Pergamon

European Journal of Cancer Vol. 33, No. 8, pp. 1268–1274, 1997

Printed in Great Britain
0959-8049/97 $17.00 + 0.00

PII: S0959-8049(97)00096-8

Original Paper

p53 Expression is of Independent Predictive Value in Lymph Node-negative Breast Carcinoma

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The aim of this study was to evaluate p53 expression, determined by immunohistochemistry, in 151 infiltrating ductal breast carcinomas with negative axillary lymph nodes, and to determine whether p53 can be considered as an independent prognostic value for overall and disease-free survival. A monoclonal antibody (DO-7) that reacts with an epitope on the N terminal portion of the human protein p53 was used to detect p53 in paraffin-embedded sections, utilising a standard avidin–biotin–peroxidase complex (ABC) technique with a microwave oven antigen retrieval. Overexpression of p53 (more than 50% of stained cells) was found in 45 cases (30%). Forty-five cases were negative and occasionally or moderately stained cells were present in 61 cases. p53 protein overexpression was significantly associated with high histological grade and tumour necrosis, high MIB-1 value (MIB-1>30%) and negative oestrogen receptor status. Univariate analysis (log-rank) showed a shorter overall survival ($P = 0.003$) in patients with high tumour p53 positivity. This statistical significance was also seen on multivariate analysis (Cox's logistic regression, $P = 0.004$). p53 protein overexpression is an independent prognostic marker in node-negative breast carcinoma for overall survival and should be used with other prognostic factors. © **1997 Published by Elsevier Science Ltd.**

Key words: p53, immunohistochemistry, oncoprotein, breast carcinoma

Eur J Cancer, Vol. 33, No. 8, pp. 1268–1274, 1997

INTRODUCTION

AXILLARY LYMPH node status is currently the most important prognostic factor for breast cancer. However, 20–30% of women with node-negative tumours experience recurrence within 5 years. Thus, the search for additional tumour markers with prognostic value has increased considerably in the last few years, especially in node-negative tumours [1].

The discovery of tumour suppressor genes has provided a new approach to our understanding of tumour cell biology. One of the best known tumour suppressor genes is the *TP53* gene [2–4]. Point mutations or deletion of the wild type gene have been suggested as a key event in the development of malignancy [5, 6]. The wild type p53 protein is present in the nuclei of all mammalian cells where it appears to be involved in the regulation of cell proliferation, acting as a suppressor of cell growth [7–9]. In the normal cell, the concentration of the wild type p53 protein is generally below the detection level for immunohistochemical procedures. However, tumours with the mutant form of the *TP53* gene may express high levels of p53 protein with a significantly prolonged half-life, which can reach the threshold of immunoreactivity [10]. Although, in some studies, a correlation between overexpression of p53 protein and mutation of the *TP53* gene has been found, others have found discrepancies [11, 12]. It is believed that, in some cases, wild type p53 can be overexpressed or stabilised by non-mutational factors. Overexpression of p53 could interfere with cell-cycle regulatory functions of the wild type protein. This mutated *TP53* acts as an oncogene [7, 13, 14]. Overexpression of p53 in breast [15–19], ovarian [20, 21], lung [10, 22], bladder [23–25] and colon carcinomas [26, 27] has been associated with aggressive tumours and may

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Received 5 Sep. 1996; revised 9 Dec. 1996; accepted 17 Dec. 1996.

be an independent, unfavourable prognostic marker. However, the prognostic value of p53 in breast carcinoma is controversial [19, 28–36].

In this study, we examined the incidence of p53 in node-negative breast carcinomas. The relationship between p53 expression and clinical and prognostic factors, including proliferation markers and receptor status, was also studied. The predictive value of p53, in relation to overall survival and disease-free survival, was calculated.

MATERIALS AND METHODS

Formalin-fixed, paraffin-embedded blocks of 174 node-negative breast carcinomas, diagnosed between 1981 and 1986 at the Hospital Covadonga, were studied. We excluded from this group those patients who were lost to follow-up (10), and those with *in situ* carcinomas (11), or lobular carcinomas (2), thus limiting our study to 151 patients with infiltrating ductal carcinomas of the breast with negative axillary lymph nodes at the time of diagnosis. The patients' ages ranged from 29 to 88 years, with a mean age of 57 years. Follow-up was available in all patients with a median follow-up duration of 75 months. All patients were treated with partial, or modified radical mastectomy and axillary node dissection. Histopathological confirmation of node status was available for all patients. 41 patients received adjuvant radiotherapy. 30 patients received anti-oestrogen (tamoxifen) alone, and 50 radiotherapy plus tamoxifen. Chemotherapy (methotrexate/cyclophosamide/5-fluorouracil) was received by 13 patients. Only 2 patients were treated with chemotherapy and tamoxifen before mastectomy. Tumour types were classified according to the WHO classification. Histological grades of tumours were scored according to Bloom and Richardson criteria.

p53 protein expression evaluation

A monoclonal mouse anti-human p53 protein DO-7 (Dako, Carpentaria, California, U.S.A.) was used that reacts with an epitope on the p53 protein, which is resistant to formalin fixation. The antibody was diluted 1:50, incubated with the slides for 1 h, at room temperature, followed by a standard avidin–biotin–peroxidase complex (ABC) technique. A microwave oven heating technique was used as the retrieval method. The tissue sections were placed in 10 mM citrate buffer at pH 6.0, and were treated in the microwave oven (750 W) for 10 min (the buffer was boiled), turning the oven on three times for 5, 2.5 and 2.5 min, with a pause of 5 and 3 min between each period. The section was kept immersed in the buffer throughout the treatment, refilling the container with distilled water if necessary. The tissue sections were left at room temperature for 20 min after the last treatment. Diaminobenzidine-hydrogen peroxidase was employed as the chromogen and a light haematoxylin counterstain was used.

Immunostaining was scored by evaluating the percentage of p53 immunopositive tumour cell nuclei. The tumours were classified into four groups as follows: negative tumours: no stained neoplastic cells; low immunoreactivity: 1–10% stained cells; moderate immunoreactivity: 10–50% stained cells and high immunoreactivity: more than 50% stained cells.

For statistical analysis, staining was evaluated according to two immunostaining patterns previously described [37]: pattern I: high immunoreactivity (>50% cells) and pattern II: negative, low and moderate immunoreactivity.

Evaluation of proliferation markers

A recently available Ki-67 proliferating marker, MIB-1 (Immunotech, Marseille, France) was used. A microwave oven heating technique was also employed. An ABC technique, with a 1:50 antibody dilution with 1 h incubation was used. MIB-1 immunostaining results were scored, counting at least 500 cells in more than 10 high-power representative fields. Cases with less than 10% of positive cells were considered MIB-1 low labelling index (LI). Between 10% and 30% was considered as moderate MIB-1 LI and those cases with more than 30% of positive cells were considered high MIB-1 LI.

Oestrogen and progesterone receptor status evaluation

Oestrogen (Dako, Carpentaria, California, U.S.A.) (ER) and progesterone (Novocastra Laboratories, Newcastle U.K.) (PgR) receptors were evaluated immunohistochemically in formalin-fixed paraffin-embedded tumours. The microwave oven heating technique was used. A histoscore was employed for immunostaining according to staining intensity (I) and percentage (P) of stained cells: histoscore = $I \times P$. Staining intensity was evaluated as follows: negative (no staining at all); low (+) = 1; moderate (++) = 2 and strong (+ + +) = 3. Cases with less than 10 histoscore were considered as negative.

Controls

A positive control was used in all cases, usually a known positive breast tumour for p53, oestrogen and progesterone receptors and tonsil for MIB-1. Negative controls consisted of substitution of primary antibody by PBS; all other steps were followed unchanged.

Statistical analysis

Statistical analysis was performed using SPSS/WIN statistical software. The relationship between immunohistochemical staining and morphological variables was evaluated by means of a univariate chi-square test for association. Analysis of disease-free and overall survival were performed with the use of the Kaplan–Meier method. Tests for differences between curves were made with the log-rank tests. For the survival study, p53 and MIB-1 staining cases were divided into two groups: p53 (pattern I) and MIB-1 (high LI) overexpression cases and the remaining cases.

Multivariate analysis was carried out using a Cox's logistic regression, considering breast cancer deaths as events for relative risks. The following variables were included in the analysis: p53, histological and nuclear grade, hormonal receptor status, MIB-1, tumour size and tumour necrosis. Significance was established at the $P < 0.05$ level.

RESULTS

p53 pattern I nuclear staining (high immunoreactivity) was seen in 45 of 151 cases of breast carcinomas (30%) (Figure 1). For tumours with pattern II immunoreactivity 45 cases were totally negative (30%), 49 (32%) showed low and 12 (8%) had moderate immunoreactivity. Normal breast epithelium and stroma were negative.

Table 1 shows the correlation between tumour parameters and p53 positivity. p53 positivity was associated

Figure 1. p53 pattern I nuclear immunoreactivity. All or nearly all cells show nuclear staining (×200).

Table 1. Correlation between p53 protein expression and biological and clinical parameters

	$p53^+$	$p53^-$	*P*	Chi
Histological grade				
I	0	35		
II	6	44		
III	39	27	< 0.0001	49.49
Nuclear grade				
I	0	45		
II	16	39	< 0.0001	36.96
III	29	22		
Receptor status*				
ER^+	9	70	< 0.0001	27.27
ER^-	35	34		
PgR^+	4	36		
PgR^-	40	65	< 0.001	10.81
Proliferation marker*				
Low MIB-1 LI	4	58		
Moderate MIB-1 LI	9	30	< 0.0001	49.53
High MIB-1 LI	31	14		
Tumour size (cm)*				
Less than 1.5 cm	7	22		
Between 1.6 and 2 cm	18	54	NS	3.44
More than 2 cm	19	29		
Necrosis				
No necrosis	6	80		
Focal	9	14	< 0.0001	66.17
Moderate	12	11		
Extensive	18	1		

*Data incomplete. ER, oestrogen receptor; PgR, progesterone receptor; LI, labelling index.

with high histological and nuclear grade ($P < 0.0001$) and especially with tumour necrosis ($P < 0.0001$); all cases with extensive tumour necrosis were pattern I stained. No association was found with tumour size. A significant association was observed between p53 positive staining and ER ($P < 0.0001$) and PgR negativity ($P = 0.001$).

The log-rank between survival curves was significant ($P = 0.003$) with a short survival for cases with pattern I staining (Figure 2). No association was found between disease-free survival and p53 ($P = 0$, 1) (Figure 3).

For MIB-1 evaluation (146 cases studied), every stained nucleus was considered positive, independent of intensity. A low MIB-1 LI was observed in 62 cases (45%), moderate in 39 cases (27%) and high LI in 45 cases (31%). Table 2 shows MIB-1 correlation with tumour parameters. A significant association was found between high LI and high histological and nuclear grade ($P < 0.0001$) as well as with tumour necrosis ($P < 0.0001$). No association was found with tumour size (Table 2). A significant inverse correlation was found with ER ($P < 0.0001$) and PgR content ($P = 0.0003$). The log-rank between survival curves was significant ($P = 0.02$) with a short survival for cases with moderate and high LI (Figure 4).

ER (148 cases studied) was positive in 53% of tumours and PgR (145 cases) in 28%. Both ER and PgR were significantly associated with histological (ER: $P < 0.001$; PgR: $P = 0.003$), nuclear grade (ER: $P < 0.0001$; PgR: $P = 0.003$) and tumour necrosis (ER: $P < 0.0001$; PgR: $P = 0.0005$).

A strong significant association was observed between p53 and MIB-1 ($P < 0.0001$).

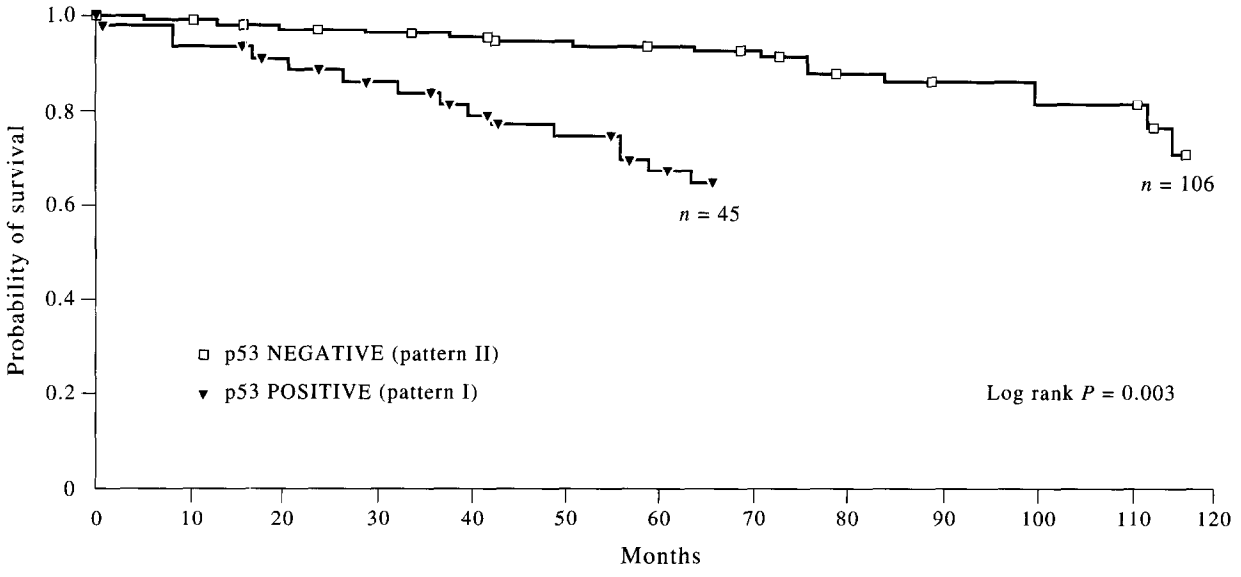

Figure 2. Overall survival of patients relative to immunohistochemical staining for p53.

A multivariate analysis was conducted to evaluate whether the correlation of p53 with survival depended on the association with the other factors indicative of a poor prognosis. The results are shown in Table 3. Overexpression of p53 protein was the strongest independent predictor of overall survival.

Cox's analysis has shown that p53 overexpression has an independent prognostic value for overall survival with a relative risk of 2.93 (95% CI 1.41–6.09).

DISCUSSION

p53 protein expression is the most widely studied of the tumour suppressor genes, present in 26–54% of primary invasive breast carcinoma [16, 28, 33, 37–39]. In intraductal tumours, the percentage has been reported to be 15–26% [15, 36]. These differences could be related to the type of monoclonal antibody [35, 38] or the immunohistochemical procedure and tissue used. Another difference among different studies is the lack of standardised immunohistochemical criteria to classify a tumour as p53 positive or negative. In the present study, 70% of the breast cancers were positive for p53; we found a greater number of positive tumours than other studies, probably due to the fact that we used, for formalin-fixed paraffin-embedded tissue, a microwave oven heating technique as antigen retrieval. For this reason, we used a high cut-off (50% of stained cells) in statistical analysis. Most of studies use 20–30% of stained cells as the cut-off point [19, 33, 35].

Our observations in this study of 151 node-negative breast carcinomas suggest a significant association of tumour p53 protein expression with a lack of ER and PgR reactivity and high nuclear grade. These results agree with those reported by others [16, 33, 35, 38]. A strong association between p53 immunoreactivity and necrosis has also been found by Poller and associates [15] in intraductal carcinomas. In the series of Ostrowsky and associates [28], although p53 positivity was seen in patients with a worse prognosis, no statistical significance was found. However,

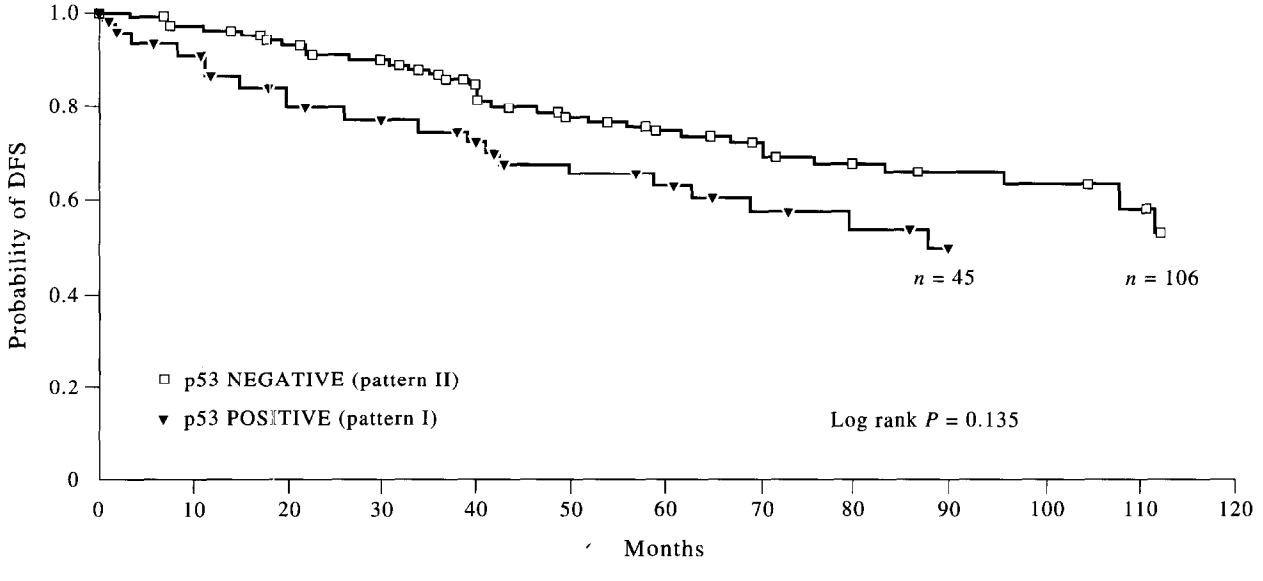

Figure 3. Disease-free survival (DFS) of patients relative to immunohistochemical staining for p53.

Table 2. Correlation between MIB-1 proliferation index and biological and clinical parameters

	MIB-1 LI				
	Low	Moderate	High	*P*	Chi
Histological grade					
I	26	5	2		
II	23	19	6		
III	13	15	37	< 0.0001	49.42
Nuclear grade*					
I	30	10	2		
II	24	16	13	< 0.0001	40.31
III	8	13	30		
Receptor status*					
ER^+	46	20	10	< 0.0001	30.63
ER^-	14	19	35		
PgR^+	26	9	4		
PgR^-	34	29	41	< 0.001	15.71
Tumour size (cm)*					
Less than 1.5 cm	13	8	8		
Between 1.6 and 2 cm	34	19	16	NS	4.92
More than 2 cm	15	12	19		
Necrosis					
No necrosis	52	19	11		
Focal	7	11	4	< 0.0001	59.44
Moderate	2	6	15		
Extensive	1	3	15		

*Data incomplete. ER, oestrogen receptor; PgR, progesterone receptor; LI, labelling index.

Barnes and associates [17] found a considerably worse prognosis in patients with p53 protein expression in the majority of their tumour cells.

The use of p53 protein overexpression may be used, according to some authors, as a diagnostic tool for malignancy, especially in cytological samples. This use may be possible only in those cases with pattern I immunostaining in which almost 100% of cells are stained, but not in cases with pattern II immunostaining because weak and focal staining has been detected in benign lesions such as fibroadenomas, benign fibrocystic disease and epithelial hyperplasia without atypia.

A significant association of MIB-1 with high histological grade, high mitotic count and an inverse relationship with ER were confirmed in our study. The strong association with p53 is in keeping with those reported by Bosari and 38associates [33], Cattoretti and co-workers [38] and Barbareschi and colleagues [16]. We also found a significant association with tumour necrosis, progesterone receptor and survival. The semiquantitative values of MIB-1 immunostaining observed in our study are higher than those reported in other studies [40], these being possibly due to the monoclonal antibody used and especially to the use of the microwave oven heating technique.

The different results obtained with p53 overexpression in breast cancer might be explained by the role of p53 protein in cancer biology. The literature on the prognostic value of p53 immunoreactivity is very controversial. Similar to our results, most studies have found a correlation between p53 expression and high tumour grade and other established indicators of poor prognosis, such as hormone receptors, high proliferation index and necrosis. Our results confirm the

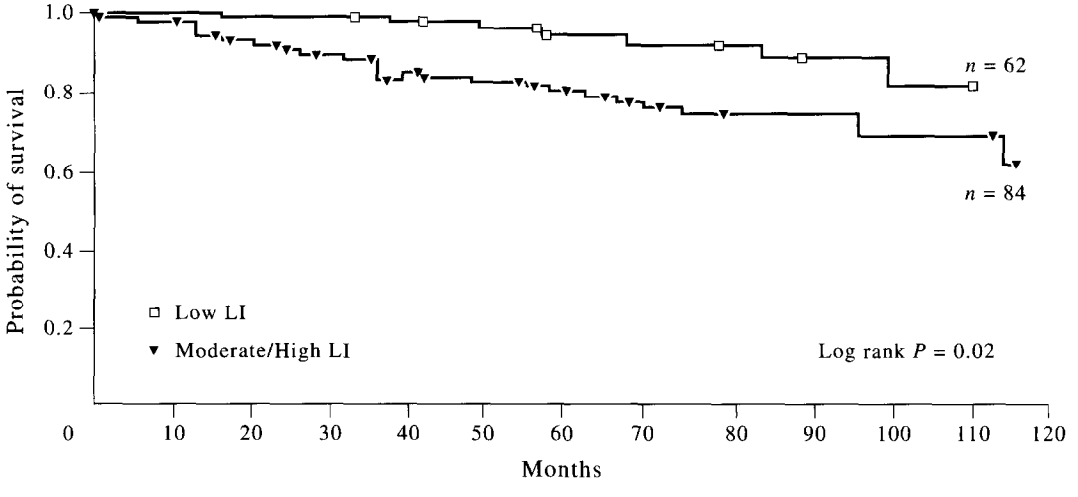

Figure 4. Overall survival of patients relative to immunohistochemical staining for MIB-1.

Table 3. p53 expression in patients with breast cancer

Factor	Overall survival		DFS	
	Univariate *P* value	Multivariate *P* value	Univariate *P* value	Multivariate *P* value
p53	0.003	0.004	0.135	0.463
HG	0.207	0.245	0.062	0.408
NG	0.008	0.154	0.493	0.862
ER	0.066	0.598	0.042	0.041
PgR	0.185	0.650	0.112	0.507
MIB-1	0.01	0.182	0.23	0.618
Size	0.066	0.128	0.151	0.110
Necrosis	0.01	0.184	0.093	0.415

DFS, disease-free survival; HG, histological grade; NG, nuclear grade; ER, oestrogen receptor; PgR, progesterone receptor.

independent prognostic significance of p53 overexpression reported by other investigators [19, 31, 36, 41, 42] in lymph node negative tumours and are in keeping with those studies which found an association or a trend between high p53 expression and a shorter disease-free survival [33, 34]. Although many authors have found p53 overexpression to be a useful prognostic marker, many others have only found a strong correlation with p53 immunostaining and other classical prognosticators, particularly histological grade and absence of oestrogen receptor [18, 28, 30, 35, 43].

We conclude that p53 protein expression is an additional independent biological marker in node-negative breast carcinoma. The present study shows that patients with high p53 reactivity (pattern I) have a poor prognosis. p53 expression could be used with other established prognostic factors to identify high-risk groups in node-negative breast cancer.

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Acknowledgements—The authors thank Aurora Fernández-García and Inés Arguelles from the Section of Immunohistochemistry for immunohistochemical staining.