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Abnormal *p53* immunoreactivity and prognosis in node-negative breast carcinomas with long-term follow-up

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Summary. The expression of the *p53* gene product was investigated immunocytochemically in a retrospective series of 164 formalin-fixed paraffin-embedded invasive breast carcinomas with pathologically proven negative lymph nodes. Overall, 78 tumors (48%) showed a variable degree of *p53* immunoreactivity. Among these, 38 cases were low expressors (1–10% *p53* immunoreactive tumor cells), 21 moderate expressors (10–50% immunoreactive cells) and 19 high expressors (> 50% immunoreactive cells). Abnormal *p53* expression correlated significantly with tumor size, histological and nuclear grade, DNA ploidy, mitotic rate and proliferation index, and with the lack of estrogen receptors. Disease-free and adjusted survival analysis of the 124 node-negative patients with long term (more than 10 years) follow-up, however, did not reveal an independent prognostic role for *p53* expression. These data suggest that the evaluation of *p53* immunoreactivity may only play a role in a multiparametric prognostic assessment of node-negative breast carcinoma.

Key words: *p53* gene – Breast carcinoma – Prognosis – Immunocytochemistry

Introduction

The *p53* gene, coding for a 375-amino-acid nuclear phosphoprotein, has been extensively investigated in the past decade (for review see Lane and Benchimol 1990). It was formerly considered to be a dominant oncogene, capable of inducing cell transformation, either alone or in association with an activated *ras* gene (Eliyahu et al. 1984; Jenkins et al. 1984; Parada et al. 1984). Recent experimental studies, however, have demonstrated that wild-type *p53* can actually suppress cell transformation, whereas mutated *p53* genes play a role in tumorigenesis (Finlay et al. 1989; Friedman et al. 1990). Thus, accumulating evidence has clearly established *p53* as a tumor suppressor gene, functionally comparable to the *Rb* susceptibility gene. Allelic losses of chromosome 17p, where the *p53* gene has been mapped (Isobe et al. 1986), and genetic alterations of this gene, including mutations and/or deletions, are frequently detected in many common human malignancies (Fearon et al. 1987; Yokota et al. 1987; Mackay et al. 1988; Nigro et al. 1989). Wild-type *p53* protein has a short half-life (6–30 min) and it is expressed at very low levels in normal tissues (Rogel et al. 1985). Conversely, mutant *p53* proteins are more stable and have a prolonged half-life (Finlay et al. 1988). Accordingly, the intranuclear accumulation of *p53* protein, conveniently investigated by immunocytochemistry, is thought to reflect the occurrence of a mutation of the *p53* coding sequence (Rogel et al. 1985; Iggo et al. 1990; Bartek et al. 1991).

p53 abnormalities, as shown by molecular biology techniques or by immunocytochemistry, occur frequently in breast carcinomas (Cattoretti et al. 1988; Bartek et al. 1990; Thompson et al. 1990; Chang et al. 1991; Davidoff et al. 1991a; Varley et al. 1991). Alterations of *p53* have been found in "in situ" carcinomas (Davidoff et al. 1991b; Walker et al. 1991), are maintained throughout cancer progression (Davidoff et al. 1991b), and have been related to unfavorable prognostic variables such as stage, metastatic involvement, lack of steroid hormone receptors, aneuploidy, and high proliferative activity (Cattoretti et al. 1988; Davidoff et al. 1991c). Despite the rapid accumulation of data on *p53* alteration in human tumors, particularly in breast carcinoma, its prognostic role in well-defined series of patients still needs to be assessed.

The current investigation was aimed to evaluate the prevalence of abnormal *p53* immunoreactivity in a series of breast carcinomas and to define its relationships with several prognostic variables. Furthermore, we have investigated whether *p53* immunoreactivity could be an independent prognostic indicator in node-negative breast cancer patients. Two series of previously charac-

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terized breast cancer patients (Bosari et al. 1992; Lee et al. 1992) provided the framework for the current study.

Materials and methods

The study population included two separate series of patients with invasive breast carcinoma treated surgically at the Lahey Clinic Medical Center, Burlington, Massachusetts, USA. The first series comprises 124 patients treated with modified radical mastectomy between 1973 and 1982. All patients had histologically proven negative axillary nodes, were without clinical evidence of metastases and did not receive postoperative adjuvant therapy. Patient survival and clinical status were obtained from clinic records, from contact with the patients' physicians, or both. The patients were followed up for at least 10 years. The second series comprises 40 patients with negative nodes treated between 1988 and 1990. Overall, 31 patients were premenopausal whereas 133 patients were postmenopausal. The prognostic role of *p53* expression was evaluated only in the first series of patients.

The original pathological material was reviewed, and conventional histological variables were determined, including tumor size, histological grade, nuclear grade, mitotic rate, and peritumoral lymphatic and blood vessel invasion (Bloom and Richardson 1957; Elston 1987; Lee et al. 1990).

The tumors studied included 148 carcinomas of duct cell type (not otherwise specified), 11 lobular carcinomas, 4 colloid carcinomas and 1 metaplastic carcinoma.

For all tumors, one block was selected for flow cytometry based on abundance of tumor cells and good morphological preservation. Tissues were prepared according to the technique of Hedley et al. (1983). Briefly, 50 μm-thick sections were dewaxed, rehydrated, and mechanically and enzymatically dissociated to yield nuclear suspensions. Nuclei were stained with propidium iodide and counted on a Becton-Dickinson (San Jose, Calif., USA) flow cytometer. At least 10000 events were measured in each specimen.

One paraffin block was selected for immunocytochemistry in each case, based on good morphological preservation. For the immunolocalization of *p53* protein, sections were stained with the monoclonal antibody PAb 1801 (Oncogene Science, Manhasset, N.Y., USA) according to Roncalli et al. (1992). Briefly, dewaxed sections were rehydrated and treated with 0.05% saponin in distilled water for 30 min at room temperature (r.t.) and with 5% normal horse serum for 20 min at r.t. before being subsequently incubated with: (a) PAb1801 mAb diluted 1:4000 in phosphate-buffered saline containing 5% normal horse serum, overnight at 4° C; (b) biotinylated horse anti-mouse immunoglobulin serum (Vector, Burlingame, Calif., USA) diluted 1:200, for 30 min at r.t.; and (c) alkaline phosphatase-labeled streptavidin (Dakopatts, Glostrup, Denmark) diluted 1:100, for 30 min at r.t. Alkaline phosphatase activity was developed with the McGadey reagent (nitro blue tetrazolium and bromo-chloro-indolyl phosphate) containing 1 mM levamisole, for 1 h at r.t. The stained slides were evaluated independently by two of the authors; in the few cases in which the evaluation provided different results, a consensus interpretation was reached after re-examination. Only nuclear staining was considered positive; tumors showing exclusively cytoplasmic staining were regarded as negative. The same authors assessed the percentage of tumor cell nuclei with definite *p53* immunoreactivity and the tumors were classified into four groups as follows: negative tumors, 0–1% immunoreactive neoplastic cells; low expressors, 1–10% immunoreactive cells; moderate expressors, 10–50% immunoreactive cells; high expressors, > 50% immunoreactive cells.

Statistical differences between variables were analyzed using unpaired *t*-tests or Wilcoxon rank sum analysis as appropriate. Contingency tables were analyzed with Miettinen's modification of the Fisher exact test. Disease-free and adjusted survival distribution were calculated by the product-limit method of Kaplan and Meier. The statistical significance of differences between distributions was analyzed by the Tarone-Ware method. Probability values are two-tailed, with $p < 0.05$ regarded as statistically significant.

Results

Overall, of the 164 tumors evaluated, 78 (48%) demonstrated variable levels of *p53* immunoreactivity whereas 86 (52%) did not express any immunocytochemically detectable *p53* protein. The prevalence of *p53*-immunoreactive tumors was very similar in the two series of patients. Staining was observed exclusively in the neoplastic tissues (Fig. 1), with the benign breast tissue surrounding the tumors being consistently negative (Fig. 2). In *p53*-immunoreactive tumors, the intraductal in situ component, when present, was frequently stained, particularly in the "comedo carcinomas" (Fig. 3). Among the 78 *p53*-immunoreactive tumors, 38 cases (49% of

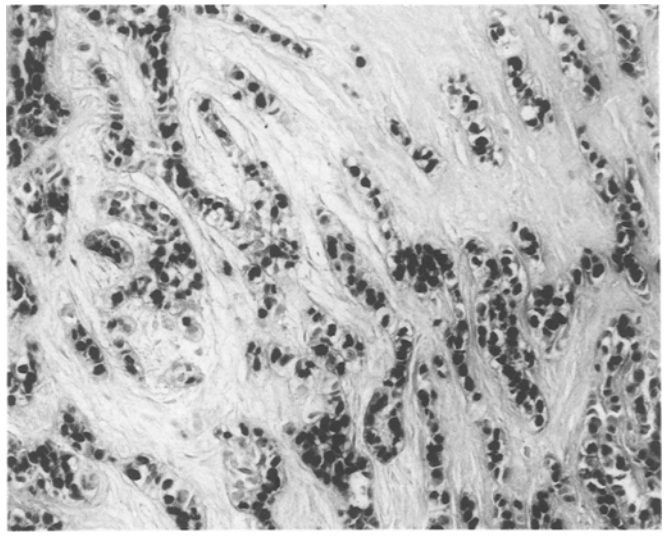

Fig. 1. Invasive breast carcinoma with diffuse staining for *p53* protein (high expressor). × 250

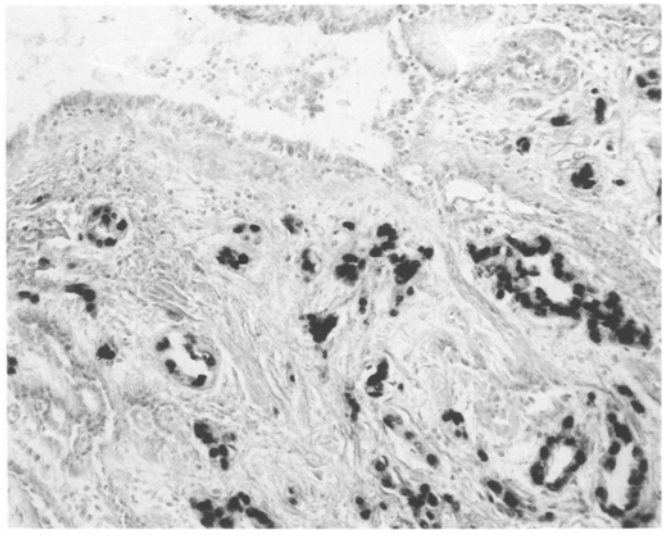

Fig. 2. Immunoreactivity for *p53* is present in neoplastic cells but not in the adjacent benign breast tissue. × 250

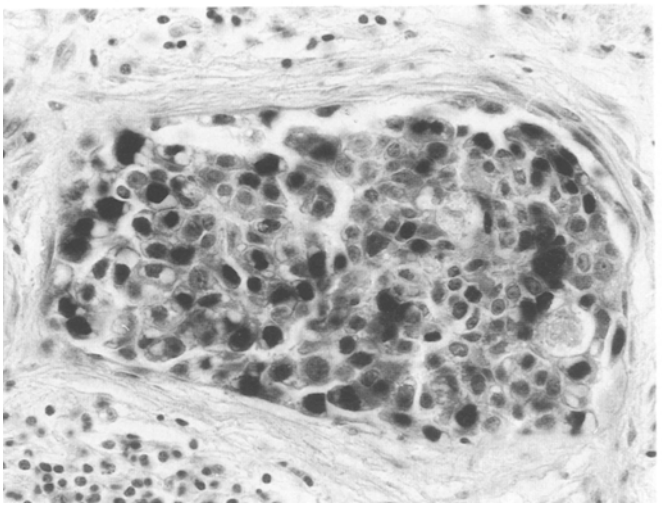

Fig. 3. Intraductal component at the periphery of an invasive carcinoma, showing intense *p53* immunoreactivity. ×400

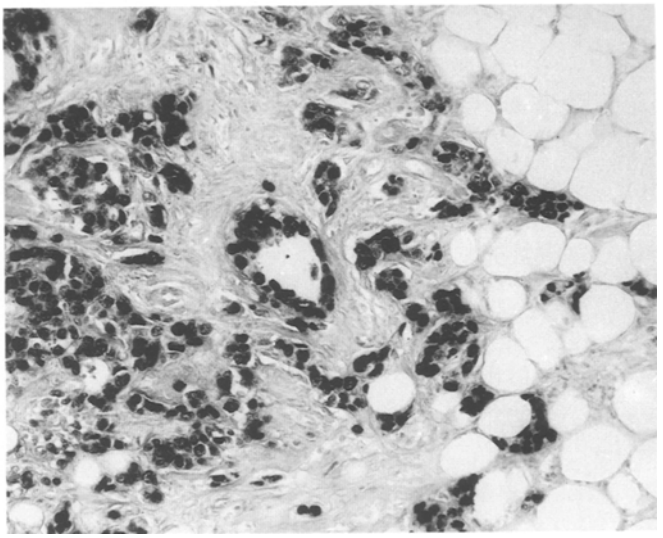

Fig. 4. Tumor cells immunoreactive for *p53* infiltrating into fat lobules at the edges of the neoplasm. ×250

Table 1. Comparison between *p53* immunoreactivity and prognostic parameters

		p53−	*p53*+	
TS	<10 mm	18 (95%)	1 (5%)	
	11–20 mm	78 (78%)	22 (22%)	$P=0.015$
	21–50 mm	26 (62%)	16 (38%)	
	>50 mm	1 (50%)	1 (50%)	
HG	well	14 (93%)	1 (7%)	
	mod	45 (88%)	6 (12%)	$P=0.0034$
	poor	53 (65%)	29 (35%)	
NG	well	12 (92%)	1 (8%)	
	mod	56 (84%)	11 (16%)	$P=0.019$
	poor	56 (67%)	28 (33%)	
MR	low	64 (91%)	6 (9%)	
	mod	31 (82%)	7 (18%)	$P=0.0001$
	high	29 (53%)	26 (47%)	
LBVI	−	88 (79%)	23 (21%)	ns
	+	36 (68%)	17 (32%)	
ER	+	57 (86%)	9 (14%)	$P=0.0016$
	−	24 (59%)	17 (41%)	
PR	+	14 (87%)	2 (13%)	ns
	−	18 (75%)	6 (25%)	
Ploidy	D	59 (86%)	10 (14%)	
	T	22 (73%)	8 (27%)	$P=0.0056$
	A	39 (64%)	22 (36%)	

p53−, Negative tumors and low expressors; p53+, moderate and high expressors; TS, tumor size; HG, histological grade; NG, nuclear grade; MR, mitotic rate; LBVI, peritumoral lymphatic and blood vessel invasion; ER, Oestrogen receptors; PR, progesterone receptors; D, diploid; T, tetraploid; A, aneuploid; ns, not significant

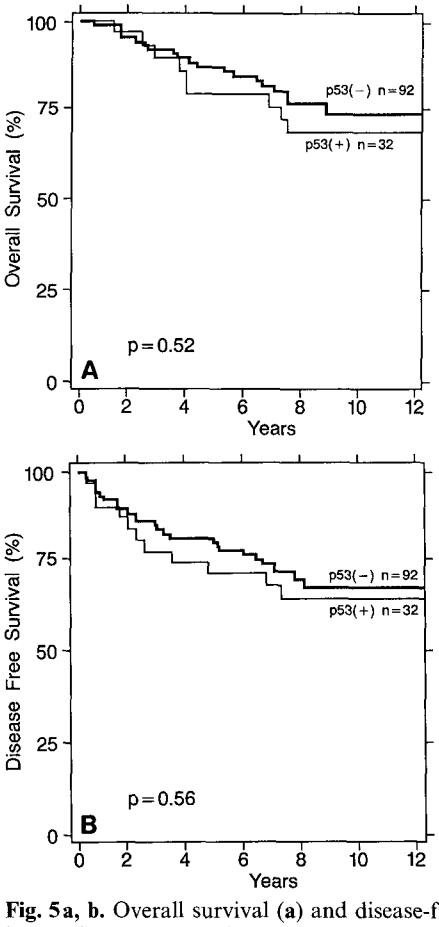

Fig. 5a, b. Overall survival (**a**) and disease-free survival (**b**) curves by *p53* immunoreactivity *p53*−, negative tumors and low expressors; *p53*+, moderate and high expressors

p53-immunoreactive neoplasms) were low expressors, 21 (27%) were moderate expressors and 19 (24%) were high expressors. When low *p53* expression was found, it was most often encountered at the infiltrating edges of the tumors (Fig. 4). No significant differences in *p53* immunoreactivity were seen between carcinomas of different histological type or between those occurring in pre- and postmenopausal patients.

Data were analyzed comparing *p53*-positive with *p53*-negative cases, as well as moderate plus high expressors

with *p53*-negative plus low expressors. The latter subdivision proved the most rewarding for clinicopathological correlation. Analysis of all patients showed a statistically significant association of the moderate and high *p53* expressors with several prognostic variables (Table 1). Larger tumors, poor histological and nuclear grade, high mitotic rate, lack of estrogen receptors and aneuploidy were significantly more frequent among *p53*-immunoreactive neoplasms. Tumors showing lymphatic and blood vessel invasion and tumors lacking progesterone receptors were also more frequently associated with *p53* immunoreactivity, but the trend did not reach statistical significance. *p53*-immunoreactive tumors also demonstrated a higher S-phase fraction than the negative tumors ($10.9\% \pm 5.2$ versus $7.8\% \pm 6.9$; $P = 0.0157$).

For adjusted and disease-free survival analysis 124 patients were evaluated. Sixty-two tumors (50%) were *p53* negative, 30 (24%) were low expressors, 16 (13%) moderate expressors and 16 (13%) high expressors. Patients with tumors showing moderate and high expression of *p53* demonstrated slightly worse disease-free and adjusted survival when compared with all other cases. Adjusted survival and disease-free survival curves are shown in Fig. 5a and 5b respectively. Mean survival time for *p53* moderate and high expressors was 8.8 years versus 10.8 years for the other patients. The recurrence rate was 38% for moderate and high expressors and 29% for all others. None of these trends reached statistical significance.

Discussion

The results of the present investigation confirm the suitability of formalin-fixed paraffin-embedded tissues for immunocytochemical evaluation of the *p53* gene product, as previously reported (Barbareschi et al. 1992; Roncalli et al. 1992).

Immunoreactivity for *p53* was found in about half the cases investigated, although the degree of staining varied considerably. Positive nuclear staining was strictly confined to neoplastic cells, in both intraductal and infiltrating components of tumors.

The immunocytochemical detection of the *p53* gene product has been shown to depend on the presence of an abnormal protein due to a mutation in the *p53* gene coding sequence (Bartek et al. 1990; Davidoff et al. 1991a). The occurrence of *p53* mutant proteins in breast carcinoma is common, ranging from 20% to over half the tumors studied (Cattoretti et al. 1988; Thompson et al. 1990; Chang et al. 1991; Davidoff et al. 1991a, c; Varley et al. 1991; Walker et al. 1991). These differences may reflect the heterogeneity of the patient populations investigated, which included cases with different disease stage and histological grade. The variable prevalence of *p53* immunoreactivity may also depend on the choice of different antibodies used for immunocytochemical studies. Walker et al. (1991) tested five antibodies, recognizing different epitopes of the *p53* protein, and found that PAb 240, PAb 1801, C19 and JG8 stained the highest number of breast carcinomas (53%), whereas PAb 421 detected *p53* protein in only 31% of tumors. Finally, the reported variable prevalence of *p53* immunoreactivity may be partially explained by the different criteria adopted for evaluating the immunocytochemical studies. For instance, Cattoretti et al. (1988) reported 45.5% of the tumors as *p53* immunoreactive (using PAb1801) irrespective of the number of immunostained cells. In contrast, Davidoff et al. (1991c) considered positive only those tumors with intense, widespread staining of all tumor cells, and thus classed only 27% of tumors as immunoreactive using the same monoclonal antibody. Variable expression of the *p53* gene product within the same tumor cell population was also observed in our series. Indeed, in our investigation, the overall incidence of *p53*-immunoreactive tumors is similar to that reported by Cattoretti et al. (1988), but the incidence of moderate and high expressors is close to that reported by Davidoff et al. (1991c). These discrepancies further emphasize the need for standard immunocytochemical techniques and interpretation criteria, particularly if data from different studies have to be compared. Although the heterogeneity of *p53*-immunoreactive cells remains to be explained, it is not a result of formalin fixation since the same degree of variable expression can be noted in frozen tissues (Chang et al. 1991; Barbareschi et al. 1992). The occurrence of increased *p53* immunoreactivity at the infiltrating edges of the tumors is unclear but has also been noted in colorectal tumors (Purdie et al. 1991). Interestingly, proliferating cells, demonstrated by Ki67 immunostaining, are also more frequently observed at the edges of tumors (Lee et al. 1992). A large number of microvessels are frequently found at the periphery of breast carcinoma, and tumor-related angiogenesis may play a role in these phenomena (Bosari et al. 1992).

Some studies have reported that abnormal *p53* expression is frequently associated with unfavorable prognostic variables. Cattoretti et al. (1988) have shown a significant association of *p53*-immunoreactive tumors with aneuploidy, high proliferation index and lack of estrogen receptors. Other investigators also reported a higher prevalence of *p53* immunoreactivity in tumors of patients with more advanced disease stage and with lymph node metastases (Davidoff et al. 1991c; Walker et al. 1991).

To the best of our knowledge, the present study is the first that evaluates the prognostic role of *p53* immunoreactivity in a well-defined population of node-negative patients with long-term follow-up. Since the use of postoperative adjuvant systemic therapy in node-negative breast carcinoma patients remains controversial (DeVita 1989; McGuire 1989), additional prognostic factors may help in identifying those patients at high risk for recurrence, thus facilitating patient management. In our series, cases with moderate to high *p53* expression were significantly more frequent among tumors showing aneuploidy, larger size, poor histological and nuclear grade, and absence of estrogen receptors. Mitotic rate and high proliferative fraction, measured by flow cytometry, also correlated with *p53* expression. Although these variables have been associated with a worse prognosis (McGuire et al. 1990), *p53* immunoreactivity had

only a minor direct effect on the adjusted and disease-free survival. These trends, however, did not reach statistical significance in the patient population studied, which was followed up for at least 10 years. A much larger number of node-negative breast cancer patients should be investigated to provide more definite results, but the small differences in disease-free and adjusted survival reported herein make it unlikely that *p53* immunoreactivity will be a powerful prognostic factor. The results of the current study, however, suggest that the assessment of *p53* immunoreactivity in node-negative breast cancer patients may still be a useful adjunct to other more established parameters (McGuire et al. 1990).

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