclear phosphoprotein p53 is expressed in normal cells and appears to be involved in the regulation of cellular proliferation (1, 2). The level of p53 protein is regulated through the cell cycle by two mechanisms: (*i*) p53 RNA transcription increases by 10- to 20-fold late in the G_1 phase (3, 4) and (*ii*) normal p53 protein is rapidly eliminated by virtue of its short half-life (6–30 min) (5–7). These two mechanisms serve to give the cell a short pulse of protein that is then quickly degraded. Levels of the protein, therefore, are normally low, even in actively dividing cells.

Mutant p53 genes can cooperate with the *ras* oncogene to transform primary rodent fibroblasts (8, 9). In general, mutant forms of the p53 protein that are transforming have several properties that distinguish them from wild type. They fail to bind to the simian virus 40 tumor antigen (10) but do form stable complexes with hsc70 (11), a constitutively expressed member of the heat shock family. Perhaps as a result of this complex with hsc70, mutant p53 proteins have an increased half-life that, in part, gives rise to high steady-state levels of the protein in cells transformed with a mutant p53 gene (8).

Altered p53 genes with proven *in vitro* transforming activity have been isolated from several sources including carcinogen-treated rodent cells (12) and primary human colorectal cancers (13). In addition, a variety of point and deletion mutations have been described for the p53 gene in primary tumors and cell lines derived from tumors (14). Although most of these mutations have not been tested for transforming activity, they almost invariably alter amino acid residues that have been highly conserved during evolution (15), suggesting that they are oncogenic mutations.

Several lines of evidence suggest that p53 may act as a recessive oncogene or tumor suppressor, much like the retinoblastoma gene. (*i*) Mutations that activate the oncogenic potential of p53 have been found over a large region of the protein, suggesting that these mutations serve to eliminate a normal function of the protein (8, 14). (*ii*) The wild-type gene is capable of suppressing transformation of rodent cells by mutant p53 plus ras (16, 17). (*iii*) Mutant p53 protein is capable of forming oligomeric complexes with wild-type p53 (12, 18). This binding may inactivate the function of the normal protein and could explain how mutant p53 is able to transform cells in the presence of wild-type protein. (*iv*) The human p53 gene is located on the short arm of chromosome 17 in a region very frequently deleted in many different human tumors (19). Allelic loss on 17p is often accompanied by a mutation in the remaining copy of the p53 gene, thus eliminating all wild-type p53 in those cells. Nigro *et al.* (14) have proposed that mutation occurs first during tumor progression and promotes cell growth by a dominant negative effect on the wild-type protein. These mutations are then followed by deletion of the wild-type gene, leading to a further loss of growth control.

The extremely high frequency (60–70%) of allelic deletion on 17p in many types of cancer would, therefore, predict an equally high frequency of p53 mutation, as appears to be true in colorectal cancer (14). We have examined primary human breast cancer to assess the generality of this hypothesis. Greater than 60% of breast cancers have been shown to be allelically deleted for sequences on the distal end of 17p (20, 21). In addition, a separate study has found that 45% of breast cancers overexpress the p53 protein (22). Since p53 mutations that are oncogenic also increase the stability of the protein, tumors that have elevated steady-state levels of the protein likely harbor activating mutations in the gene. Therefore, it appeared that p53 activation in breast cancer would follow the same pattern as colorectal cancer. We report, however, that deletion on 17p is not a good predictor of mutation but that widespread overexpression of the p53 protein does indicate the presence of a mutation in breast cancer.

MATERIAL AND METHODS

Tissue. Pieces from each breast biopsy or mastectomy at Duke University Medical Center were collected after surgical removal, immediately flash frozen, and stored at −120°C. Specimens from 49 consecutive patients whose tumors were diagnosed as primary invasive breast carcinoma by a member of the Department of Pathology were studied. Forty-five of these tumors were infiltrating ductal, 3 were infiltrating lobular, and 1 was medullary carcinoma. Peripheral blood

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Abbreviation: RFLP, restriction fragment length polymorphism.
‡To whom reprint requests should be addressed.

leukocytes were collected from each of these patients as a source of normal cells.

Immunohistochemistry. Sections (8 μm) of each breast cancer specimen were cut, air-dried, and fixed in acetone for 10 min. Immunologic detection using the anti-p53 monoclonal antibody PAb 1801 (Ab-2, Oncogene Science, Manhasset, NY, at 500 ng/ml) was performed on all specimens. PAb 1801 reacts specifically in Western blots, immunoprecipitation, and immunohistochemical procedures with a denaturation-stable epitope between amino acids 32 and 79 (ref. 23 and J.R.M., unpublished data). In addition, selected tissues were reacted with PAb 240 (Ab-3, Oncogene Science) at 2 μg/ml and PAb 421 (hybridoma supernatant) diluted 1:10 in phosphate-buffered saline. PAb 240 recognizes a common conformational epitope between amino acids 156 and 355 of mutant p53 proteins and PAb 421 recognizes an epitope between amino acids 370 and 378 of both mutant and wild-type p53 (24, 25). Binding of these antibodies was visualized using the ABC (Vector Laboratories) immunoperoxidase system according to the manufacturer's recommendations. Medium from the mouse myeloma cell line NS-1 was used as a control. Immunohistochemical staining was also performed on sections from each specimen with the anti-cytokeratin monoclonal antibodies AE1 + 3 (Hybritech) at 2 μg/ml. Confirmation of pathologic diagnoses and evaluation of immunohistochemical staining were performed by P.A.H.

Northern Blot Analysis. Sections stained with AE1 + 3 from each tumor were examined to assess the amount of breast epithelium in each sample relative to other cell types. Tissue blocks were trimmed to minimize the number of lymphocytes in each sample. RNA was extracted by the guanidium thiocyanate/phenol method (26). Northern blot analysis was performed as described (27).

Sequencing. Total RNA (1 μg) was used as a template for p53 cDNA synthesis with murine leukemia virus reverse transcriptase (BRL). The reaction was performed at 37°C for 30 min in 1× *Taq* polymerase buffer (New England Biolabs) containing an antisense oligonucleotide primer (25 pmol) from exon 10 (5′-CCTGGGCATCCTTGAGTT; all oligonucleotide primers were synthesized at the Duke Comprehensive Cancer Center), 2.5 mM $MgCl_2$, all four dNTPs (each at 125 μM), and 10 units of placental RNase inhibitor (Stratagene). Exons 4 through 10 were then amplified from this cDNA by using the polymerase chain reaction (PCR; ref. 28) with an oligonucleotide primer from exon 4 (5′-GGGACAGCCAAGTCTGTGACT), more 1× *Taq* polymerase buffer, and 2.5 units of *Taq* DNA polymerase (New England Biolabs). This mixture underwent thermocycling for 30 cycles as follows: 94°C for 1 min, 60°C for 1 min, and 72°C for 3 min. The 712-base-pair product was gel-purified and reamplified using the same thermocycling conditions with 5 pmol of each primer, all four dNTPs (each at 200 μM), and 2.5 units of *Taq* DNA polymerase in 1× *Taq* polymerase buffer. This product was purified away from primers and unincorporated nucleotides by gel filtration through a Sepharose CL-6B (Pharmacia) spin column, ethanol precipitated, and resolubilized in water. This material was the template for dideoxynucleotide sequencing using Sequenase 2.0 (United States Biochemical). Oligonucleotides flanking exon 5, 6, 7, or 8 (5′-TACTCCCCTGCCCTC-AACAAG, 5′-CATCGCTATCTGAGCAGCGCT; 5′-GTCTGGCCCCTCCTCAG, 5′-CTCAGGCGGCTCATAGG; 5′-GTTGGCTCTGACTGTAC, 5′-CCGAATTCAGTCTTCCAGTGTGATGATG; or 5′-TTCCGTCCCAGTAGATTACCA, 5′-TGGTAATCTACTGGGA, respectively) were used to prime the reactions in which the primer–template mixture was first boiled and labeled on ice for 10 min with [^{32}P]dATP. The termination reactions were performed at 45°C for 10 min. The reaction products were electrophoresed on a polyacrylamide gel, which was then soaked in 10% (vol/vol) acetic acid/12% (vol/vol) methanol, dried, and set with Kodak XAR-5 film overnight. Mutations were confirmed by repeating the sequencing procedure from new samples of RNA. Sequencing of tumor DNA was performed as for RNA, with the elimination of the reverse transcriptase reaction. An antisense oligonucleotide primer from exon 9 (5′-AGCTGGTGTTGT-

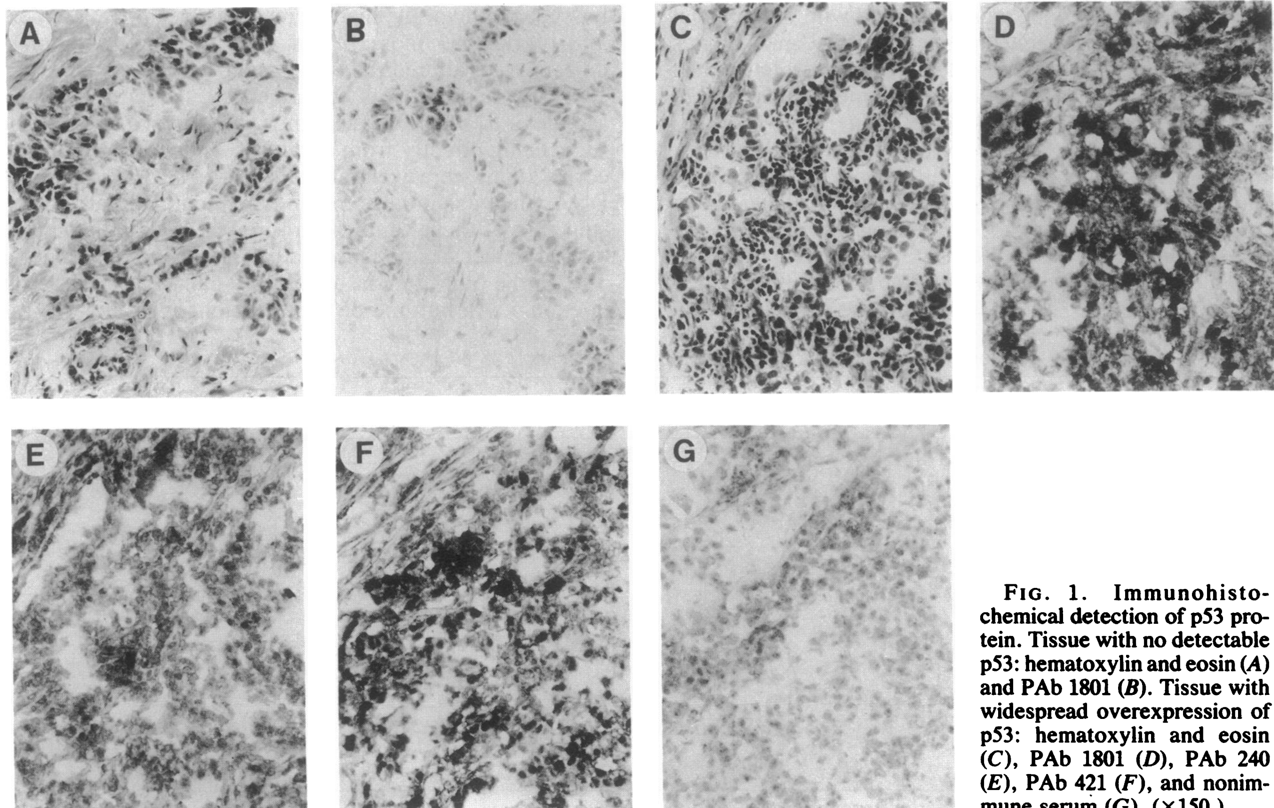

FIG. 1. Immunohistochemical detection of p53 protein. Tissue with no detectable p53: hematoxylin and eosin (*A*) and PAb 1801 (*B*). Tissue with widespread overexpression of p53: hematoxylin and eosin (*C*), PAb 1801 (*D*), PAb 240 (*E*), PAb 421 (*F*), and nonimmune serum (*G*). (×150.)

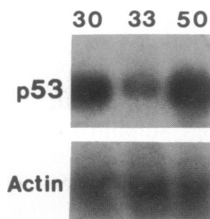

FIG. 2. Northern blot analysis of p53 transcripts in tumors expressing undetectable (lane 30), sparse (lane 33), or widespread (lane 50) p53 protein.

TGGGCAGTG) was used to prime the amplification reactions and a 2.7-kilobase product was recovered and reamplified for sequencing.

Southern Blot Analysis. Allelic deletion of the p53 gene was determined through Southern blot analysis by comparing DNA from peripheral leukocytes and tumor tissue. Tumor tissue was sectioned and stained and the original block was then trimmed to maximize the amount of malignant epithelium. The sample was then digested with 1% SDS/proteinase K (500 μg/ml) and extracted with phenol and ether before being precipitated in ethanol and resolubilized in 10 mM Tris·HCl/1 mM EDTA, pH 7.5. Normal DNA was similarly prepared from isolated leukocytes from each patient. Normal and tumor DNAs (5 μg) were digested separately with restriction endonucleases *Bgl* II and *Msp* I, fractionated by agarose gel electrophoresis, and transferred to Biotrans (ICN). These blots were then hybridized with the following ^{32}P-labeled probes that detect restriction fragment length polymorphisms (RFLPs) at or near the p53 locus: (*i*) p53 cDNA, (*ii*) MCT35.1, and (*iii*) HF12-2 (American Type Culture Collection). The p53 cDNA probe detects a polymorphic *Bgl* II site within intron 1 of the p53 gene itself (29), and the other two are anonymous probes that detect polymorphisms at *Msp* I sites closely linked to the p53 locus (30). After hybridization the blots were exposed to Kodak XAR film overnight with intensifying screens at −80°C.

RESULTS

Expression of p53 in Primary Human Breast Cancers. Three patterns of nuclear staining were observed with immunohistochemical analysis of the 49 breast cancer specimens in this study. (*i*) No p53 protein was detected in normal human breast tissue derived from reduction mammoplasties or in 26 (53%) of the invasive tumors (Fig. 1*B*). (*ii*) Twelve cancer specimens (24%) had detectable p53 protein but only in a very small percentage of the malignant epithelial cells. Positively staining cells in this category were generally localized in one area of the section but were not closely clustered. (*iii*) Eleven of the tumors (22%) exhibited intense nuclear staining throughout the malignant epithelium (Fig. 1*D*), suggesting very high levels of p53 expression in these tissues. Of the different histologic types, 9 of 45 infiltrating ductal, 1 of 3 infiltrating lobular, and the medullary carcinoma expressed high levels of p53. Overexpression of p53 was confirmed by reacting sections from each of these 11 tumors with two other PAbs, 240 and 421, each of which recognizes an epitope different from that recognized by PAb 1801 (31). Staining patterns were similar with each of the three monoclonal antibodies, although staining with PAb 240 tended to be less intense in each case than with the other two antibodies (Fig. 1 *E* and *F*). PAb 240 has also been shown to recover less p53 than the other anti-p53 antibodies by immunoprecipitation (24).

Relative Levels of p53 RNA. The abundant protein expression observed in some breast cancers might have been due to increased steady-state levels of p53 mRNA. To test this possibility, we compared the amount of p53 RNA in tissues with widely varying levels of the protein. By Northern blot analysis, no correlation was observed between the level of p53 RNA and the level of protein expression in tumors that expressed high and low levels of the protein. A representative blot of three of these tumors is shown in Fig. 2. Tissue 30 had no detectable p53 protein, tissue 33 had sparse staining, and tissue 50 had widespread protein expression. Although significant variation was observed in the amount of p53 message, the RNA did not vary with the protein levels in a consistent manner. A similar lack of correlation was found between expression of p53 protein and the amount of message in human breast cancer cell lines (J.R.M., unpublished data). In all cases, a single species of message was observed of ≈2.5 kilobases. These findings suggested that overexpression of the p53 protein was due to a posttranscriptional mechanism.

Sequence of the p53 Message in Tumors with Elevated Levels of the Protein. Increased stability leading to higher steady-state levels of p53 protein has been observed to result from mutations that change the coding sequence in a highly conserved region of the gene (8). Seven breast cancer specimens that exhibited widespread overexpression of p53 protein, including the one medullary carcinoma, were selected for sequencing of the highly conserved regions within exons 5–8 of the p53 message. In each case, a mutation was detected that altered the coding sequence of the gene (Table 1). Each mutation was confirmed by sequencing a second sample of RNA from each tumor and, for two specimens, by sequencing directly from amplified tumor DNA. No misincorporations were ever detected, starting from either RNA or DNA while using these methods, which eliminate a cloning step. Six of these tumors harbored a single point mutation that produced a nonconservative amino acid change (Fig. 3*A*). In the other tumor, an 18-base-pair deletion was found in exon 5. All but one of the mutations were located in the

Table 1. Alterations of p53 in primary human breast cancers

Patient	No. of p53 alleles	Expression	Mutation	Codon	Amino acid change
1	1	−	None detected	—	—
3	2	+	CCT → GCT	278	Pro → Ala
5	1	−*	None detected	—	—
11	1	+	TAC → TGC	163	Tyr → Cys
13	1	+	CGG → CAG	248	Arg → Gln
17	1	+	CGG → CTG	282	Arg → Leu
22	1	−*	None detected	—	—
29	1	−	None detected	—	—
30	1	−	None detected	—	—
33	1	−*	None detected	—	—
43	2	+	ATG → ATA	237	Met → Ile
50	1	+	Deletion	175–180	In-frame
52	2	+	GGC → GAC	245	Gly → Asp

*In these tissues there was staining of occasional cells.

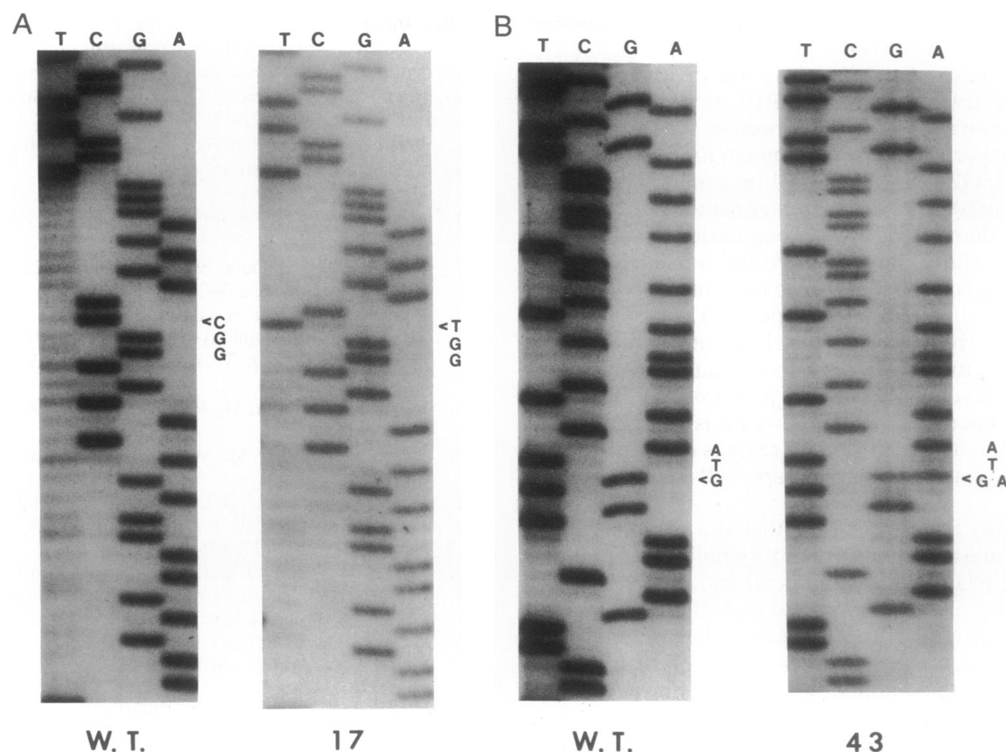

FIG. 3. Direct sequencing of p53 cDNA from two breast cancers overexpressing the p53 protein. (*A*) Mutation in codon 282 detected in tissue 17. (*B*) Mutation in codon 237 detected in tissue 43. Sequencing shows the presence of the wild-type (W.T.) nucleotide in cDNA derived from this tissue.

evolutionarily conserved regions between codons 126 and 290. These regions are ≈95% conserved at the amino acid level between frog and man. The mutation in p53 from tissue 11 at codon 163 was not within one of these domains, although it did alter a tyrosine residue that is conserved in frogs, rodents, and humans (14). The location of these mutations strongly suggests a causal relationship with overexpression of the protein. The sequence analysis also showed that both wild-type and mutant alleles were present and expressed in three of the seven tumors that were sequenced. In these three tumors, the autoradiograms had equally intense bands representing both mutant and wild-type nucleotides (Fig. 3*B*).

Genomic Analysis of 17p Deletions. It has been implied that allelic deletion of p53 is a necessary step toward the full oncogenic activation of the gene (14). Finding a high percentage of mutated tumors that retained the wild-type allele (three out of seven) prompted us to examine our series of 49 tumors for deletion of sequences at or near the p53 locus (Table 2). Loss of heterozygosity in tumor DNA was found in five (56%) of nine patients informative for a RFLP located in the p53 gene. Two other probes (HF12-2 and MCT35.1) that detect RFLPs near the p53 gene were also used to increase the percentage of informative cases. These two probes detected 16 (64%) deleted tumors out of 25 informative cases. The overall frequency of allelic loss at or near the p53 locus, combining the results from all three markers, was 61% (17 of 28 informative cases). In tissues that were informative for more than one probe, the deletion status was consistent in each case. Of the 7 overexpressed tumors that were sequenced, 5 were informative for at least one of the RFLPs and the number of alleles that had been observed in the sequence analysis was confirmed.

Table 2. Allelic deletion on 17p in breast cancer

Probe	Deleted	Heterozygous
p53	5	9
MCT35.1	11	17
HF12-2	8	14
Total* (n = 49)	17†	28

Probes p53 and MCT35.1 localize to 17p13.1 and HF12-2 localizes to 17p13 (30).
*Some cases were heterozygous at more than one locus.
†Of these 17 deleted tumors, 8 overexpressed the p53 protein.

Sequence of the p53 Message in Deleted Tumors Without Elevated Levels of the Protein. Of the 17 tumors that had detectable 17p deletions by RFLP analysis, 9 did not have widespread overexpression of the p53 protein. Deletion and mutation have been closely associated in colorectal cancer. From our sequencing analysis, it appeared that mutation of the p53 gene in breast cancer results in overexpression of the protein. Therefore, it was of interest to determine if deleted tumors that did not overexpress the protein also harbored mutations. RNA from 6 tumors of this type was sequenced from codons 126 through 290, the region that contains all of the activating mutations so far described for the p53 gene (8). Each of these tumors contained p53 mRNA that was identical to the reported wild-type sequence (32) (Table 1). Three of these tumors did not express any detectable p53 protein by immunohistochemistry, whereas the other three cases expressed the protein in only occasional malignant cells. Because these tumors contained a low percentage of expressing cells, mutation of the remaining p53 gene in these tissues would not have been detected if present only in these infrequent cells.

DISCUSSION

In this study we have examined the relationship of p53 protein expression, gene mutation, and allelic loss in 49 primary human breast cancers. Eleven (22%) of these tumors expressed elevated levels of the p53 protein and, when seven of these tumors were sequenced, we detected a p53 gene

mutation in each. Each of these mutations altered the coding sequence in a highly conserved region of the gene (Table 1), suggesting that these mutations may be involved in the activation of the transforming potential of p53. A similar correlation between protein overexpression and gene mutation has recently been described in human lung cancer (33).

Cattoretti *et al.* (22) using PAb 1801 on similarly prepared breast cancer tissues found that a greater percentage (45%) of their specimens demonstrated overexpression of p53 (22). However, in their study, "overexpression" included tissues that contained only a few cells with positive nuclear staining. We too observed staining of this type, in 12 (24%) of our tumor specimens. However, we restricted our definition of "overexpression" to include only those tumors displaying widespread high-level expression of p53 protein (Fig. 1*D*). Subsequent sequence analysis supported this distinction, as no mutations of the highly conserved regions of the p53 gene were found in tissues containing rare cells with nuclear staining.

Nigro *et al.* (14) have suggested that the presence of a deletion on chromosome 17p frequently predicts a mutation in the remaining p53 allele in colorectal and other types of human cancer. Therefore, we expected that allelic loss on 17p would also correlate with overexpression of the protein since, in our series of breast cancers, overexpression of p53 was always associated with a mutation. We found, however, that allelic loss at or near p53 was a much more frequent event than overexpression. This suggested that allelic deletion on 17p was not always associated with a mutation in the remaining p53 allele. Direct sequence analysis of six deleted tumors not overexpressing p53 confirmed that, in each case, the highly conserved regions of the p53 gene matched the wild-type sequence.

These findings raise the issue of whether allelic loss of the p53 gene is necessary for or related to the oncogenic activation of p53 in human breast carcinoma. Since mutant p53 protein can participate in cell transformation *in vitro*, even in the presence of the wild-type protein (8, 9), allelic loss may not be required to activate the oncogenic potential of p53. The more stable mutant p53 gene product may complex or compete with the wild-type protein and inactivate it in a transdominant manner (12, 18). Sequence analysis revealed the presence of coexisting mutant and wild-type alleles in three of seven overexpressing tumors in our series, suggesting that the complete loss of wild-type p53 is not an essential step for the activation of this gene in breast cancer.

Because the frequency of allelic loss on chromosome 17p is so high in breast cancer, it likely confers a growth advantage that is selected for during tumor formation. It is possible that the loss of one-half the amount of wild-type p53 is enough to provide some selection for those cells harboring deletions. Transfection experiments have demonstrated that high levels of mutant p53 are required to efficiently transform rodent fibroblasts (8). These high levels may be needed to titrate out the wild-type protein, perhaps through oligomeric binding. Therefore, the amount of available wild-type protein may be an important parameter in cell-cycle regulation and 2-fold differences could prove to be significant.

Alternatively, selection for allelic deletion on 17p could be due to the loss of another, as yet unidentified, recessive oncogene. Recent studies of allelic loss in primary breast cancer have shown that there may be more than one minimum deletion unit on the short arm of chromosome 17 and that the p53 locus is not always included in deletions that encompass the telomeric end of the chromosome (34, 35). In addition, p53 mutations have been identified concurrent with 17p deletions that do not include the p53 locus itself (34). Therefore, deletions in this region of the chromosome may, at least partially, be unrelated to the activation of the p53 gene in breast cancer.

We thank Arnold Levine for the PAb 421 hybridoma cell line, Nancy Glover for technical assistance, and Dennis Pittman for preparation of the manuscript. This work was supported, in part, by American Cancer Society Grant IN-158C and National Cancer Institute Grant 1 F32 CA08899-01 awarded to A.M.D. J.D.I. is the recipient of a Clinical Oncology Career Development Award from the American Cancer Society.

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