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# p53 Gene Mutation, Tumor p53 Protein Overexpression, and Serum p53 Autoantibody Generation in Patients with Breast Cancer

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**Objectives:** Autoantibodies against the p53 tumor suppressor protein have been detected in the serum of a proportion of patients with various cancers. The generation of such antibodies has been proposed to be due to either tumor p53 protein accumulation or to the type of p53 gene mutation. These hypotheses are examined in the present study.

**Design and methods:** Using immunofluorometric assays, we studied 195 patients with primary breast cancer for the presence of p53 antibodies in serum and p53 protein accumulation in the corresponding tumor. Seventeen patients (9%) were p53 antibody-positive and 77 (40%) overexpressed p53. Ten of the 17 p53 antibody-positive patients had tumor p53 accumulation and 7 were negative for p53. Statistical analysis revealed a weak association between the presence of p53 antibodies and p53 protein accumulation ($p = 0.05$). Direct DNA sequencing of exons 1–11 of the p53 gene was performed for 16 p53 antibody-positive and 16 p53 antibody-negative patients.

**Results:** Five of the seropositive and eight of the seronegative patients had a p53 gene mutation. Four of the five mutations in the p53 antibody-positive patients affected a Tyr residue, whereas none of the gene abnormalities in the seronegative patients had such an effect.

**Conclusions:** We conclude that p53 antibodies tend to develop in patients with tumor p53 accumulation, but p53 accumulation is neither sufficient nor necessary for the generation of the immune response. Further, p53 antibody-positive patients do not have higher frequency of p53 gene mutations than p53 antibody-negative patients, but the former patient group is associated with a Tyr substitution in the protein product. 

KEY WORDS: p53 tumor suppressor; breast cancer; tumor markers; autoantibodies; gene mutation.

## Introduction

The p53 gene is localized on the short arm of chromosome 17 and it encodes a 393-amino acid phosphoprotein, which is present at very low levels in normal cells. This molecule appears to play a major role in the maintenance of genomic integrity (1). Following DNA damage, p53 can either arrest the cells at the G1 phase of the cell cycle, thus providing time for the damage to be repaired (2–4), or induce apoptosis (5). Both pathways prevent replication of damaged DNA and further accumulation of mutations. Cells containing biologically inactive p53 protein are devoid of such protective mechanisms and they are genetically unstable. Genetic mutation is the most common pathway for p53 inactivation. Being mutated in approximately 50% of all tumors, p53 is currently considered the most frequently altered gene in human tumorigenesis. Since the discovery of this gene, more than 4000 abnormalities have been reported, the majority of them being missense point mutations that result in single amino acid substitutions. These mutations cluster between exons 5–9, which correspond to highly conserved domains of the protein (6). Many mutants have a different conformation and a longer lifetime compared to the wild-type protein. Increased lifetime causes mutant p53 protein accumulation in the tumor cells, which is detectable by conventional immunohistochemical or other immunologic methods.

Crawford et al. (7) reported for the first time the presence of autoantibodies against p53 in serum of patients with breast cancer. During the past few years, analysis of a large number of sera from patients with various malignancies revealed that the most immunogenic tumors are those of the lung (8–13), ovary (9,10,14–16), colon (9,10,17), breast

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(7,9,10,18–21), head and neck (22), and esophagus (23). The positivity rates for autoantibodies correlate with the frequency of p53 mutations in these tumors (10,24). The clinical significance of p53 autoantibodies in cancer patients has recently been studied. In lung cancer, p53 antibodies have been proposed as early markers for diagnosis, because they were detected in serum samples of two asymptomatic heavy smokers without demonstrated cancer, who developed lung cancer 5 and 15 months later (25). In ovarian cancer, p53 antibodies are detected with a frequency of 24% and are indicators of unfavorable prognosis because they are associated with high histologic grade and shorter disease-free survival (15). In addition, p53 autoantibodies were found in the ascites fluid of patients with advanced ovarian cancer (16). In colon (17) as well as in head and neck cancer (22), serum p53 antibodies are also associated with unfavorable prognosis. In breast cancer, which has been studied more extensively, positivity rates vary between different groups from 5%–26% (7,10,18–21). However, there is a general agreement that the presence of p53 autoantibodies indicates disease aggressiveness and poor clinical outcome in this cancer. Contrary to the literature cited above, Vojtesek and colleagues (26) reported absence of serum p53 antibodies in breast cancer patients, claiming that the antibodies described in previous studies are most likely specific for another 53 kDa protein, which is unrelated to p53.

The mechanisms leading to p53 immunogenicity still remain unclear. In 1992, Davidoff *et al.* (18) suggested that a 70 kDa heat shock protein (HSP70) was responsible for the antigenic presentation of mutant p53 to the immune system. Another study proposed that the development of such antibodies was dependent on the type of the mutation of the p53 gene; all p53 antibody-positive patients had p53 missense mutations, whereas no antibodies were detected in sera from patients whose tumor had stop, splice, splice/stop, or frameshift mutations (8). Soussi's group (19,27) has shown that these antibodies are specific for the amino and carboxy terminus of the p53 protein, two regions that are devoid of mutations (11). The epitope localization was found to be similar in animals hyperimmunized with human p53. Given the similarities between the immune response against p53 in patients and hyperimmunized animals, they suggested that p53 accumulation is a major trigger for the development of the immune response in cancer patients.

In this study, we examined whether tumor p53 protein accumulation or p53 mutational status were associated with the development of p53 autoantibodies in serum of breast cancer patients.

## Materials and methods

### Patient population

One hundred ninety-five patients with primary breast cancer operated on at the Department of Gynecologic Oncology, University of Turin, Italy, between January 1992 and May 1993, were included in this study. The ages of the patients were between 29 and 93 years, with a median of 57 years. The clinical stages were as follows: 44% stage I, 48% stage II, and 8% stage III or IV. The tumor size ranged from 0.1 to 15 cm, with a median of 2 cm. Of the 195 patients, 185 had their axillary lymph nodes examined at surgery; the median number of nodes examined was 15. Of the 185 patients, 99 had cancer metastasis to their axillary lymph nodes. The major histologic types were invasive ductal (56%) and invasive lobular (16.5%). The rest included ductal *in situ* (2%), medullary (2%), papillary (4.5%), tubular (6.5%), inflammatory (3.5%), tubulo-lobular (4%), muciparous (2.5%), and others (2.5%). All patients donated a presurgical serum sample. Snap-frozen tumor tissue was collected at surgery. Seventy-five patients donated serum 6 months after surgery. All serum and tissue specimens were stored at −70° C until analysis.

### Cell culture and production of recombinant p53 protein

Sf9 insect cells were cultured in supplemented Grace's insect medium (Invitrogen, San Diego, CA) containing 10% fetal calf serum (FCS). They were propagated in monolayers at 27° C to approximately 90% confluency. The cells were infected with recombinant baculovirus for both viral amplification and protein expression according to standard protocols (28). Briefly, a low titer stock of baculovirus vector containing the full-length wild-type human p53 cDNA (a gift of Dr. T. Soussi, Institute Curie, Paris, France) was used to infect log phase Sf9 cells; 5 days later the supernatant, which contained the amplified virus, was harvested and used to reinfect Sf9 cells for the production of recombinant p53 protein. Infected cells were collected after 48 h, washed three times in phosphate-buffered saline (PBS), and lysed in a lysis buffer (10 mmol/L Tris, pH 8.0, 1 mmol/L EDTA, 150 mmol/L NaCl, 10 mL/L Nonidet NP-40 surfactant, 10 g/L sodium deoxycholate, 1 g/L sodium dodecyl sulfate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mg/L pepstatin, and 10 mg/L each of leupeptin and aprotinin) on ice for 30 min. The soluble extract was collected after ultracentrifugation at 100,000×g for 30 min at 4° C, and aliquoted for storage at −80° C. The presence of p53 protein was confirmed by Western blot analysis, using the mouse monoclonal p53 specific antibody DO-1, which recognizes an epitope between amino acids 21–25 of the p53 protein. Probing with DO-1 produced a single band at 53 kDa, and demonstrated that this antibody has no cross-reactivity with any other protein present in the extract.

### Immunofluorometric assay for p53 protein

p53 in tumor extracts was quantitatively analyzed with a time-resolved immunofluorometric procedure

TABLE 1
Primers for PCR Amplification of p53 Exons

| Primer code | Sequence[1] | Size, bases | Gene | Concentration |
|---|---|---|---|---|
| 5 × 1 | CGGATTACTTGCCCTTACTTGTAC | 24 | 711 | 0.4 |
| 3 × 1 | CCCCAGCCCCAGCGATTTT | 19 | 1041 | 0.4 |
| 5 × 2 | ACCCAGGGTTGGAAGCGTCT | 20 | 11639 | 0.6 |
| 3 × 2 | GACAAGAGCAGAAAGTCAGTCC | 22 | 11899 | 0.6 |
| 5 × 3,4 | CATGGGACTGACTTTCTGCT | 20 | 11874 | 0.8 |
| 3 × 3,4 | AAAGAAATGCAGGGGGATACGG | 22 | 12367 | 0.8 |
| 5 × 5 | CACTTGTGCCCTGACTTT | 18 | 13009 | 0.4 |
| 3 × 5 | CCTGGGGACCCTGGGCAA | 18 | 13291 | 0.4 |
| 5 × 6 | TGTTCACTTGTGCCCTGACT | 20 | 13005 | 0.4 |
| 3 × 6 | GGAGGGCCACTGACAACCA | 19 | 13493 | 0.4 |
| 5 × 7 | GGCGACAGAGCGAGATTCCA | 20 | 13890 | 0.8 |
| 3 × 7 | GGGTCAGCGGCAAGCAGAGG | 20 | 14175 | 0.8 |
| 5 × 8 | GACAAGGGTGGTTGGGAGTAGATG | 24 | 14350 | 0.6 |
| 3 × 8 | GCAAGGAAAGGTGATAAAAGTGAA | 24 | 14669 | 0.6 |
| 5 × 9 | GCGGTGGAGGAGACCAAGG | 19 | 14609 | 0.4 |
| 3 × 9 | AACGGCATTTTGAGTGTTAGAC | 22 | 14817 | 0.4 |
| 5 × 10 | TGATCCGTCATAAAGTCAAACAA | 23 | 17477 | 0.4 |
| 3 × 10 | GTGGAGGCAAGAATGTGGTTA | 21 | 17866 | 0.4 |
| 5 × 11 | GGCACAGACCCTCTCACTCAT | 21 | 18540 | 0.4 |
| 3 × 11 | TGCTTCTGACGCACACCTATT | 21 | 18795 | 0.4 |

[1]All sequences are given in 5′ to 3′ direction; [2]numbers refer to Genbank accession # 54156 and are the starting points of primer annealing.

previously described elsewhere (29). Briefly, the tumor extracts were incubated for 3 h at 37° C with a mouse monoclonal anti-p53 antibody (PAb240, mutant-specific) in goat anti-mouse-coated polystyrene microtitre wells. After washing of the wells, a rabbit CM-1 polyclonal anti-p53 antibody was added. A goat anti-rabbit antibody conjugated to alkaline phosphatase was used for the detection of the immunocomplex. For quantification, an arbitrary p53 standard solution established in our laboratory was used as described (30). All p53 concentrations in the extracts, in arbitrary Units/L, were transformed to units of p53 per gram of total protein (U/g) to compensate for the amount of cells extracted per tumor. For statistical analysis, we chose a cutoff level of 3 U/g as previously described (30).

### IMUNOFLUOROMETRIC ASSAY FOR P53 ANTIBODIES

An ELISA assay was used for the detection of p53 autoantibodies in serum (31,32). The mouse monoclonal p53 specific antibody DO-1 was used for coating microtiter wells. The DO-1 antibody was saturated with recombinant wild-type p53 protein and patient serum diluted tenfold was added to the immunocomplex. Any serum anti-p53 antibodies bound to recombinant p53 were subsequently detected with a goat anti-human IgG immunoglobulin conjugated to alkaline phosphatase. All sera were analyzed in parallel with extracts from Sf9 cells infected with the baculovirus carrying the wild-type p53, and with extracts from noninfected Sf9 cells (blank). The fluorescence signals between the two analyses were compared, and when their ratio was higher than 2, the samples were considered positive. This is an arbitrary cutoff point. The precision of this assay is $< 15\%$.

### STATISTICAL ANALYSIS

The chi-square test and the Wilcoxon Rank Sums test were used to determine the statistical significance of differences in distributions. The $p$ values were calculated by the statistical software SAS (SAS Institute Inc., Cary, NC).

### PCR AND MUTATIONAL ANALYSIS

DNA fragments were amplified in 50 μL reaction mixtures containing approximately 500 ng of genomic DNA, 10 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 1.5–2.5 mmol/L $MgCl_2$, 200 μmol/L deoxynucleoside triphosphates (dNTPs), and 2 units of Taq DNA polymerase (Boehringer Mannheim, Laval, Quebec, Canada). A detailed description of the PCR primers used to amplify each of the 11 p53 exons as well as the concentrations at which these primers were used are given in Table 1. The temperature cycling protocol for PCR, on a Perkin Elmer 9600 DNA Thermal Cycler (Perkin Elmer Inc., Foster City, CA), consisted of denaturation at 94° C for 20s, annealing at 60° C (exons 1, 3, 4, 5, 6, 9, 10, and 11), 62° C (exon 7), or 63° C (exons 2 and 8) for 30s, and extension at 72° C for 30s. The cycling was repeated 30 times. Each PCR was initiated with a 3-min denaturation at 94° C and terminated with a 3-min extension at 72° C. PCR products were subjected to Exonuclease I digestion and shrimp alkaline phosphatase hydrolysis according to the ThermoSequenase protocol (Amersham Life Sciences

TABLE 2
Cy5 Labeled Primers for DNA Sequencing of p53 Exons

| Primer | Sequence[1] | Size, bases | Gene Position[2] |
|---|---|---|---|
| 3 × 1S | AGCCGAGAGCCCGTGAC | 18 | 1002 |
| 5 × 1S | GCCTCGCAGGGGTTGAT | 18 | 762 |
| 3 × 2S | GCTAGGGGGCTGGGGTTGG | 19 | 11849 |
| 5 × 2S | CCAGGGTTGGAAGCGTCTC | 19 | 11641 |
| 3 × 3S | ATGGGTGAAAAGAGCAGT | 18 | 12014 |
| 4 × 4S | ATACGGCCAGGCATTGAA | 18 | 12351 |
| 5 × 4S | TGGTCCTCTGACTGCTCT | 18 | 11987 |
| 3 × 5S | CCTGGGACCCGTTGGTCG | 18 | 13286 |
| 5 × 5S | TCTTTGCTGCCGTGTTCC | 18 | 12975 |
| 3 × 6S | CCACCCTTAACCCCTCC | 17 | 13477 |
| 5 × 6S | TGGTTGCCCAGGGTCCCC | 18 | 13271 |
| 5 × 7S | CTCCCCTGCTTGCCACA | 17 | 13933 |
| 3 × 7S | TCAGCGGCAAGCAGAGG | 17 | 14172 |
| 3 × 8S | CATAACTGCACCCTTGG | 17 | 14638 |
| 5 × 8S | ATGGGACAGGTAGGACC | 17 | 14390 |
| 3 × 9S | GGAAACTTTCCACTTGA | 17 | 14794 |
| 5 × 9S | GGAGGAGACCAAGGGTGC | 18 | 14614 |
| 3 × 10S | CATGAAGGCAGGATGAG | 17 | 17766 |
| 5 × 10S | CCATCTTTTAACTCAGGT | 18 | 17512 |
| 3 × 11S | CAAGCAAGGGTTCAAAG | 17 | 18773 |
| 5 × 11S | AGACCCTCTCACTCATG | 17 | 18545 |

[1]All sequences are given in 5′ to 3′ direction; [2]numbers refer to Genbank accession # 54156 and are the starting points of primer annealing.

Inc., Arlington Heights, IL) in order to remove excess of primers and dNTPs from the reaction mixture. The sequencing reactions were performed using the ThermoSequenase fluorescent labeled primer cycle sequencing kit (Amersham) according to the manufacturer's instructions. Sequence analysis was performed with an automated laser fluorescence sequencing apparatus (A.L.F. Express DNA Sequencer; Pharmacia Biotech, Uppsala, Sweden). The sequencing primers used are shown in Table 2. All sequencing primers were labeled at their 5′-end with the fluorescent dye Cy5. All PCR and sequencing primers were designed using the computer software Oligo 5.0 (National Biosciences Inc., Plymouth, MN) according to the p53 sequence deposited in Genbank by Chumakov *et al.* (accession # 54156).

## Results

### p53 AUTOANTIBODIES IN SERA OF PATIENTS WITH BREAST CANCER

Presurgical serum samples from 195 patients with primary breast cancer were analyzed for the presence of p53 antibodies with an immunofluorometric method (31,32). Seventeen of the patients (9%) were positive (Table 3). Seventy-five patients had postsurgical serum samples, taken 6 months after surgery. Six of them were p53 antibody positive before surgery. In five of the six patients p53 antibody levels decreased after surgery. In one (patient 276), the antibody titer remained the same (Table 3). None of the 69 patients who were p53 antibody negative before surgery became positive after surgery.

In order to demonstrate that the analytes measured in serum of the cancer patients were antibody specific for p53, seven highly positive presurgical sera were spiked with increasing concentrations of

TABLE 3
Serum p53 Antibody Titers, Tumor p53 Protein Levels, and p53 Gene Mutational Status in 17 p53 Antibody-Positive Patients

| Patient Code | Antibody Titer[1] | | Tumor p53 Levels (U/g)[3] | p53 Mutational Status | | | |
|---|---|---|---|---|---|---|---|
| | Presurgical Serum | Postsurgical Serum | | Exon | Base Substitution | Codon | Amino Acid Substitution |
| 204 | 20 | ND[2] | 214 | 7 | C—T | 248 | Arg—Trp |
| 222 | 21 | ND | 13 | ND | | | |
| 228 | 2.9 | 1.5 | 5 | WT[4] | | | |
| 238 | 40 | ND | 4 | 5 | A—G | 163 | Tyr—Cys |
| 267 | 19 | 5.2 | < 3 | 6 | A—G | 220 | Tyr—Cys |
| 276 | 2.3 | 2.3 | < 3 | WT | | | |
| 286 | 13 | ND | < 3 | WT | | | |
| 298 | 2.1 | 1.7 | < 3 | WT | | | |
| 299 | 21 | ND | < 3 | WT | | | |
| 309 | 2.2 | 1.7 | 102 | 6 | A—G | 220 | Tyr—Cys |
| 323 | 5.5 | ND | 3 | WT | | | |
| 328 | 6 | ND | 40 | WT | | | |
| 335 | 2.9 | ND | 16 | WT | | | |
| 342 | 2.0 | ND | 11 | WT | | | |
| 351 | 29 | ND | < 3 | 4 | C—G | 107 | Tyr—stop codon |
| 363 | 8 | 4.2 | 7 | WT | | | |
| 381 | 3.2 | ND | < 3 | WT | | | |

[1]Ratio between the fluorescence signals obtained in the presence and absence (blank) of p53; [2]ND: the analysis was not done (postsurgical serum not available); [3]p53 ≥ 3 U/g define tumor as positive; [4]WT: wild-type.

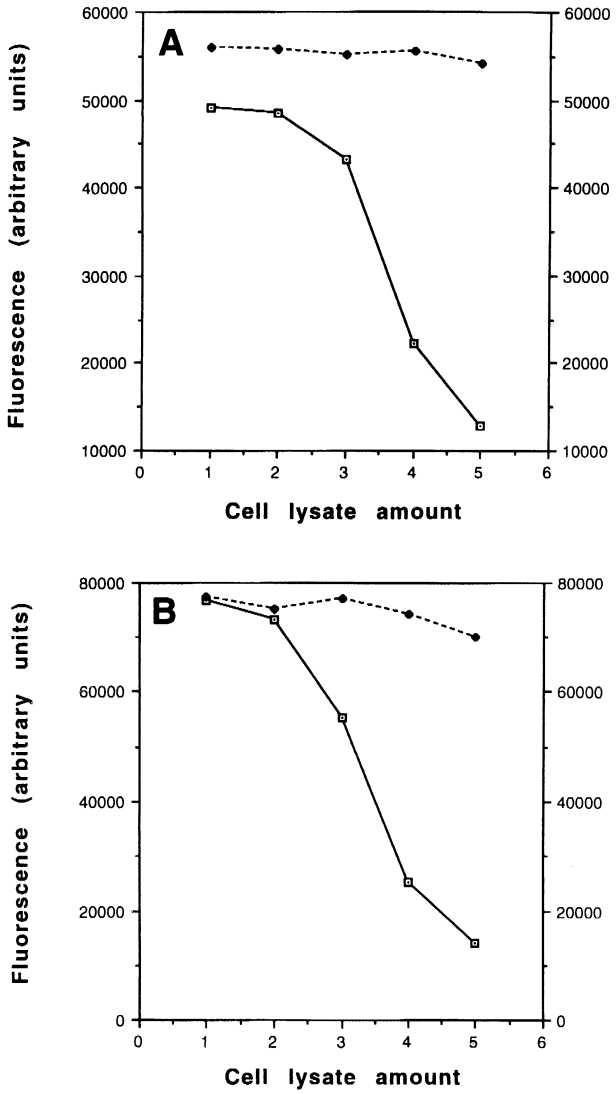


Figure 1 — Specificity of serum antibodies for p53 protein. Two p53 antibody-positive sera, (A) and (B), were spiked with increasing amounts of lysates from Sf9 cells expressing (solid lines) or not expressing (broken lines) p53 protein, and reanalyzed for the presence of p53 antibodies. Incubation of the two sera with increasing amounts of p53-containing lysates resulted in the capture of p53 protein by the serum antibodies, thus lowering their concentration in serum (measured as arbitrary units of fluorescence signal). Amount of lysate added: 1 = no lysate; 2 = $10^3$-fold-diluted lysate; 3 = $10^2$-fold diluted lysate; 4 = 10-fold-diluted lysate; 5 = undiluted lysate.

cell lysates from Sf9 cells previously infected or not infected with baculovirus carrying the p53 gene. After incubation, these sera were reanalyzed for p53 antibodies. Results for two samples encoded 238 and 267 (Table 3) are presented in Figure 1. Incubation of the sera with increasing concentrations of lysates containing p53 protein resulted in progressive reduction of the antibody titer, whereas incubation with lysates devoid of p53 protein resulted in no significant change of the antibody titer. These data demonstrate that the antibodies detected in human sera specifically react with p53 protein. The same conclusion was reached when Western blot analysis was performed. Membrane strips with protein extracts from infected or noninfected Sf9 cells were immunoblotted with patient sera positive for p53 antibodies. A 53-kDa band was recognized only in strips containing cell lysates from infected Sf9 cells (data not shown).

### Association between serum p53 antibodies and tumor p53 overexpression

Breast tumor extracts were analyzed with an immunofluorometric procedure for p53 protein. Using a cutoff level of 3 arbitrary units per gram of total protein (U/g), 77 tumors were positive for p53 (40%). The levels ranged from 3–419 U/g. Ten of the 17 p53 antibody-positive patients had tumors containing $\geq$ 3 U/g of p53 protein, whereas 7 had p53 $<$ 3 U/g. With chi-square analysis (Table 4A), no association was found between p53 protein overexpression and p53 antibody presence although there was a trend ($p = 0.09$). Comparison of the median tumor p53 concentration between the p53 antibody-positive and p53 antibody-negative groups by the Wilcoxon Rank Sums test (Table 4B), revealed that the p53 antibody-positive group had significantly higher p53 protein in their tumors ($p = 0.05$). Further, a possible correlation between tumor p53 protein levels and serum p53 antibody titers was examined in 17 p53 antibody-positive patients (Figure 2). No such correlation was found (Pearson correlation coefficient $r = 0.04$).

### Association between p53 antibodies and other clinicopathologic variables

The relationships between p53 autoantibodies and breast tumor clinicopathologic features are shown in Table 5. The presence of p53 antibodies was not associated with patient age ($p = 0.62$), disease stage ($p = 0.37$), tumor histologic type ($p = 0.83$), or involvement of axillary nodes ($p = 0.20$). p53 autoantibodies were significantly associated with larger tumor size and absence of steroid hormone receptors. Among the breast cancer patients with tumor size $<$ 2 cm, 4% had p53 antibodies, whereas among those with tumor $>$ 2 cm, 12% developed antibodies ($p = 0.04$). Eighteen percent of the estrogen receptor-negative patients were p53 antibody-positive, but only 3% of the estrogen receptor-positive patients were positive for p53 antibodies ($p < 0.01$). Similarly, 16% of the progesterone receptor negative patients were antibody positive, whereas only 3% of the progesterone receptor positive patients developed antibodies ($p < 0.01$).

### Association between serum p53 antibodies and p53 gene mutational status

In order to investigate whether the type of the mutation in the p53 gene was responsible for p53

TABLE 4
Associations Between p53 Antibodies (p53 Abs) in Serum and p53 Protein in the Tumor of Breast Cancer Patients

| | **Serum p53 Ab (+) (n = 17)** | **Serum p53 Ab (−) (n = 178)** | **Chi-square, *p*** |
|---|---|---|---|
| Tumor p53 (+) (n = 77) | 10 (13%) | 67 (87%) | 0.09 |
| Tumor p53 (−) (n = 118) | 7 (6%) | 111 (94%) | |

| | **Tumor p53 (U/g)** | | |
|---|---|---|---|
| | **Serum p53 Ab (+) (n = 17)** | **Serum p53 Ab (−) (n = 178)** | **Wilcoxon, *p*** |
| 0 | 0.5 | 0.7 | 0.05 |
| 25% | 2.3 | 1.5 | |
| 50% | 4.4 | 2.4 | |
| 75% | 12.8 | 4.2 | |
| 100% | 214 | 419 | |

protein immunogenicity, direct DNA sequencing of all 11 p53 exons was performed in DNA isolated from tumors of p53 antibody-positive and negative patients. Tumor DNA from 16 of the 17 p53 antibody-positive patients were analyzed; one tumor specimen was depleted. The same analysis was done with tumor DNA from 16 p53 antibody-negative patients who were preselected to have p53 protein overexpression in their tumors. The mutational status of the p53 gene for the p53 antibody-positive and -negative patients is presented in Tables 3 and 6, respectively. Of 16 p53 antibody positive patients, 5 exhibited a mutation in the p53 gene. One mutation was found in exon 4, 1 in exon 5, 2 in exon 6, and 1 in exon 7. Examples are shown in Figure 3. Four abnormalities were missense point mutations and represented transitions and one was a nonsense mutation. Three of the four point missense mutations resulted in substitution of Tyr to Cys, whereas the fourth changed an Arg to Trp. In all cases, both mutant and wild-type allele coexisted (Figure 3), suggesting patient heterozygosity, contamination of tumor with normal tissue, or tumor cell heterogeneity. For all but one case (patient 267) with a missense mutation, the presence of the mutation was associated with protein overexpression in the tumor. The tumor with the nonsense mutation was negative for p53 protein. For another two p53 antibody-positive patients (276 and 363), a polymorphism was identified within exon 6. For the remaining nine patients, no mutation was found. Four of these were

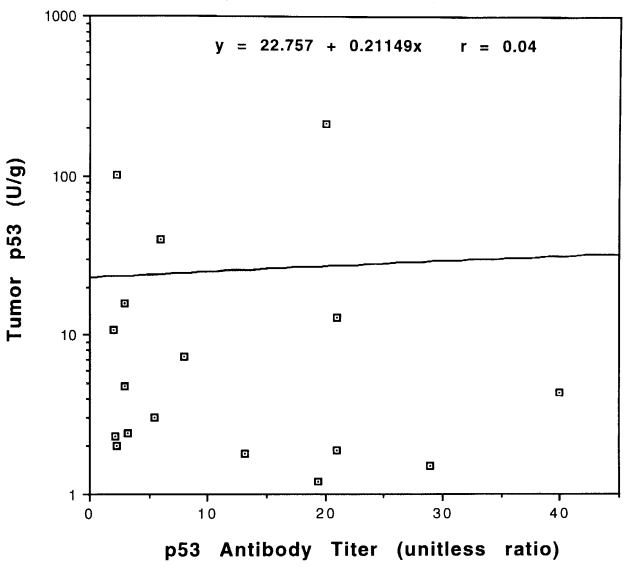


Figure 2 — Correlation between tumor p53 levels and serum p53 antibody titers in p53 antibody-positive breast cancer patients. $r$ = Pearson correlation coefficient. No correlation exists between these two parameters.

TABLE 5
Associations Between p53 Antibodies and Clinicopathological Features in Breast Cancer Patients

| Feature | Number of Patients (%) p53 Ab (−) | Number of Patients (%) p53 Ab (+) | *p* Value |
|---|---|---|---|
| Age (yr) | | | |
| < 50 | 52 (93%) | 4 (7%) | |
| ≥ 50 | 126 (91%) | 13 (9%) | 0.62 |
| Stage | | | |
| I–II | 161 (91%) | 17 (9%) | |
| III–IV | 17 (100%) | 0 (0%) | 0.37 |
| Tumor size (cm) | | | |
| < 2.0 | 78 (96%) | 3 (4%) | |
| ≥ 2.0 | 100 (88%) | 14 (12%) | 0.04 |
| Node | | | |
| Negative | 76 (88%) | 10 (12%) | |
| Positive | 90 (94%) | 6 (6%) | 0.20 |
| Histology | | | |
| Invasive ductal | 79 (91%) | 8 (9%) | |
| Others | 99 (92%) | 9 (8%) | 0.83 |
| ER[1] | | | |
| Negative | 59 (82%) | 13 (18%) | |
| Positive | 117 (97%) | 4 (3%) | <0.01 |
| PR[1] | | | |
| Negative | 76 (84%) | 14 (16%) | |
| Positive | 100 (97%) | 3 (3%) | <0.01 |

[1]Cutoff level is 10 fmol/mg.

TABLE 6
Tumor p53 Protein Levels and p53 Gene Mutational Status in p53 Antibody-negative Patients

| Patient Code | Tumor p53 Levels (U/g) | p53 Mutational Status | | | |
|---|---|---|---|---|---|
| | | Exon | Base Substitution | Codon | Amino acid Substitution |
| 209 | 35 | 8 | C—T | 283 | Arg—Cys |
| 211 | 8 | WT[1] | | | |
| 217 | 7 | WT | | | |
| 233 | 6 | WT | | | |
| 240 | 14 | 7 | G—A | 245 | Gly—Asp |
| 262 | 15 | WT | | | |
| 266 | 419 | 8 | G—C | 280 | Arg—Thr |
| 301 | 49 | 8 | C—T | 278 | Pro—Ser |
| 318 | 19 | WT | | | |
| 322 | 161 | WT | | | |
| 338 | 14 | WT | | | |
| 360 | 197 | 7 | G—C | 249 | Arg—Thr |
| 370 | 12 | 6 | A—G | 213 | Arg—Arg |
| 376 | 30 | 7 | G—A | 245 | Gly—Ser |
| 378 | 42 | 7 | deletion | | |
| 384 | 40 | 5 | A—G | 132 | Lys—Glu |

[1]WT: wild-type.

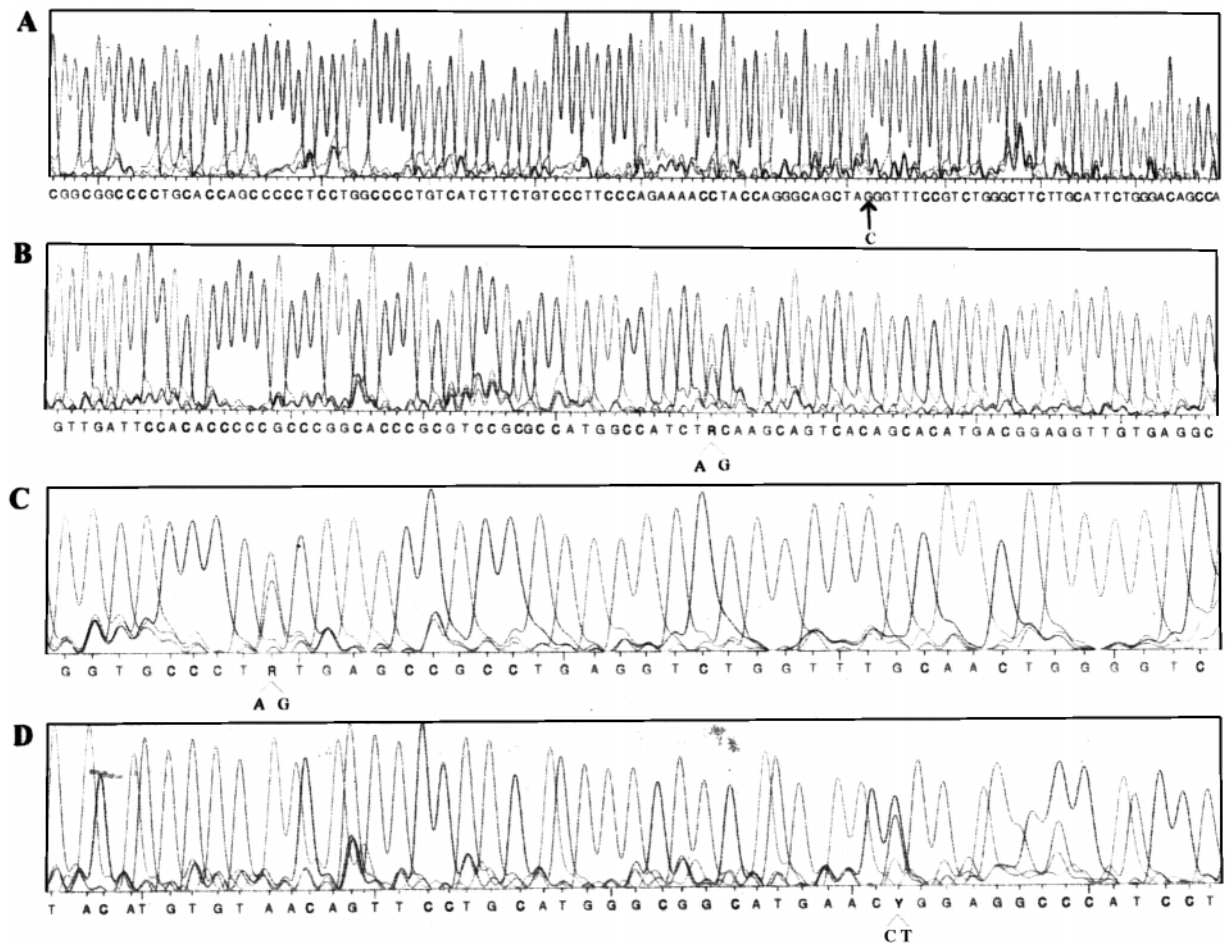


Figure 3 — DNA sequencing of the p53 gene from p53 antibody-positive patients. (A) C—G point nonsense (Tyr—stop codon) mutation in exon 4, patient 351; (B) A—G point missense (Tyr—Cys) mutation in exon 5, patient 238; (C) A—G point missense (Tyr—Cys) mutation in exon 6, patient 267; (D) C—T point missense (Arg—Trp) mutation in exon 7, patient 204. R = A or G; Y = C or T.

negative for p53 protein in the tumor, whereas the other five overexpressed p53 (Table 3).

Of the 16 p53 antibody-negative patients, 8 had an abnormal p53 gene (Table 6). Seven of the abnormalities were missense point mutations and the eighth was a deletion. One mutation was found in exon 5, 4 in exon 7, and 3 in exon 8. Five of the point mutations were transitions and two were transversions. In another 2 cases, a polymorphism was identified within exon 6. For the remaining 6 patients, no mutation was detected in any of the 11 p53 exons, despite the fact that the corresponding tumor expressed high levels of p53 protein.

## Discussion

p53 mutations and their implications in human tumorigenesis attracted much attention over the past 10 years. Every aspect concerning the p53 gene and its protein product has been thoroughly studied, in efforts aiming to clarify the role of this molecule in malignant transformation. Until recently, the discovery that some patients with cancer develop an immune reaction against p53 (7) did not attract much attention. p53 autoantibodies are developed in a proportion of cancer patients and they can be detected in serum. This led researchers to investigate the utility of these antibodies as markers for disease diagnosis or prognosis. Recently, it was suggested that p53 antibodies may represent a new and sensitive tool for detection of preneoplastic and microinvasive bronchial lesions in patients with high risk for developing lung cancer, for example, heavy smokers (25). It has also been proposed that serum p53 antibodies may facilitate the early diagnosis of cancer in a subset of individuals who are at high cancer risk, for example, patients with chronic obstructive pulmonary disease (33) or workers with occupational exposure to vinyl chloride (34). In addition, p53 autoantibodies were detected in sera of nonaffected individuals with family history of breast (35) and ovarian cancers (Angelopoulou *et al.*, unpublished data). Furthermore, it has been shown that these serum antibodies represent indicators of unfavorable prognosis in patients with breast (20, 21), colon (17), head and neck (22), and ovarian cancers (14,15).

Although much work has been done towards identifying the clinical value of p53 antibodies in serum, the molecular mechanisms that render this self protein immunogenic, leading to generation of autoantibodies in some patients still remain obscure. One hypothesis suggests that mutant p53 accumulation in the tumor is the major trigger for the development of the humoral immune response. Accumulation of mutant p53 in tumors may induce self-immunization, which would result in the development of these antibodies. However, many recent studies reported the presence of p53 antibodies in sera of patients with undetectable levels of p53 in their tumor (22,35), as well as presence of these antibodies in patients with wild-type p53 gene (12, 23). These reports suffer from the limitation of small number of patients studied. In the present work, we address this question with a group of 195 breast cancer patients.

Using immunofluorometric assays, we analyzed 195 breast cancer patients for the presence of p53 antibodies in serum and p53 protein in the corresponding tumor. Seventeen patients (9%) were p53 antibody-positive. Also, 78 patients (40%) had p53 accumulation in their tumor. p53 antibody-positive patients tended to have tumors overexpressing p53, but the association between the two parameters was of borderline significance by chi-square analysis ($p = 0.09$) and by the Wilcoxon test ($p = 0.05$). No correlation was identified between p53 protein levels in the tumor and p53 antibody titers in the serum.

The presence of p53 autoantibodies with absence of the antigen from the corresponding tumor has also been observed by others. There are a few possible explanations: (i) the immune system may be triggered by minute amounts of p53 protein; and (ii) another mechanism that is not related to protein accumulation but rather to an effective presentation of p53 to the immune system might be operating. Binding of wild-type p53 to cellular or viral proteins may elicit an immune response due to altered protein processing as shown by Dong and colleagues (36).

If p53 protein overexpression plays a role in p53 immunogenicity, it remains to be explained why some patients with high p53 levels do not develop p53 autoantibodies, as observed in the present study and by others (22,35). Antibody response might result from the loss of tolerance induced by accumulation of p53 protein in the tumor cells. This nuclear protein may be either released during tumor cell necrosis or translocated to the cell surface, where it can be detected by immunocompetent cells. The degree of tumor cell necrosis may play a role in triggering an immune response. The way that p53 is presented to the cell surface may also explain its immunogenicity. It has been suggested that p53 is presented to the immune system via HSP70 (18). However, not all p53 mutants can form complexes with, and thus be presented by, HSP70. Lastly, the status of the patient's immune system should not be neglected. In some cancer patients the immune system may have been suppressed, leading to p53 antibody levels too low to be detected.

Another factor related to the p53 antibody response may be the site of mutation within the p53 gene. It was postulated that certain types of gene mutations might lead to a conformational change that would render the protein product immunogenic. In the present study, unlike other investigations, all 11 p53 exons were searched for the presence of a mutation in order to identify possible abnormalities outside hot spot regions that would probably render p53 immunogenic. We found that 31% of p53 antibody-positive patients had a mutation in the p53 gene. This is in contrast to other reports that dem-

onstrated that the majority of these patients carry a mutation in the p53 gene. The abnormalities detected here were missense point mutations in exons 5, 6, and 7, and one nonsense mutation in exon 4. The identification of a nonsense mutation in a p53 antibody-positive patient contrasts with data by Winter and colleagues (8), who suggested that patients with stop, splice/stop, or frameshift mutations do not develop p53 autoantibodies.

Mutational analysis of a selected group of p53 antibody-negative patients who had p53 protein overexpression revealed that 50% of them had a mutant p53 allele. Accumulation of wild-type p53 is not unusual, especially in breast cancer (37,38). The mutations appeared to cluster in exons 7 and 8, with only one exception of a mutation identified in exon 5. These results are in accord with data from other investigators showing that p53 antibodies could not be detected in sera of patients whose tumors had a mutation in exon 8, despite the fact that these tumors expressed high levels of p53 antigen. The failure to develop an antibody response to exon 8 mutants could occur due to low immunogenicity of these mutants or due to a defect in antigen processing or presentation by tumor cells.

The proposal that as yet unidentified p53 gene mutations in regions outside the hot spot areas would be responsible for triggering the immune system is not supported by our data. All but one mutations in p53 antibody-positive patients were identified in core domains of the protein. p53 autoantibodies are directed against immunogenic epitopes that are present in both wild-type and mutant p53 proteins, in which no mutations are generally detected, namely the N- and C-terminals (11,19,27). It is not clear how core domain mutations elicit an autoimmune response. An explanation for this has been discussed (23,39), implying that the conformation of the p53 protein may cause differential proteolysis that could yield one or more peptides for major histocompatibility complex (MHC) presentation. Mutant-specific peptides would not necessarily be derived from the site of mutation, but simply reflect altered proteolytic cleavage due to altered protein structure.

Four of the five mutations found in the p53 gene of antibody-positive patients affected a Tyr residue in the protein product and three of these changed a Tyr to Cys. Both amino acids are known to play a role in the secondary structure of proteins through formation of hydrogen (Tyr) or disulfide (Cys) bonds. It is possible that the substitution of Tyr by Cys affects protein conformation in such a way so that cryptic immunogenic epitopes are exposed and trigger the immune response.

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