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# IMMUNOFLUOROMETRICALLY DETERMINED p53 ACCUMULATION AS A PROGNOSTIC INDICATOR IN ITALIAN BREAST CANCER PATIENTS

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**The prognostic value of p53 protein accumulation in breast cancer, especially as detected by methods other than immunohistochemistry, has not been established unequivocally. A sensitive immunofluorometric assay of p53 protein employing DO-1 and CM-1 antibodies was used in this study to assay extracts of 171 breast carcinomas from northern Italy. p53 over-expression, demonstrated in 36 (21%) tumours, was associated with lack of oestrogen receptor (ER) expression but was not related to patient age, stage, lymph node status, tumour size, histologic type, grade or progesterone receptor (PR) expression status in contingency tables. An increased risk for cancer relapse of p53-positive patients compared to p53-negative patients was determined using multivariate Cox regression analysis, which also showed that p53 protein over-expression was an independent predictor of reduced disease-free survival in node-positive and $ER^+$ patients but not in node-negative or $ER^-$ individuals. The equivalent analysis for assessing the impact of p53 status on overall survival was not statistically significant, possibly reflecting the short patient follow-up. Our results suggest that an immunoassay of p53 protein, applicable to cytosolic extracts prepared for steroid hormone receptor analyses, may provide information for breast cancer prognosis.** ***Int. J. Cancer (Pred. Oncol.) 79*:147–152, 1998.**
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Carcinoma of the breast results from multiple somatic mutations, many of which affect the balance between cell proliferation and programmed cell death. Regulating both processes is the product of the most frequently altered gene in diverse human cancers, the *p53* tumour-suppressor gene (Hollstein *et al.,* 1991). DNA damage associated with exposure to a variety of agents induces p53 to act as a tetrameric transcriptional regulator which enhances the expression of genes containing specific p53-binding sites and interacts with a variety of transcription factors to inhibit the expression of other genes (Zambetti and Levine, 1993). Products of p53-regulated genes play roles in the induction of cell-cycle arrest, apoptosis and DNA repair (Levine, 1997). In light of evidence demonstrating that cells lacking normal p53 function have a selective growth advantage and are more resistant to ionizing radiation and many widely used anti-cancer drugs than cells with wild-type p53 function (Levine, 1997), tumour harboring mutated *p53* might be expected to be more aggressive clinically and, therefore, associated with poor cancer prognosis.

A number of studies have linked *p53* gene mutation as well as the associated accumulation of conformationally altered mutant p53 protein to many clinical and pathologic features used as traditional breast cancer prognostic indicators (Isola *et al.,* 1992; Seshadri *et al.,* 1996). However, whether the presence of *p53* abnormalities can predict reduced survival of breast cancer patients independently of these established factors remains unresolved. Even when the same technique, immunohistochemistry, was employed to assess p53 protein over-expression, different studies have yielded discordant conclusions with respect to the prognostic value of p53 in breast cancer (Thor *et al.,* 1992; Allred *et al.,* 1993; Lipponen *et al.,* 1993; Silvestrini *et al.,* 1993; Pietilainen *et al.,* 1995; Rosen *et al.,* 1995). Methodologic differences between these studies, illustrated by the various reported combinations of fixation and post-fixation procedures, immunoreagents and often subjective scoring criteria for the interpretation of immunostaining results, have likely contributed to this disagreement. More quantitative, and therefore more objective and consistent, p53 protein analysis may be facilitated by ELISA methods, one of which has been applied to extracts of breast tumour tissues prepared for routine steroid hormone receptor analyses and has demonstrated utility for the identification of breast cancer patients at increased risk for relapse and death (Borg *et al.,* 1995; de Witte *et al.,* 1996). Sensitive and well-characterized ELISAs of p53 protein, applicable to cytosolic breast tumour extracts, also have been described by our group (Hassapoglidou *et al.,* 1993; Levesque *et al.,* 1995*b*) and revealed associations between p53 over-expression and a number of other breast cancer prognostic indicators (Levesque *et al.,* 1995*a*). Since follow-up information was unavailable for the patients in that study, the purpose of the work reported here is to extend our previous observations of the prognostic potential of ELISA-detected p53 protein accumulation to a series of breast cancer patients from the Piedmont region of northern Italy for whom detailed survival information was collected.

## PATIENTS AND METHODS

### *Breast cancer patients*

One hundred and seventy-one patients with primary breast cancer were included in the study. These patients were accrued consecutively, given that their resected tumours were of sufficient mass for p53 analysis (see below), and represented approximately 70% of all new cases of breast cancer diagnosed and treated in the Department of Obstetrics and Gynecology and the Department of Gynecologic Oncology at the University of Turin from January 1988 to December 1991. Patients with bilateral lesions, Paget's disease of the breast or disseminated disease at the time of diagnosis or within 2 months after surgery and patients who received only palliative treatment were excluded from the study. Ages of the patients ranged from 25 to 91 years, with a median of 55 years. Of the patients, 32% were under the age of 50 years, and 68% were at the age of 50 years or over. For all patients, the surgical procedure consisted of either modified mastectomy or conservative surgery in conjunction with post-operative irradiation of the breast. Axillary lymph node dissection was performed on all

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but 11 patients, for whom this procedure was not performed due to their advanced age (>75 years). The mean number of resected lymph nodes examined histopathologically for the presence of malignancy was 15 (±6 SD). Histopathologic examination of tumour tissues confirmed the diagnosis of primary breast carcinoma in all cases.

Clinical staging was performed according to the International Union Against Cancer Post-surgical Tumour-Node-Metastasis classification system (Spiessl *et al.*, 1989). Of the 171 patients, 45%, 47% and 8% had stages I, II and III, cancer, respectively. Each breast cancer specimen also was graded histologically and typed according to the criteria described by Bloom and Richardson (1957) and Hopton *et al.* (1989). Of the 115 patients for whom histologic grade was known, 8% had low (I), 63% had moderate (II) and 29% had high (III) grades. Ductal carcinomas accounted for the majority (71%) of specimens, while the remaining specimens were of lobular (14%), medullary (5%), papillary (2%), tubular (2%), tubulolobular (3%) or other (3%) histotypes. In the statistical analysis, because of the small numbers of patients with histotypes other than ductal carcinoma, specimens were grouped into 2 categories (ductal *vs.* non-ductal). Tumour sizes, recorded as the maximum diameter of fresh mastectomy specimens, ranged from 0.7 to 6 cm and had a median size, identical to the mean size, of 2.0 cm. Fifty-two percent of patients had axillary lymph node invasion; node status was unknown for 11 patients. Oestrogen receptor (ER) and progesterone receptor (PR) concentrations were determined by dextran-coated charcoal methods (Thorpe, 1987), and, using cut-off levels of 10 fmol/mg of protein, 66% and 59% of tumour specimens were positive for ER and PR, respectively.

Pre-operatively, no patients had been treated for breast cancer. Adjuvant tamoxifen treatment (30 mg/day for 5 years) was administered to lymph node-positive, post-menopausal patients, and adjuvant CMF chemotherapy (600 mg/mq cyclophosphamide, 40 mg/mq methotrexate, 600 mg/mq 5-fluorouracil, i.v., every 21 days for 8 courses) was given to node-positive, pre-menopausal patients. Node-negative patients received no additional treatment following surgery. Of the 171 patients, 57% were treated with adjuvant therapy, including tamoxifen (37%), chemotherapy (16%) or both (4%).

For each patient, follow-up was scheduled once every 3 months during the first 2 years after surgery, followed by examination at 6-month intervals for 3 years and annually thereafter. The overall follow-up time ranged from 7 to 67 months, with a median follow-up of 33 months, during which 42 patients underwent cancer relapse and 27 died.

*Preparation of tissue extracts*

Tumour specimens were snap-frozen in liquid nitrogen immediately after surgical removal and stored at −80°C until extraction. Approximately 200 mg of each specimen, containing at least 70% tumour cells as evaluated histopathologically, were pulverized to a fine powder at −80°C, after which the cells were lysed for 30 min on ice with 1 ml of lysis buffer (50 mM Tris, pH 8.0, containing 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40 surfactant and 1 mM phenylmethyl sulfonyl fluoride). Lysates were centrifuged at 15,000 *g* at 4°C for 30 min to collect the supernatants, which were assayed directly for total protein content using a commercially available kit based on the bicinchoninic acid method (Pierce, Rockford, IL) and for p53 protein.

*Immunofluorometric assay of p53*

p53 protein immunoreactivity in the breast tumour extracts was measured with an ELISA technique described and evaluated in detail elsewhere (Levesque *et al.*, 1995*b*). Briefly, this method involves the capture of soluble p53 protein onto microtiter wells coated with mouse DO-1 monoclonal antibody (MAb) (gift of Dr. D.P. Lane, Dundee, UK), followed by the detection of bound immunocomplexes by subsequent additions of rabbit polyclonal CM-1 anti-serum (Novocastra, Newcastle upon Tyne, UK) and alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Jackson ImmunoResearch, West Grove, PA). Enzyme activity was detected by monitoring the formation of fluorescent complexes between the dephosphorylated reaction product diflunisal and EDTA-chelated $Tb^{3+}$, using time-resolved fluorometry (Christopoulos and Diamandis, 1992). Tumour extracts and assay calibrator solutions, ranging in concentration from 0.15 to 75 ng/ml and prepared by dilutions of recombinantly expressed p53 protein, were assayed in parallel and as duplicates. Concentrations of p53 protein exceeding the detection limit of approximately 0.04 ng/ml were divided by the total protein contents of the extracts to adjust for variable extraction efficiencies. Adjusted p53 concentrations of 0.40 ng/mg were considered p53-positive, as followed from our previous study (Levesque *et al.*, 1995*a*).

*Statistical analysis*

The distributions of demographic, clinical and pathological variables, including age, clinical stage, histologic grade and type, nodal status, tumour size, ER, PR and adjuvant treatment, were compared between p53-positive and p53-negative patients using contingency tables and $\chi^2$ tests. The relationships between each of the study variables and relapse-free survival or overall survival were evaluated by the hazards ratios (relative risks for relapse or death) and their 95% confidence intervals, which were calculated from fitted Cox proportional hazard regression models. Multivariate Cox regression models were used to assess the impact of p53 protein positivity on patient survival while controlling for other variables that may also affect survival, such as patient age (less than 50 years or 50 years and older), clinical stage (I, II or III), nodal status (positive or negative), tumour size (less than 2.0 cm or 2.0 cm and greater), steroid hormone receptor status (presence or absence) and adjuvant treatment (none, tamoxifen or chemotherapy with or without tamoxifen). In addition to the analysis of all patients together, analyses were performed separately for each subgroup of patients classified by the status of nodal involvement or ER-positivity. Kaplan-Meier relapse-free and overall survival curves were constructed to demonstrate the survival differences between p53-positive and p53-negative patients, and the statistical significance of each difference was assessed by the log-rank test. Computer software SAS (SAS Institute, Cary, NC) and EGRET (Statistics and Epidemiology Research Corp., Seattle, WA) were used for these analyses, as well as 2-sided tests of significance throughout.

## RESULTS

*Relationships between p53 protein status and other prognostic variables*

The distribution of the total protein-adjusted p53 protein concentrations in the 171 breast tumour extracts, shown in Figure 1, was positively skewed, ranged from 0 to 97 ng/mg and had a median of 0.13 ng/mg. Application of a cut-off point for p53-positivity of 0.40 ng/mg distinguished 21% of the specimens as p53-positive. The distributions of the other clinical and pathological variables among the p53-positive and p53-negative patients, who had median p53 concentrations of 0.30 and 0.06 ng/mg, respectively, are shown in Table I. Although p53-positive patients more frequently had tumours which were negative for ER expression, no associations were found between p53 status and patient age at surgery, clinical stage, lymph node status, tumours size, histologic type/grade and PR expression status. Furthermore, patients treated with tamoxifen, chemotherapy alone or chemotherapy in combination with tamoxifen or who received only palliative care post-operatively did not differ with respect to p53-positivity rate. Without considering the length of follow-up time, there were trends suggesting that p53-positive patients were more likely to relapse or die than p53-negative patients (31% *vs.* 18% for cancer relapse and 33% *vs.* 19% for death).

*Association between p53 status and breast cancer patient survival*

During their follow-up periods, 13 and 9 of the 36 p53-positive patients had cancer relapse or died, respectively, while of the 135

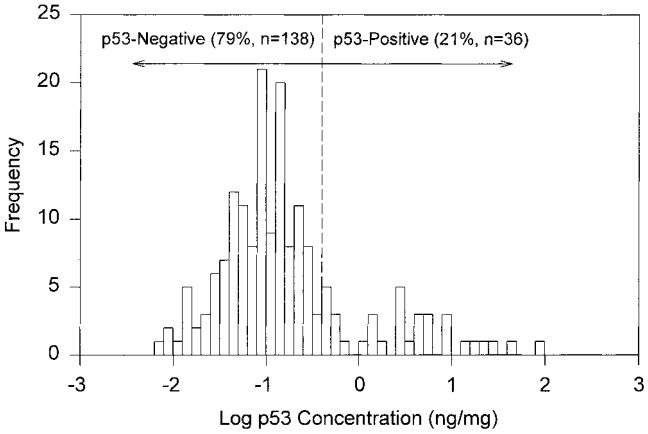


**FIGURE 1** – Frequency distribution of logarithmically transformed p53 protein concentrations in the 171 breast tumour extracts. Dashed line indicates the cut-off point for p53-positivity, 0.40 ng/mg.

**TABLE I** – ASSOCIATIONS BETWEEN p53 STATUS[1] AND OTHER CLINICO-PATHOLOGIC VARIABLES

| Variable | p53-negative patients (%) | p53-positive patients (%) | *p* value[2] |
|---|---|---|---|
| Age (years) | | | |
| <50 | 43 (79.6) | 11 (20.4) | |
| ≥50 | 92 (78.6) | 25 (21.4) | 0.88 |
| Stage[3] | | | |
| I | 58 (76.3) | 18 (23.7) | |
| II | 63 (78.8) | 17 (21.2) | |
| III | 13 (92.9) | 1 (7.1) | 0.38 |
| Nodal status[4] | | | |
| Negative | 60 (77.9) | 17 (20.1) | |
| Positive | 66 (79.5) | 17 (20.5) | 0.81 |
| Tumour size (cm)[5] | | | |
| <2.0 | 42 (80.8) | 10 (19.2) | |
| ≥2.0 | 91 (77.8) | 26 (22.2) | 0.66 |
| Histologic type | | | |
| Ductal | 98 (80.3) | 24 (19.7) | |
| Others | 37 (75.5) | 12 (24.5) | 0.49 |
| Histologic grade[6] | | | |
| I | 9 (100.0) | 0 (0.0) | |
| II | 58 (79.5) | 15 (20.5) | |
| III | 24 (72.7) | 9 (27.3) | 0.20 |
| Oestrogen receptors[7] | | | |
| Negative | 37 (66.1) | 19 (33.9) | |
| Positive | 93 (84.6) | 17 (15.4) | <0.01 |
| Progesterone receptors[8] | | | |
| Negative | 50 (73.5) | 18 (26.5) | |
| Positive | 79 (81.4) | 18 (18.6) | 0.23 |
| Adjuvant treatment | | | |
| None | 56 (76.7) | 17 (23.3) | |
| Tamoxifen | 52 (81.3) | 12 (18.7) | |
| Chemotherapy ± tamoxifen | 27 (79.4) | 7 (20.6) | 0.81 |
| Relapse | | | |
| No | 106 (82.2) | 23 (17.8) | |
| Yes | 29 (69.1) | 13 (30.9) | 0.07 |
| Death | | | |
| No | 117 (81.3) | 27 (18.7) | |
| Yes | 18 (66.7) | 9 (33.3) | 0.09 |

[1]p53 expression status based on a cut-off level of 0.40 ng/mg.–[2]*p* values calculated from $\chi^2$ tests.–[3]Clinical stage unknown for 1 patient.–[4]Nodal status unknown for 11 patients.–[5]Tumour size unknown for 4 patients.–[6]Grade unknown for 56 patients.–[7]ER status unknown for 5 patients and based on a cut-off level of 10 fmol/mg.–[8]PR status unknown for 6 patients and based on a cut-off level of 10 fmol/mg.

p53-negative patients, 29 relapsed and 18 died. In the univariate analysis, the risk for relapse was significantly higher in patients with p53-positive tumours than in patients whose tumors had p53 concentrations below 0.40 ng/mg, and the hazards ratio (HR) of 1.99 indicated a more than 100% increased risk for relapse (Table II). The corresponding hazards ratio (HR = 2.51) for overall survival, however, was of borderline statistical significance, likely due to the small number of events for death. Figure 2 shows the Kaplan-Meier survival curves representing disease-free survival (DFS) and overall survival (OS). Significantly higher risks for the outcome events were demonstrated also in patients with stage II (HR = 2.65, $p < 0.01$ for DFS, n = 156) or stage III (HR = 4.68, $p < 0.01$ for DFS and HR = 4.43, $p = 0.01$ for OS, n = 90) disease compared to patients with stage I disease, node-positive compared to node-negative (HR = 3.70, $p < 0.01$ for DFS and HR = 4.13, $p < 0.01$ for OS, n = 160) and tumour size greater than or equal to 2.0 cm compared to smaller tumours (HR = 10.21, $p < 0.01$ for DFS and HR = 3.18, $p = 0.06$ for OS, n = 169). In contrast, patients with ages greater than 50 years had a reduced risk for relapse (HR = 0.52, $p = 0.03$, n = 171) compared to younger patients (<50 years); ER-positivity was similarly associated with a better prognosis (HR = 0.34, $p < 0.01$ for DFS and HR = 0.22, $p < 0.01$ for OS, n = 166). Statistically significant hazard ratios for either outcome were not found between $PR^+$ and $PR^-$ patients, nor were they found for relapse or death between grade III and grade I patients or between grade II and grade I patients or for overall survival with respect to patient age, tumour size or between stage II and stage I patients (data not shown).

After adjusting for all of the variables in the multivariate analyses, p53-positivity remained a significant predictor of disease relapse (Table II). In the same Cox model, it was also demonstrated that late stage (HR = 2.11, $p = 0.04$) and high histologic grade (HR = 2.78, $p = 0.04$) were independent markers of breast cancer relapse, while high grade (HR = 3.90, $p = 0.01$) and ER-positivity (HR = 0.23, $p = 0.02$) were independent predictors of patient death.

*p53 effect on disease-free survival of patients classified by nodal status and ER status*

Since patients with axillary lymph node metastases differ substantially from node-negative patients with respect to their prognosis and post-operative treatment, Cox models at both univariate and multivariate levels were developed to evaluate the effect of p53 on relapse-free survival of patients within each of the 2 groups (Table III). In node-positive patients, but not in node-negative patients, p53 protein accumulation above the cut-off point was associated with increased risk for relapse, an effect sustained after controlling for the other variables. Since it was shown in the multivariate analysis of all study subjects that both p53 and ER were independent, although oppositely impacting, markers of

**TABLE II** – ASSOCIATIONS BETWEEN p53 STATUS[1] AND DISEASE-FREE AND OVERALL SURVIVAL

| p53 status | HR[2] | 95% CI[3] | *p* value |
|---|---|---|---|
| Disease-free survival | | | |
| Univariate analysis (n = 171) | | | |
| Negative | 1.00 | | |
| Positive | 1.99 | 1.03–3.84 | 0.04 |
| Multivariate analysis[4] (n = 152) | | | |
| Negative | 1.00 | | |
| Positive | 2.51 | 1.16–5.44 | 0.02 |
| Overall survival | | | |
| Univariate analysis (n = 171) | | | |
| Negative | 1.00 | | |
| Positive | 2.10 | 0.93–4.76 | 0.07 |
| Multivariate analysis[4] (n = 152) | | | |
| Negative | 1.00 | | |
| Positive | 2.43 | 0.96–6.14 | 0.06 |

[1]p53 expression status based on a cut-off level of 0.40 ng/mg.–[2]HR is hazard ratio estimated by the Cox proportional hazards regression model.–[3]CI is the confidence interval of the estimated HR.–[4]Included in the multivariate model were age, nodal status, tumour size, clinical stage, histologic type, ER, PR and adjuvant treatment.

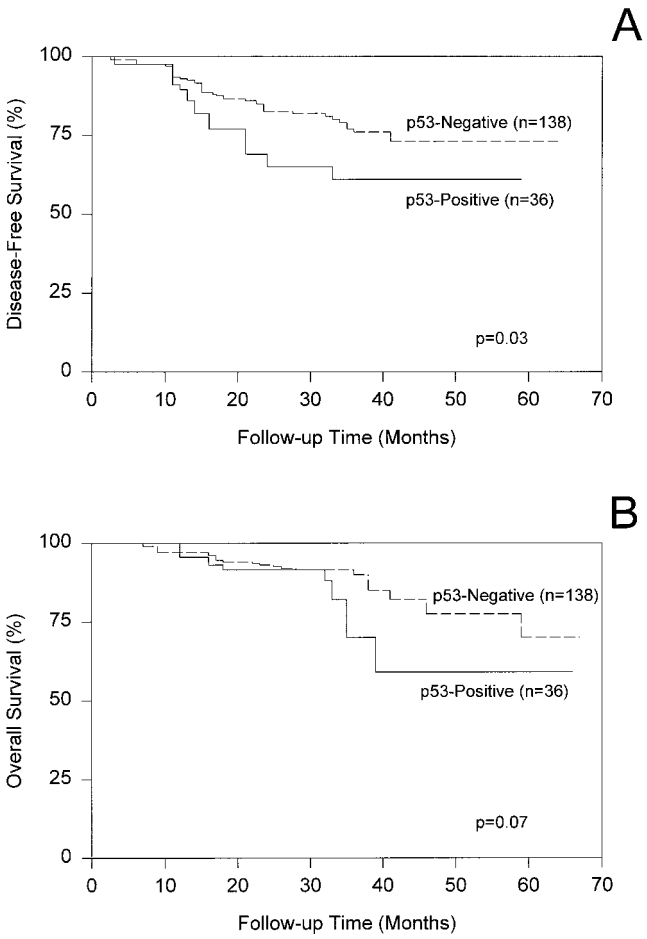


**FIGURE 2** – Kaplan-Meier analysis of disease-free survival *(a)* and overall survival *(b)* of the 171 p53-negative and p53-positive patients. *p* values were calculated by log-rank tests.

**TABLE III** – ASSOCIATIONS BETWEEN p53 STATUS[1] AND DISEASE-FREE SURVIVAL OF PATIENTS STRATIFIED BY LYMPH NODE STATUS AND OESTROGEN RECEPTOR STATUS[2]

| p53 status | HR[3] | 95% CI[4] | *p* value |
|---|---|---|---|
| Node-positive patients | | | |
| Univariate analysis (n = 83) | | | |
| Negative | 1.00 | | |
| Positive | 2.74 | 1.28–5.86 | 0.01 |
| Multivariate analysis[5] (n = 77) | | | |
| Negative | 1.00 | | |
| Positive | 4.68 | 1.89–11.64 | <0.01 |
| Node-negative patients | | | |
| Univariate analysis (n = 77) | | | |
| Negative | 1.00 | | |
| Positive | 1.00 | 0.21–4.83 | 0.99 |
| Multivariate analysis[5] (n = 75) | | | |
| Negative | 1.00 | | |
| Positive | 0.49 | 0.09–2.64 | 0.41 |
| $ER^+$ patients | | | |
| Univariate analysis (n = 110) | | | |
| Negative | 1.00 | | |
| Positive | 2.37 | 0.84–6.68 | 0.10 |
| Multivariate analysis[6] (n = 103) | | | |
| Negative | 1.00 | | |
| Positive | 7.44 | 2.03–27.23 | <0.01 |
| $ER^-$ patients | | | |
| Univariate analysis (n = 56) | | | |
| Negative | 1.00 | | |
| Positive | 1.35 | 0.56–3.22 | 0.50 |
| Multivariate analysis[6] (n = 49) | | | |
| Negative | 1.00 | | |
| Positive | 1.61 | 0.57–4.52 | 0.37 |

[1]p53 expression status based on a cut-off level of 0.40 ng/mg.–[2]ER status based on a cut-off level of 10 fmol/mg.–[3]HR is hazard ratio estimated by the Cox proportional hazards regression model.–[4]CI is the confidence interval of the estimated HR.–[5]Included in the multivariate model were age, tumour size, clinical stage, ER and PR.–[6]Included in the multivariate model were age, tumour size, clinical stage, PR and nodal status.

breast cancer prognosis and that p53-positivity was associated with the absence of ER, the hazards ratio for relapse between p53-positive and p53-negative patients was calculated separately in the 2 groups defined by ER status, and the results are shown in Table III. In $ER^-$ patients, the increased risk for relapse was not statistically significant, while in the $ER^+$ group, patients whose tumours were p53-positive had reduced disease-free survival compared to p53-negative patients, an effect significant at a multivariate level.

## DISCUSSION

Despite the large body of literature that has accumulated over the last decade, there is no consensus regarding the impact of *p53* alterations on breast cancer prognosis. Although such disagreement may constitute evidence that the clinical implications of *p53* are minimal for this disease, it may simply reflect the wide variety of approaches used in these studies. Most of these have been immunohistochemical methods, which have varied with respect to p53-specific monoclonal and polyclonal antibodies, fixation and antigen retrieval details and the particular scoring scheme used to assess the results of immunostaining. The majority of these studies, therefore, have yielded qualitative or semi-quantitative descriptions of p53 protein over-expression. The coupling of densitometric image analysis to conventional immunostaining techniques (Charpin *et al.,* 1996) and the application of ELISA-type assays to extracts prepared from breast tumor tissues (Borg *et al.,* 1995; Levesque *et al.,* 1995*a;* de Witte *et al.,* 1996) have permitted more precise, sensitive and objective p53 protein analyses. ELISAs may offer another advantage over standard immunohistochemistry in that they may be applied directly to breast tumor cytosols prepared for routine ER and PR concentration measurements (Hassapoglidou *et al.,* 1993), thus obviating the requirement for additional tumour material. A small number of immunoassays of p53 protein, most of them similiar in overall design, have been described (Hassapoglidou *et al.,* 1993; Borg *et al.,* 1995; Levesque *et al.,* 1995*b*) but not extensively used to relate p53 expression levels to breast cancer prognosis (Borg *et al.,* 1995; de Witte *et al.,* 1996).

In the present study, we employed a time-resolved immunofluorometric assay of p53 protein which had previously demonstrated suitability for assay in extracts prepared from breast (Levesque *et al.,* 1995*b*) and lung (Levesque *et al.,* 1997) carcinomas as well as from non-diseased breast tissue (Levesque *et al.,* 1995*b*). It was by the application of a cut-off point used in a previous study, obtained from visual inspection of a histogram of protein-adjusted p53 protein concentrations determined by ELISA in 200 breast tumour specimens (Levesque *et al.,* 1995*a*), and not by a retrospective ''minimum *p*-value approach'' (Altman *et al.,* 1994) or by any *a priori* assumption of the normal level of p53 protein expression in breast tumour tissue, that the cut-off point of 0.40 ng/g was chosen. This value had been used in our previous work (Levesque *et al.,* 1995*a*); it exceeded the 100th percentile of p53 concentrations in non-diseased tissues removed at cosmetic breast reduction surgery (Levesque *et al.,* 1995*b*) and led to a p53-positivity rate (21%) within the range of values reported by others using immunohistochemical methods (Isola *et al.,* 1992; Thor *et al.,* 1992; Rosen *et al.,* 1995). Patients within p53-negative and -positive groups were first compared with respect to the status of the other prognostic factors

for which information was available in order to identify potential confounding influences. Unlike other studies which had shown p53 protein over-expression to be associated variably with younger patient age (Allred *et al.,* 1993; Seshadri *et al.,* 1996), axillary lymph node metastasis (Pietilainen *et al.,* 1995), larger tumour size (Pietilainen *et al.,* 1995), high histologic grade (Isola *et al.,* 1992; Thor *et al.,* 1992; Lipponen *et al.,* 1993), ductal histotype (Lipponen *et al.,* 1993) and lack of expression of ER and/or PR (Allred *et al.,* 1993; Silvestrini *et al.,* 1993; Rosen *et al.,* 1995), a statistically significant relationship in our series of breast cancer patients was revealed only between p53 and ER expression. Because these same workers had been unable to demonstrate some of these associations, demographic and clinical differences between study populations, differences in population size and duration of follow-up as well as the different methods and categorization criteria used to ascertain p53 expression status and the status of the other prognostic markers might have accounted for the discordant conclusions.

One implication of the lack of demonstrable associations between p53 and the other factors, many of which were themselves prognostic of disease-free or overall survival, was that an apparent relationship between p53 and survival might not have simply reflected an association between p53 and any of these other variables. In fact, the significantly elevated risk for breast cancer relapse in p53-positive patients was maintained in the multivariate model adjusted for most of the other variables, including the adjuvant treatment given. The corresponding risk for death, however, attained only borderline statistical significance, even when p53 status was considered singly in the univariate model. Interestingly, this increased risk for recurrence or distant metastases was restricted to patients within lymph node-positive and ER-positive subgroups, findings shared by others (Cunningham *et al.,* 1994; Pietilainen *et al.,* 1995) but contradictory to earlier reports suggesting that p53 abnormalities might further stratify lymph node-negative patients at risk of a poor outcome (Thor *et al.,* 1992; Allred *et al.,* 1993; Silvestrini *et al.,* 1993). The clinical implications of these findings are unclear, but intriguing possibilities exist. The diagnosis of axillary lymph node involvement is generally believed to foretell an unfavourable outcome, warranting adjuvant treatment. Given that p53 may be an important functional mediator of the response to chemotherapeutic agents and radiation and that the majority of patients in this subgroup were treated with chemotherapy, the poor survival associated with positive p53 expression status may have resulted from treatment failure. Conversely, node-positive, p53-negative individuals might potentially represent more successfully treated breast cancer patients. The decision to administer endocrine therapy rests largely on the demonstration of ER expression. Our results suggest that $ER^+$ patients, who may have better prognoses, may be stratified into high- and low-risk groups by p53 accumulation status. Since the impact of p53 over-expression on patient survival was small in comparison to the influences of disease stage, grade, nodal involvement and tumour size and since time to relapse was the only outcome statistically related to p53 status, the prognostic value of p53 protein accumulation in our study could be considered relatively weak. A larger patient population followed up for a longer period of time, however, might have provided enhanced statistical power, particularly with respect to the impact of p53 status on overall survival; during their respective follow-up periods, only 27 of the 171 patients (16%) died and 42 (25%) suffered breast cancer relapse. In fact, using the method of Schoenfeld and Richter (1982), we estimated that our ability to detect statistically significant (at $p < 0.05$) differences in either disease-free or overall survival of the patients in our series ranged from 60% to 70%. Moreover, the limitations of this study, in terms of the short median follow-up of 33 months combined with the moderate size of the patient series, also might have prevented statistically significant differences in survival to be demonstrated between p53-negative and -positive patients in axillary lymph node-negative and $ER^-$ subgroups. In light of this possibility, the results of this study should be interpreted cautiously.

p53 protein over-expression, detected by a sensitive ELISA method, was a significant indicator of poor disease-free survival in a series of 171 breast cancer patients from northern Italy. A statistical trend relating p53-positivity and reduced overall survival also was observed. Future studies employing a much larger patient cohort are presently under way and may help to clarify the prognostic value of p53 protein over-expression in breast cancer.

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