The p53 Gene in Breast Cancer: Prognostic Value of Complementary DNA Sequencing Versus Immunohistochemistry

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**Background:** Mutations in the p53 tumor suppressor gene (also known as TP53) have been detected in a wide variety of human cancers. In breast cancer, the presence of p53 gene alterations has been associated with worse prognosis. **Purpose:** We compared a complementary DNA (cDNA)-based sequencing method and an immunohistochemical (IHC) method for their abilities to detect p53 mutations in breast cancer specimens. In addition, we determined the prognostic value of information obtained when these two methods were used. **Methods:** Specimens from 316 primary breast tumors were evaluated for the presence of mutant p53 protein by use of the mouse monoclonal antibody Pab 1801 (that recognizes both wild-type and mutant forms of p53) and standard IHC methods. In addition, the entire coding region of p53 genes expressed in these tumors was screened for mutations by combining reverse transcription, the polymerase chain reaction, and DNA sequencing. Probabilities for overall survival (OS), breast cancer-corrected survival (BCCS; death from breast cancer is the considered event), and relapse-free survival (RFS) were estimated by use of the Kaplan–Meier method, and survival curves for different patient subgroups were compared by use of the logrank method. All reported *p* values are from two-sided tests. **Results:** Sixty-nine (22%) of 316 tumors had p53 gene mutations detected by the cDNA-based sequencing method; only 31 (45%) of these mutations were located in evolutionarily conserved portions of the p53 coding region. Sixty-four tumors (20% of the total) had elevated levels of p53 protein as detected by IHC, suggesting the presence of mutations. Of the sequencing-positive tumors (i.e., p53 mutant), 23 exhibited negative IHC reactions, indicating that IHC failed to detect 33% of the mutations. Furthermore, 19 of the IHC-positive tumors were sequencing negative (i.e., p53 wild-type), suggesting a 30% false-positive frequency with IHC. Four tumors (1.3% of the total) could not be analyzed by the cDNA-based sequencing method, and three tumors (1% of the total) could not be analyzed by IHC. The 5-year estimates for RFS, BCCS, and OS were significantly shorter for patients with p53 sequencing-positive tumors than for patients with sequencing-negative tumors (*P* = .001, *P* = .01, and *P* = .0003, respectively). Patients with IHC-positive tumors showed reduced survival in all three categories when compared with those with IHC-negative tumors, but the differences were not statistically significant. **Conclusions:** Use of a cDNA-based sequencing method to determine the status of the p53 gene in primary breast cancers yielded better prognostic information than IHC performed with the Pab 1801 monoclonal antibody. [J Natl Cancer Inst 1996;88:173-82]
cumulation of mutant p53 protein in cells due to conformational changes in the p53 polypeptide that result in increased stability (4,9). Wild-type p53 protein has a short half-life and is not usually detected by IHC. However, the accuracy of detecting p53 mutations by IHC has been questioned. A recent study (20) comparing five different anti-p53 antibodies revealed positive IHC staining in 18%-35% of the tumors examined if individual antibodies were used and in 45% of the tumors if a cocktail of two of the antibodies (i.e., antibody 240 and Pab 1801) was used. Even if the true mutation status of the p53 gene is not revealed by IHC, it has been suggested that this methodologic approach may provide information more reflective of the functional status of the p53 protein than that provided by molecular biological techniques such as DNA sequencing (21) and, therefore, might be more effective in defining patients with poor prognosis.

In this study, we compared the prognostic value of screening for p53 gene mutations in breast cancer specimens using IHC (employing the monoclonal antibody Pab 1801) with that of complementary DNA (cDNA)-based sequence analysis of the complete p53 coding region. The cDNA sequences used in this study were determined as part of an effort to evaluate p53 gene status in the primary tumors of 316 patients with breast cancer in relation to adjuvant therapy and prognosis (22).

Materials and Methods

Study Materials and Patient Population

Tumor material from 316 consecutive patients operated on for primary invasive breast cancer in Uppsala County (Sweden) from January 1, 1987, through December 31, 1989, was used in this study. The tumors were collected at the Department of Pathology, University of Uppsala, Akademiska sjukhuset, Sweden. This department contained the only laboratory for histopathology in Uppsala County during the study period. All breast cancer samples were sent directly, unfixed and fresh, from the operation theater to the Department of Pathology.

Patient age at diagnosis ranged from 28 to 94 years, with a median age of 63 years. Tumor sizes ranged from 2 to 130 mm, with a median size of 20 mm. Lymph node metastases were detected in 97 (31%) of the 316 patients.

Of the 316 patients, 111 were diagnosed via a mammography screening program initiated in 1988, which invited all women in Uppsala County aged 40-74 years to participate.

Patient records were reviewed with regard to relapse information and date of death without knowledge of the p53 status of the tumors. Details about primary adjuvant therapy, including radiotherapy, and relapse therapy were also recorded. All fatal outcomes were studied, and causes of death were divided into three categories: 1) death caused by breast cancer, 2) death with signs of active breast cancer, and 3) death from other causes without signs of relapse. These clinical data were analyzed together with prospectively recorded data on lymph node status and p53 data from IHC and cDNA sequencing.

Therapy and Clinical Follow-up

Locoregional therapy. Primary surgical therapy consisted of either sector resection or modified radical mastectomy; in both instances, this was combined with axillary lymph node dissection. Axillary lymph node status information is missing for 13 patients for whom nodal exploration was not performed because of high age or the presence of other serious concomitant disease.

Patients with lymph node-positive disease or tumors larger than 20 mm in diameter located in the medial or central area of the breast received locoregional radiotherapy, except for 19 women who did not receive such therapy because of high age or the presence of concomitant or metastatic disease. Patients who underwent sector resection were routinely given radiotherapy, except for those who participated in two randomized studies exploring the efficacy of postoperative radiotherapy. (One of these studies has been published (23).)

Systemic adjuvant therapy. Systemic adjuvant therapy was offered routinely to all patients with lymph node-positive disease. Premenopausal women received intravenous adjuvant polychemotherapy consisting primarily of six to nine courses of intravenous cyclophosphamide, methotrexate, and 5-fluorouracil. When radiotherapy was given concurrently, only cyclophosphamide was administered. Tamoxifen was given to postmenopausal women with lymph node-positive disease. Tamoxifen was also given to women with stage II tumors who were lymph node negative as part of a randomized trial. Individualized therapeutic strategies were used for patients with primary inoperable disease or metastatic disease.

Follow-up. All patients treated for breast cancer in Uppsala County were seen on a regular outpatient basis at increasing intervals of time each year for at least 5 years; after 5 years, they were seen on a yearly basis until 10 years of follow-up had been completed. A few patients were followed at institutions other than the University of Uppsala; they were referred back to the University of Uppsala (Department of Oncology) on relapse, since this institution has the only clinic for oncology in Uppsala County. The routine follow-up evaluation consisted of a clinical examination. Blood tests and x-ray procedures were performed when indicated. Women aged 40-74 years had mammography checkups through the screening program.

Handling of Tumor Material

Each freshly isolated tumor was divided into two equal portions by use of a disposable scalpel. Slices were taken from the outer, viable, cellular region of one half and frozen in isopentane for later DNA analysis and sequencing and for estrogen- and progesterone-receptor determinations. Corresponding slices from the other half were prepared for histopathologic examination. The part of the tumor that was frozen was stored at -70 °C until analysis.

Sequence-Based Analysis of p53 Status

RNA preparation and isolation. A 1.5-mL polypropylene microcentrifuge tube containing 300 μL of extraction solution (RNAzole, Cinna Biotech Inc, Houston, TX) was placed on wet ice. A section of frozen tumor specimen (5 x 2 x 2 mm) was placed on wet ice for 5 minutes to allow RNA to phase-separate from tissue and other cellular components. Subsequently, the tube was spun in a microcentrifuge for 10 minutes (at 14 000g), and 350 μL of the aqueous, upper phase was recovered and transferred to a new tube that contained 350 μL of isopropanol. After brief vortex mixing, the new tube was placed on wet ice for 30 minutes and then subjected to microcentrifugation at 14 000g for 20 minutes. The supernatant was discarded, and the pelletted RNA was washed twice with 70% ethanol, dried briefly, and finally dissolved in 50 μL of diethyl pyrocarbonate-treated water and 1 μL of RNA guard (25 U; Pharmacia Biotech AB, Uppsala, Sweden).

cDNA synthesis. The RNA samples were heat denatured at 90 °C for 3 minutes, followed by chilling on wet ice for 3 minutes. To prepare a cDNA reaction mixture containing 10 μL of Moloney murine leukemia virus reverse transcriptase (200 U; Pharmacia Biotech AB), 2.5 μL of RNA guard (62.5 U), 37.5 μL of 2× “cDNA mix” (90 mM Tris-HCl [pH 8.3], 138 mM KCl, 18 mM MgCl2, 30 mM DTT [dithiothreitol], 3.6 mM dCTP [deoxyctydilic triphosphate], 3.6 mM dATP [deoxyadenosine triphosphate], 3.6 mM dGTP [deoxyguanosine triphosphate], and 0.152 μM of pd[N]3 random primers [approximately 2.5 pmol of primers]) to yield a final volume of 75 μL. The cDNA reaction mixture was incubated at 37 °C for 1 hour, and the reaction products were heat-denatured at 90 °C for 3 minutes and stored at -20 °C.
Polymerase chain reaction (PCR). Five microfilters of 10x PCR II buffer (Perkin-Elmer AB, Sundbyberg, Sweden), 5 pmol each of the 5' PCR primer and the 3' PCR primer (one of them being biotinylated), 1.2 µL of 25 mM MgCl₂ (Perkin-Elmer AB), 28 µL of distilled H₂O, and 0.8 µL of Taq polymerase (4 U) (AmpliTaq, Perkin-Elmer AB) were mixed together in individual 0.2-mL PCR tubes (Perkin-Elmer AB). Five microfilters of a given cDNA preparation or negative control template was added to specified tubes, yielding total PCR reaction volumes of 50 µL. The reaction mixtures were incubated in a Perkin Elmer 9600 PCR machine programmed to carry out 38 temperature cycles with the following profile: 94 °C, 15 seconds; 58 °C, 30 seconds; and 72 °C, 45 seconds. A 5-minute incubation at 72 °C was performed at the conclusion of the thermocycling program, followed by incubation at 4 °C.

Primers. PCR and DNA sequencing primers were synthesized based on the cDNA sequence of p53 messenger RNA. PCR primers were prepared by Custom Design Oligonucleotides (Pharmacia Biotech AB). Four sets of primers were used to cover the complete protein coding region of the p53 cDNA.

PCR primers (B = biotin-labeled). Fragment 1: 5'-G'GAC CAC TTT CCT TGG ATT GGC-3' and 5'-GCA AAA CAT GTG GGC GGC A-3' (covers the entire sequence of exons 2, 3, and 4, plus parts of exons 1 and 5). Fragment 2: 5'-GTT GCC TCC TGG GCT TCT GGC A-3' and 5'-GGT ACA GTC AGA GCC AAC C-3' (covers the entire sequence of exons 5 and 6 plus parts of exons 4 and 7). Fragment 3: 5'-TGG CCC CTC CTC AGC ATC TTA-3' and 5'-GCAA GGC CTT GCT C-3' (covers the entire sequence of exons 8, 9, 10, and 11 plus a part of exon 8).

DNA sequencing primers (fluorescein-labeled). Fragment 1: 5'-G'GAG GTC TGG AGT GCA-3' and 5'-GGT TGC TCG CGC A-3'. Fragment 2: 5'-GGC AGC ATC TTA-3' and 5'-GGT ACA GTC AGA GCC AAC C-3'. Fragment 3: 5'-TGG CCC CTC CTC AGC ATC TTA-3'. Fragment 4: 5'-GGG GAG CTC CAC CAC GAG CTG-3' (covers the entire sequence of exons 8, 9, 10, and 11 plus a part of exon 8).

Agarose gel electrophoresis and quality control. To control for contamination of PCR products that might have originated during the steps preceding DNA sequencing, we included negative control samples for both the RNA isolation and the cDNA preparation steps. The negative controls consisted of adding RNA to RNA extraction tubes and not adding RNA to cDNA-reaction mixtures. The presence of amplified DNA in negative-control sample tubes was taken as an indication that all samples might be contaminated and that the corresponding batch of samples had to be discarded.

The purity, quality, and quantity of amplified DNA from specimens and from controls were evaluated by subjecting 5-µL aliquots of the relevant PCR products to electrophoresis in 1% agarose gels containing 5 µg/mL ethidium bromide. The 100-base-pair ladder (0.2 µg; Pharmacia Biotech AB) was used in the gels as a reference standard.

Solid-phase DNA sequencing on combs. Sequencing reactions were performed as described by Lagerkvist et al. (24) with the use of streptavidin-coupled Sepharose HP (AutoLoad, Pharmacia Biotech AB) attached to the teeth of plastic combs (solid-phase sequencing combs). Forty microfilters of a PCR product was transferred to a "4-teeth well" containing 80 µL of capture buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, and 2.0 mM NaCl). The liquid was carefully mixed by pipetting (avoiding the generation of air bubbles), and a solid-phase sequencing comb was immersed in the resulting mixture. PCR products were captured by the comb during an incubation that lasted at least 60 minutes at room temperature. This incubation was interrupted only by occasional raising and dipping of the comb to improve PCR product capture (24).

The comb was then moved to a second "4-teeth well" containing 100 µL of 0.1 M NaOH and incubated at room temperature for 5 minutes. Subsequently, the comb was washed once with 0.1 M NaOH, once with TE buffer (10 mM Tris-HCl [pH 7.5] and 1 mM EDTA), and once with ultra-pure distilled H₂O. One hundred four microfilters of distilled H₂O, 12 µL of 10x concentrated annealing buffer (AutoRead kit, Pharmacia Biotech AB), and 4 µL of a 1 pmol/µL stock solution of fluorescein-labeled sequencing primer were mixed in a third "4-teeth well," followed by insertion of the comb. This annealing mixture, with the comb immersed in it, was heated at 55 °C for 5 minutes and then placed at room temperature for at least 10 more minutes.

Twenty microfilters of sequencing reaction mixture (containing 2 µL of 10x concentrated annealing buffer, 1 µL of extension buffer [AutoRead kit, Pharmacia Biotech AB], 4 µL of dd/dNTP mixture, 12 µL of distilled H₂O, and 1 µL of T7 DNA polymerase [2 U] (diluted in enzyme dilution buffer; AutoRead kit, Pharmacia Biotech AB)) were dispensed into individual "1-tooth wells" just prior to insertion of the comb. The comb was incubated in the wells for 5 minutes at 37 °C, and the entire well assembly (with the comb still inserted) was placed finally on wet ice.

The sample wells of an automated laser fluorescence (ALF; Pharmacia Biotech AB)-sequencing gel (containing 6% polyacrylamide and 7 M urea) were rinsed with 1 x TBE buffer (100 mM Tris, 90 mM boric acid, and 1 mM EDTA [pH 8.3]), prewarmed to 45 °C, and loaded with 15 µL of 100% formamide STOP solution (AutoRead kit, Pharmacia Biotech AB). The comb was removed from the sequencing-reaction wells and inserted into the wells of the ALF-sequencing gel for 10 minutes. The comb was then carefully removed from the gel apparatus, and ALF electrophoresis was initiated.

Sequencing evaluation and verification. Evaluation of p53 sequences was performed with the aid of a newly developed software program, Sequence Evaluator (Pharmacia Biotech AB), version 0.16, which compares the wild-type sequence of a gene with sequences obtained from sample analysis.

All mutations were confirmed by reamplifying the relevant cDNAs and sequencing the new PCR products. If a mutation was identified in a region contained within the overlapping portion of two cDNA segments, its existence was further verified by analyzing amplification products from the neighboring cDNA segment.

Immunohistochemical Analysis of p53 Status

Paraffin-embedded tumor sections on silane-coated slides (Eire Scientific Co., Porthmouth, Philadelphia, PA) were dewaxed in xylene and rehydrated in ethanol and distilled water. Pretreatment in a microwave oven at 750 W (three times for 5 minutes each) enhanced p53 antigen accessibility to antibodies. The mouse monoclonal antibody ci 1801 (12) (Biozol, Järfalla, Sweden), which recognizes both wild-type and mutant forms of p53, was used at a dilution of 1:10.

Immunostaining was performed in a Ventana ES Automated Immunohistochemistry Instrument (Annen, Helsinki, Finland). We used the manufacturer's diaminobenzidine (DAB) detection kit that includes biotin-labeled secondary antibodies directed against mouse immunoglobulins, avidin-labeled horseradish peroxidase, and DAB as the localization reagents. Negative control IHC reactions were performed by omitting the primary antibody.

Positive p53 IHC staining is seen in the nucleus of cancer cells. In our primary IHC analysis, positive p53 staining in any percentage of cancer cells was considered to be positive.

In a secondary IHC analysis, IHC-positive samples were subclassified with regard to immunostaining intensity and extent according to graded scales that ranged from 1 to 3. For intensity of staining, 1 represented weakly positive tumor cells and 3 represented strongly positive cells. For extent of staining, 1 denoted samples in which less than one third of the tumor cells had positive staining; 2 signified those with positive staining in one third to two thirds of the tumor cells, and 3 denoted those with more than two thirds positive staining. The results obtained with the two scales were multiplied against each other, yielding a single scale with steps of 1, 2, 3, 4, 5, 6, and 9, where 1 and 2 were considered to be low staining, 3 and 4 were considered to be medium staining, and 5, 6, and 9 were considered to be high staining. This classification system is derived from that described by Busch et al. (25). All slides were viewed and judged independently by two pathologists (A. Lindgren and H. Nordgren) according to this multiplied scale at different times without knowledge of clinicopathological findings and p53 mutation status.

Statistical Methods

Survival probabilities for overall, breast cancer-corrected, and relapse-free survival were estimated by use of the Kaplan-Meier method, and the equality of survival curves for different subgroups was evaluated by use of the logrank method. All P values are estimated from two-sided statistical tests. Relative hazards of dying of breast cancer were estimated by use of Cox's proportional hazards models. In the multivariate models, age at diagnosis, tumor size, estrogen- and progesterone-receptor status, and S phase proportion were taken into consideration. Hormone-receptor status was dichotomized as negative versus positive variables (cutoff point, 0.1 fmol/mg DNA) and S phase as high versus low variables (cutoff points, 7% for diploid and 12% for aneuploid tumors, respectively). In breast cancer-corrected survival, death from breast cancer was considered to be the event of interest; all other deaths were treated as censoring.
Results

Clinical Outcome

Of the 316 patients included in the study, 48 died of breast cancer, five died with breast cancer present, and 21 died of unrelated causes. For seven patients, we lack information about the cause of death. The median follow-up in this study was 57 months, with a maximum follow-up of 87 months.

Mutations Detected and IHC Results

Alterations in the p53 gene were detected by means of the cDNA-based sequencing method in tumors from 69 (22%) of the 316 patients. p53 mutations were found throughout the entire protein coding region of the gene. Twenty-nine p53 mutations were detected in patients whose tumors had metastasized to axillary lymph nodes, 37 mutations were found in node-negative patients, and three mutations were detected in patients with unknown primary lymph node status. We identified 45 missense (simple point) mutations, seven nonsense mutations (creating premature stop codons), five in-frame deletions, eight out-of-frame deletions, one in-frame insertion, and three out-of-frame insertions (Table 1; Fig. 1). Thirty-one (45%) of the 69 mutations were located in evolutionarily conserved regions of p53 (Fig. 1). From four tumors (1.3% of the total), we were unable to obtain sequence information; from three others (1% of the total), we lack immunohistochemical data (Table 2). Thus, tumors from 309 patients were available for this comparative study. Since follow-up data were missing for one patient, 308 patients could be included in survival analyses.

Positive IHC (suggesting p53 mutation) was demonstrated in tumors from 64 (20%) of the 316 patients. Twenty-three (33%) of the sequence-positive patients (i.e., proven to have p53 mutations) were negative by IHC, whereas 19 (30%) of the IHC-positive patients were sequence negative (Table 2). Negative immunohistochemical reactions were noticed in all six tumors with mutations that created premature stop codons and in 11 (85%) of 13 tumors with deletions (Table 1; Fig. 1). Positive immunohistochemical reactions were seen in 40 (89%) of 45 tumors with point mutations (Table 1; Fig. 1).

Our primary method of IHC classification (see "Materials and Methods" section) was used to generate the IHC data described above. There was complete concordance between the two pathologists regarding the assessment of negative and positive immunohistochemical reactions with this method.

When the 64 immunohistochemically positive tumors were subclassified according to the 6-graded (1-9) scale (i.e., our secondary IHC analytical method; see "Materials and Methods" section), the pathologists agreed in 75% of the cases. With this more complex grading system, 12.5% of the tumors fell into different low-, medium-, and high-staining groups as judged by the two independent investigators, whereas assessments regarding low-staining versus medium- to high-staining were divergent in only 7.8% of cases. Within the low-staining group, however, there was a major discrepancy regarding subclasses 1 and 2, and there was only 53.8% agreement with respect to the proposed class-1 tumors.

Comparison of Survival Data

We analyzed the patient data with regard to survival and p53 mutation status as determined by IHC and by cDNA-based sequencing. Survival was illustrated as relapse-free survival (RFS; 304 patients), breast cancer-corrected survival (BCCS; 308 patients), and overall survival (OS; 308 patients).

IHC and survival. According to IHC, there was a trend of reduced OS for patients with p53-positive tumors compared with those having p53-negative tumors, but it did not reach statistical significance (P = .2) (Table 3). No statistically significant differences could be detected in any of the survival parameters between p53-positive and p53-negative tumors according to IHC (Table 3).

cDNA sequencing and survival. Highly significant differences in survival were seen between patients with sequencing-positive and sequencing-negative tumors, with worse prognosis for those with positive tumors. The 5-year OS frequency was 78% in the mutation-negative group as opposed to 55% for those with p53 mutations (P = .0003). Similar statistically significant differences were seen for RFS and BCCS (Table 3).

Positive IHC with or without positive cDNA sequencing. In 61 women with positive IHC, we saw statistically significant differences in RFS between those with sequencing-positive tumors and those with sequencing-negative tumors (Fig. 2, top panel). The 5-year RFS in this IHC-positive patient population was 86% for the sequencing-negative group and 56% for the sequencing-positive group (P = .02; Fig. 2, top panel). Differences in BCCS between sequencing-positive and sequencing-negative patients with positive IHC were also statistically significant (62 patients evaluated, data not shown). None of 19 patients with IHC-positive tumors but negative-sequencing results had died of

Table 1. Different types of p53 mutations detected by complementary DNA sequencing analysis and corresponding anti-p53 IHC analysis*

<table>
<thead>
<tr>
<th></th>
<th>Point mutations</th>
<th>Deletions</th>
<th>Insertions</th>
<th>Premature stop codons</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In frame</td>
<td>Out of frame</td>
<td>In frame</td>
<td>Out of frame</td>
<td></td>
</tr>
<tr>
<td>IHC +</td>
<td>40</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>44</td>
</tr>
<tr>
<td>IHC -</td>
<td>5</td>
<td>3</td>
<td>8</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
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<tr>
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<td>45</td>
<td>5</td>
<td>8</td>
<td>7</td>
<td>69</td>
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</table>

*IHC = immunohistochemistry.
Point Mutations

Deletions, Insertions and Stop codons

Fig. 1. Detection of mutant p53 by immunohistochemistry (IHC). Ability to detect p53 coding region point (missense) mutations (upper panel), deletions, insertions, and premature stop codons (lower panel) with the monoclonal antibody Pab 1801. Codon positions are outlined below the X axis. The numbers (n) of individual p53 alterations are given on the Y axis. The gray-shaded areas indicate evolutionarily conserved regions I-V [see (22) and references contained therein]. p53 alterations identified by complementary DNA sequencing but not by IHC are indicated by filled black bars, whereas those identified by both methods are indicated by unfilled bars; alterations with unknown IHC status are indicated by bars with alternating black and white filling.

breast cancer (BCCS) at 60 months' follow-up, whereas 10 of the 43 patients with positive-sequencing results had died at 60 months of follow-up (P = .03). There was a trend in the same direction for OS, but it was not statistically significant (Fig. 3, top panel).

Negative IHC with or without positive cDNA sequencing. In the 246 evaluable patients with negative IHC, there were statistically significant differences in all three survival categories, including RFS (243 patients; Fig. 2, bottom panel), between the sequencing-negative and sequencing-positive cases, with worse prognosis for patients with positive sequencing results. The most marked difference was seen in the OS comparison, where the 5-year survival frequency was 78% in the sequencing-negative group as opposed to 48% in the sequencing-positive group (P = .002; Fig. 3, bottom panel).

Positive cDNA sequencing with or without positive IHC. For the women with sequence-determined p53 mutations, there were no significant differences in any of the survival categories between the IHC-positive and the IHC-negative groups (Fig. 2, left panel; Fig. 3, left panel).

Negative cDNA sequencing with or without positive IHC. For the cDNA-based sequencing-negative patients, no significant differences in survival were observed between the IHC-positive and the IHC-negative groups. There was even a trend

Table 2. No. of tumors positive and negative for p53 mutation according to IHC- and complementary DNA sequence-based determinations*

<table>
<thead>
<tr>
<th>Sequence-based determination of p53 status</th>
<th>Mut</th>
<th>Wt</th>
<th>Unknown</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC p53 detection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>44</td>
<td>19</td>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>Negative</td>
<td>23</td>
<td>223</td>
<td>3</td>
<td>249</td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>69</td>
<td>243</td>
<td>4</td>
<td>316</td>
</tr>
</tbody>
</table>

*IHC = immunohistochemistry; Mut = mutation-positive tumors; Wt = wild-type p53.
Table 3. 5-year survival in relation to p53 mutation status detected by cDNA sequencing and immunohistochemistry (IHC), respectively*

<table>
<thead>
<tr>
<th>p53 status</th>
<th>cDNA sequencing</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RFS, % (n)</td>
<td>BCCS, % (n)</td>
</tr>
<tr>
<td>Positive</td>
<td>54 (66)</td>
<td>69 (66)</td>
</tr>
<tr>
<td>Negative</td>
<td>71 (238)</td>
<td>86 (242)</td>
</tr>
<tr>
<td>P</td>
<td>.001</td>
<td>.01</td>
</tr>
</tbody>
</table>

*RFS = relapse-free survival; BCCS = breast cancer-corrected survival; OS = overall survival; n = No. of patients; positive = mutation positive by sequencing or positive staining by IHC; negative = wild-type p53 or negative staining by IHC; cDNA = complementary DNA. All P values according to the logrank test.

for better survival in the group with positive IHC reactions (Fig. 2, right panel; Fig. 3, right panel).

Survival Analysis When IHC Low-Staining Tumors Were Classified as p53 Negative

In our primary IHC analysis of p53 status and survival, which did not indicate any statistically significant differences in survival between IHC-positive and IHC-negative groups, all 64 tumors with positive IHC staining were considered to be p53 positive (i.e., mutant). We then reanalyzed the data obtained from each of the pathologists, considering the IHC-positive subclasses 1-2 (i.e., the “low-staining group” in the more complex grading system, see “Materials and Methods” section) as negative cases. In comparison with our primary classification, this secondary classification improved all P values for differences in RFS, BCCS, and OS between IHC-positive and IHC-negative groups.

Fig. 2. Relapse-free survival for patients with breast cancer categorized according to p53 complementary DNA sequencing and immunohistochemistry (IHC) data. The four diagrams (top, left, bottom, or right) are based on a 2 x 2 design. Survival curves were generated according to the Kaplan–Meier method; statistical comparisons were made by use of the logrank method. w.t. = wild-type; mut = mutant; neg = negative; pos = positive.
tumors. The best $P$ values were seen for OS and, according to data from one of the pathologists, the difference in OS became statistically significant (Table 4). The $P$ values obtained from the other pathologist’s review were also improved relative to those from the primary analysis, but none of the values reached statistical significance. However, it is important to note that with this modification in IHC classification the number of positive cases was reduced to 36 and 39, as judged by the two pathologists, respectively (Table 4).

**Proportional Hazards Models**

Proportional hazards models were tested to investigate whether prognostic information generated by cDNA sequencing and IHC was positively or negatively confounded by other commonly used prognostic markers. The relative hazards for p53 alterations were of the same magnitude in both the univariate and multivariate models (Table 5). The relative hazards for positive cDNA sequencing data were higher than those for positive IHC data, with confidence intervals indicating an independent effect; the confidence intervals for the immunohistochemical data clearly included 1.0.

### Table 4. Overall survival at 5 years in relation to p53 status determined by cDNA sequencing, IHC (primary analysis*), and IHC modified by subclassification (secondary analysis†)

<table>
<thead>
<tr>
<th>Method</th>
<th>Positive</th>
<th>Negative</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequencing</td>
<td>55 (66)</td>
<td>78 (242)</td>
<td>.0003</td>
</tr>
<tr>
<td>IHC</td>
<td>65 (62)</td>
<td>75 (246)</td>
<td>.2</td>
</tr>
<tr>
<td>IHC 1</td>
<td>52 (36)</td>
<td>76 (272)</td>
<td>.01</td>
</tr>
<tr>
<td>IHC 2</td>
<td>59 (39)</td>
<td>75 (269)</td>
<td>.05</td>
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</table>

*See “Materials and Methods” section for details.
†IHC = immunohistochemistry; cDNA = complementary DNA; sequencing = cDNA sequencing; p53 status positive = mutation by sequencing or positive IHC; p53 status negative = wild-type p53 by sequencing or negative IHC; IHC 1 = IHC results by pathologist 1 (A. Lindgren) after modification with subclasses 1 and 2 considered to be negative IHC; IHC 2 = IHC results by pathologist 2 (H. Nordgren) after modification with subclasses 1 and 2 considered to be negative IHC. All $P$ values according to the logrank test.
Table 5. Results from Cox’s proportional hazards models*

<table>
<thead>
<tr>
<th>Factor</th>
<th>Univariate</th>
<th>Multivariate 1</th>
<th>Multivariate 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 mut versus wt</td>
<td>2.1 (1.1-3.8)</td>
<td>1.9 (1.0-3.7)</td>
<td>—</td>
</tr>
<tr>
<td>p53 IHC + versus IHC −</td>
<td>1.2 (0.9-1.5)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Tumor size</td>
<td>—</td>
<td>1.0 (0.99-1.02)</td>
<td>1.0 (0.99-1.02)</td>
</tr>
<tr>
<td>Node + versus node −</td>
<td>—</td>
<td>5.0 (2.6-9.9)</td>
<td>5.2 (2.6-10.1)</td>
</tr>
<tr>
<td>ER + versus ER −</td>
<td>—</td>
<td>1.0 (1.0-1.04)</td>
<td>1.0 (1.0-1.04)</td>
</tr>
<tr>
<td>PR + versus PR −</td>
<td>—</td>
<td>1.0 (0.93-1.01)</td>
<td>1.0 (0.94-1.01)</td>
</tr>
<tr>
<td>S phase, high versus low</td>
<td>—</td>
<td>1.5 (0.7-2.9)</td>
<td>1.5 (0.7-3.0)</td>
</tr>
</tbody>
</table>

*Mut = mutation; wt = wild-type; IHC = immunohistochemistry; + = positive; − = negative; node = axillary lymph nodes; ER = estrogen receptor; PR = progesterone receptor. Estimates of relative hazards with 95% confidence intervals for breast cancer-corrected survival. Multivariate model 1 estimates the effect of p53 mutation determined by complementary DNA sequencing; model 2 estimates the relative hazard for IHC data.

Discussion

This study differs from most other studies of p53 status and cancer in that the patient population, consisting of 316 women with primary breast cancer, was derived from a population-based cohort. The breast cancer specimens were examined by IHC with use of the monoclonal antibody Pab 1801, an antibody whose use on paraffin-embedded tumor samples is now widely accepted (7,16,26-29). The specimens were also analyzed by use of a cDNA-sequencing strategy in which all exons of the p53 gene were evaluated. Our cDNA sequencing approach stands in contrast with previous investigations of p53 gene alterations in human breast cancer, which have focused primarily on sequence changes in exons 5, 6, 7, and 8.

We observed that 23 breast cancers with p53 alterations detected by cDNA-based sequencing failed to generate positive IHC reactions with Pab 1801; 19 tumors were negative for mutation by cDNA sequencing but were positive by IHC. The lack of concordance between results obtained with these two methods may indicate that they measure different aspects of p53. An ideal method should be one that gives the best prognostic information in relation to currently used therapeutic approaches and the best delineation of the patient groups studied. Our data clearly demonstrate that the cDNA sequencing method is superior in these regards. Our finding should be of importance, since most published p53 status determinations have been based on IHC analyses. In view of the discrepancy between results obtained with the two methods, we will now discuss possible explanations for false-positive and false-negative results with each method.

The prognostic information generated by IHC- and cDNA sequence-based determinations of p53 status gave us reason to believe that IHC might generate false-positive as well as false-negative results. This was suggested by the observation that the patient group with negative cDNA sequencing data (i.e., their tumors had wild-type p53 genes) but positive IHC results did not seem to have a significantly worse prognosis than the corresponding IHC-negative group. The suspicion of false-positive IHC results is supported when considering the IHC-positive group, where comparison of the sequencing-positive and sequencing-negative patients showed significantly better survival for the patients with negative sequencing data. There are also signs of false-negative cases of IHC in the IHC-negative patient group, where significantly worse prognosis was observed for patients with positive cDNA sequencing data compared with those with negative sequencing data. Similarly, in the cDNA sequencing-positive group, no difference in prognosis was seen between the IHC-positive and the IHC-negative patients, supporting our conclusions.

False-negative IHC results may be generated as a consequence of premature stop codons and gross deletions in the p53 gene, since such alterations could lead to a cessation of protein synthesis and render the detection of mutations by IHC impossible. Our results are consistent with this hypothesis, since all six samples with premature stop codons showed negative IHC reactions. Similarly, 11 of 13 tumors with deletions and one of three tumors with insertions were negative by IHC. Thus, 18 of the 23 evaluable samples with aberrations detected by cDNA sequencing but IHC negative had deletions, insertions, and premature stop codons. In contrast, only four of 44 samples with positive IHC had mutations of these types (P<0.001).

Another possible explanation for negative IHC results in sequencing-positive tumors may be that the genetic alterations caused changes in or disappearance of the epitope recognized by Pab 1801, which is located between amino acids 40 and 65 (30). However, few of the alterations that we identified are located in this region. Furthermore, most of the p53 proteins truncated as a consequence of premature stop codons should still contain the Pab 1801 epitope. It has been suggested that missense mutations (19,31), as well as deletions, insertions (32), and premature stop codons (33), might produce conformational changes in the p53 polypeptide that interfere with recognition of the epitope for Pab 1801. In addition, Ohuc et al. (33) have proposed that truncation of the carboxyl terminus of p53 might reduce the stability of the mutated protein because of the loss of several important functional domains, such as the DNA binding domain, the nuclear localization signals, and the oligomerization domain. The accumulation of p53 protein would thus fail to occur, making detection by IHC impossible. In this study, all deletions and stop codon mutations identified were located downstream of codons that define the Pab 1801 epitope, supporting these theories. It is also possible that certain point mutations may not be able to stabilize the p53 protein sufficiently to be detectable by IHC (21).

The theoretical basis for the determination of p53 status by IHC is that mutant p53 protein exhibits a longer half-life than wild-type p53, which results in the accumulation of p53 protein in transformed cells (4,19). However, it is possible that the accumulation of p53 in tumor cells may, in some cases, indicate the existence of a regulatory defect rather than mutations in the protein-coding sequence of the gene. Several investigators (34,35) have found discrepancies between p53 protein expression and mutation status. In some cases, weak immunostaining could also represent normal cell cycle fluctuations in p53 protein levels, as indicated in a few reports (36,37). In our study, 13 of 19 tumors that were positive for p53 by IHC and had wild-type p53 cDNA sequences displayed weakly positive IHC signals only. For the 243 patients with negative cDNA sequencing data, we observed a nonsignificant trend of better survival for
those with IHC-positive tumors relative to those with IHC-negative tumors. One may speculate that the improved survival could be due, in part, to increased amounts of normal p53 protein, which might facilitate apoptosis induced by tamoxifen therapy, chemotherapy, or radiotherapy.

Can IHC analysis be refined to increase its resolving power? It has been shown that strong immunostaining is associated with the presence of p53 gene alterations detected by molecular biological methods to a greater extent than is low-grade IHC staining (38). It has also been demonstrated that IHC with Pab 1801 generates better prognostic information when considered as a continuous variable than as a dichotomous variable (8).

With these thoughts in mind, we made an additional analysis in which the IHC low-staining group (i.e., subclasses 1 and 2) were considered to be negative. This approach improved the significance of the P values obtained in all three measured survival categories. One of the P values, on the basis of one pathologist's data, became statistically significant (OS, IHC positive versus IHC negative), whereas in our primary analysis (where all positive reactions were considered as p53-positive cases) none of the P values were significant. This result may indicate that the diagnostic specificity of IHC is impaired by technical limitations and/or more fundamental biological properties of the p53 regulatory system. To achieve a similar level of significance as that obtained with the use of sequencing data, the number of positive cases was substantially reduced, thus lowering the sensitivity of the method and increasing the risk of false-negative cases.

Having analyzed the IHC data with regard to false-negative and false-positive results, we will now discuss the sequence-based analysis of one case study. False-positive sequencing results may occur as a consequence of contamination of samples during processing. In this study, we identified p53 gene mutations in 69 cases. Most of these alterations were found in 49 different codons resulting in 55 different mutations. Seven of the 14 remaining mutations were located in mutational “hot spots” reported by others (39). Given this diversity, we have no reason to suspect false-positive sequencing data in our study. This conclusion is further strengthened by the finding that none of the negative cDNA/PCR controls yielded any amplification products, indicating that the integrity of the tumor isolates was maintained.

False-negative sequencing reactions may, on the other hand, represent a greater risk. Theoretically, this could happen if the tumor samples used for the analysis contained relatively few malignant cells in relation to normal cells, which could cause the wild-type sequence to “drown out” the mutant sequence. The manner in which the tumor material was isolated in this study should have minimized such a risk. cDNA sequencing might also fail to detect a mutation if the alteration is located in a position disadvantageous for proper primer (cDNA or PCR) binding. This risk would be greatest at the extreme ends of the p53 coding region with our approach.

Taken together, our data indicate that direct cDNA sequence-based analysis of p53 status is superior to IHC in determining prognosis in breast cancer. If complete sequencing of the p53 coding region by use of the present method is taken as the gold standard, false-positive as well as false-negative results can occur with IHC. In terms of using p53 status in clinical decision making with regard to adjuvant therapy, both false positives and false negatives would pose problems.

References


Notes

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