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p53 in breast cancer. Its relation to histological grade, lymph-node status, hormone receptors, cell-proliferation fraction (ki-67) and c-erbB-2. Immunohistochemical study of 153 cases

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Summary. The mutation of the p53 gene is a common phenomenon in numerous human tumors, leading to the accumulation of nonfunctioning p53 protein in the cell nucleus, which can be detected by immunohistochemistry. In breast cancer, it has been suggested that the overexpression of p53 protein in the nucleus is an indicator of poor prognosis, which must be borne in mind in selecting coadjuvant treatment for each patient.

This study is an immunohistochemical analysis of p53 expression in 153 cases of mammary carcinoma, correlating it with histological grade, axillary node status, hormone receptors, cell-proliferation fraction and expression of the c-erbB-2 oncoprotein.

Of all the breast-cancer tissue analyzed, 43.79% was positive for p53. The overexpression of this protein bears a direct statistically significant relationship to histological grade, cell-proliferation fraction and c-erbB-2, and an inverse relationship to estrogen and progesterone receptors. No statistically significant relationship was found with axillary node status.

The expression of p53 in poorly differentiated tumors-commonly receptor negative and with a high proliferation fraction-may indicate greater tumor agressiveness and a high risk of relapse.

Key words: Breast cancer, Immunohistochemistry, p53, Ki-67, Hormone receptors, c-erbB-2

Introduction

Breast cancer is conventionally assessed according to morphological criteria. In most cases, a correlation has been observed between histological aspects and the clinical course of the disease; however, histology alone cannot always predict the patient's clinical outcome because of the high degree of subjectivity involved (Bacus et al., 1989).

For this reason, it has become essential to look at the expression of different factors (genetic, hormonal, etc.) to determine prognostic groups (Bacus et al., 1989), especially with regard to node-negative breast-cancer patients. These are patients in whom attempts have been made to use biological markers associated with tumor aggressiveness to identify groups with a high risk of recurrence (Bosari et al., 1992; Isola et al., 1992; Allred et al., 1993; Figueroa et al., 1993; Silvestrini et al., 1993), in order to concentrate therapeutic options in this specific group (Figueroa et al., 1993).

In breast cancer today, therefore, not only are conventional pathology indices studied (tumor size, histological grade and node status), but special immunohistochemistry techniques for the detection of hormone receptors (HR), cell-proliferation fraction (Ki-67, pcna), amplification of certain oncogenes (c-erbB-2) and, more recently, expression of p53 protein are also used.

Estrogen (ER) and progesterone (PR) receptors have conventionally been considered as highly significant in the therapy and prognosis of breast cancer. Receptor-positive patients are considered to have a better prognosis and are therefore candidates for hormone therapy, while receptor-negative patients present a lower survival rate and more tumor relapses (Mcguire, et al., 1977; Howell et al., 1984; Pertschuk et al., 1985).

The amplification of the c-erbB-2 gene causes overexpression of a protein, which is detectable by monoclonal or polyclonal antibodies. A positive reaction for this gene is breast-cancer patients has been associated with poor prognosis (Slamon et al., 1987; Venter et al., 1987), although this is not a generalized phenomenon (Barnes, 1989).

Ki-67 is an antibody used to determine cell proliferation fraction. It recognizes a nuclear antigen expressed in the G_1, S, G_2 and M phases of the human

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cell-proliferation cycle— but is absent in the G_0 phase— which can be determined in frozen tissue using monoclonal antibodies (MAb) (Gerdes et al., 1983, 1984) and, more recently, in paraffin-embedded tissue sections, using polyclonal antibodies (PAb), thus permitting retrospective studies (Key et al., 1993). It is a reliable, fast and objective indicator of the degree of tumor agressiveness (Bacus et al., 1989; Raymond and Leong, 1989). At the same time, depending on the degree of tumor differentiation, it has been shown to be of prognostic value for survival and tumor recurrence in breast cancer (Bacus et al., 1989; Gasparini et al,, 1990, 1992; Sahin et al., 1991; Veronese et al., 1993).

The p53 gene is located on the short arm of chromosome 17. It encodes a 53-kd nuclear protein (p53) found in scant amounts in normal tissue. Through the synthesis of this protein, the gene has an inhibitory effect on cell proliferation and transformation (Lane and Benchimol, 1990; Ostrowski et al., 1991; Weinberg, 1991), controlling the initiation and/or regulation of DNA replication, as well as the transcription and regulation of the response to DNA alteration (Levine et al., 1991; Marshall, 1991). Mutations of the p53 gene have been reported in different human tumors (Nigro et al., 1989). Such mutations favour the expression of more stable, nonfunctioning, mutant forms of the protein with a longer half-life, which accumulate in the nucleus and can be detected by immunohistochemistry (Iggo et al., 1990; Jaros et al., 1992). These modified proteins have additional functions that favour their oncogenetic potential (Dittmer et al., 1993; Fishella et al., 1993; Miller et al., 1993).

There is a correlation between the detection of mutations by molecular-biology methods, like single-strand conformation polymorphism (SSCP), and the detection of the protein by immunohistochemistry. Genetic alterations occur mainly on exons 5, 7 and 8, and somewhat less on 6 and 9. In some cases the accumulation of the protein may be caused by factors other than mutation (Thor et al., 1992; Bhargava et al., 1994; Hurlimann et al., 1994). In general, there is a correlation between the accumulation of p53 protein and mutation of the p53 gene.

This study is an immunohistochemical analysis of p53 expression in 153 cases of mammary carcinoma, correlating it with histological grade, axillary node status, hormone receptors, cell-proliferation fraction and expression of the c-erbB-2 oncoprotein.

Materials and methods

A total of 153 cases of mammary carcinoma were studied in Verge de la Cinta Hospital in Tortosa from 1985 to 1992, all in women from 32 to 88 years of age, of which 33 (21.57%) were premenopausal and 120 (78.43%) postmenopausal.

Fresh tissue from biopsy or tumorectomy was received in the Department of Pathology immediately after surgical removal. Part of the tumor was frozen in liquid nitrogen, and the rest was fixed in buffered formalin for up to 18 hours and then embedded in paraffin.

Histopathological characteristics

Histological grade and type

The histological grade was determined in sections stained with haematoxylin-eosin according to the criteria established by Bloom and Richardson (1957), modified by Elston (1988), and histological type according to the WHO classification (1981).

Metastatic lymph nodes

Axillary clearance was performed in 118 cases to determine the presence or absence of lymph-node metastasis (pN).

Immunohistochemical study

The methodology previously described (Sirvent et al., 1994) was used for the determination and assessment of hormone receptors and c-erbB-2.

Wax-embedded, formalin-fixed samples were used for the immunohistochemical determination of cell proliferation (Ki-67) and p53 protein. Briefly, sections measuring 4-6 µm were taken from blocks for each tumor; these were dewaxed and rehydrated, and placed in sodium citrate buffer solution at pH 6. The p53 specimens were subjected to five 2-minute pulses in a microwave oven (650-700 W) with a one-minute rest between each pulse— not allowing them to boil— and the Ki-67 specimens received two 5-minute pulses. They were left to cool for 20 minutes, and then underwent immunohistochemical analysis. The primary antibodies were polyclonal in the case of Ki-67 (Dako, AO47, Denmark) and monoclonal for p53 (Dako, DO7, M7001, Denmark) at dilutions of 1:50 and 1:25, respectively. The technique was completed using a StreptABComplex/HRP Duet kit (Dako, Denmark).

The cell-proliferation fraction was assessed by counting 500 cells, starting at random, expressing the number of positive cells as a percentage. The p53 protein was assessed according to the intensity of staining (I), with values from 0 to 2, and the number of positive cells was quantified as 1 when the percentage of positive cells was from 1 to 24%, 2 at 25-49% positive cells, 3 at 50-74% positive cells, and 4 at 75-100% positive cells. A histoscore was calculated by the formula, I x no. + cells; the range was from 0 to 8 (values of 5 and 7 were not found).

Statistical analysis

The Spearman test was used for comparative analysis of the different parameters.

Results

Histopathological characteristics, hormone receptors, cell-proliferation fraction and c-erbB-2

Of the 153 tumors analyzed, the most common histological type was infiltrating ductal carcinoma with 117 cases (76.4%), followed by lobular (8.5%) and medullary carcinomas (6.5%). The remaining histological types occurred at a rate of less than 3% (Table 1). Histological grade I tumors were more common (43.7%) than grades II and III (38.5% and 17.6%, respectively). Of the 118 cases in which the axillary nodes could be analyzed, 60 (50.8%) presented metastases in a varying number of nodes, ranging from 1 to 22. Positive ER stains were found in 63.4% of the tumors with a score above 100 (Fig. 1), and therefore were considered ER-positive, whereas 44.4% were PR-positive (Fig. 2). The percentage of tumor cells stained for Ki-67 ranged from 0 to 63 (X=11.6 and SD=13.85) (Fig. 3). The tumors were divided arbitrarily into two groups according to whether the percentage of positive cells was ≤10 or greater. Nearly 40% of the tumors had a cell-positive rate higher than 10%. Finally, nearly 31% of the carcinomas analyzed were positive for c-erbB-2 on the cytoplasmic membrane (Fig. 4).

The statistical analysis showed a direct relationship between the histological grade and the cell-proliferation fraction determined by Ki-67 (p=0.000) and c-erbB-2 (p=0.0037), while an inverse relationship was demonstrated with both types of HR (p=0.000). These receptors also showed a statistically significant inverse relationship with Ki-67 (p=0.000) and with c-erbB-2 (p=0.000).

On the other hand, there was a direction relation ship between Ki-67 and c-erbB-2 (p=0.0016).

In summary, the most differentiated tumors tended to be hormone-receptor positive, and frequently had a low cell-proliferation fraction and did not express c-erbB-2. On the contrary, the less differentiated tumors were more commonly hormone-receptor negative, and were found to have a high percentage of cells positive for Ki-67 and expression of c-erbB-2.

No statistically significant relationship was found between axillary node status and the rest of the variables analyzed.

Table 1. Menopausal status, histological type, differentiation grade, lymph-node status, hormonal receptors, p53, ki-67 and c-erbB-2.

	NUMBER OF CASES	PERCENTAGE
Menopausal status		
Premenopausal women	33	21.57%
Postmenopausal women	120	78.43%
Histological type		
Invasive ductal	117	76.49%
Lobular	13	8.50%
Medullar	10	6.53%
Invasive ductal carcinoma with a predominant intraductal component	4	2.62%
Mucinous	3	1.96%
Tubular	2	1.30%
Ductal + mucinous	2	1.30%
Invasive + ductal + lobular in situ	1	0.65%
Papillar	1	0.65%
Differentiation grade		
I	67	43.79%
II	59	38.56%
III	27	17.65%
Lymph-node status		
Negative	58	49.15%
Positive	60	50.85%
Not known	35	
Estrogen recptors		
Histoscore ≥100 (+)	97	63.40%
Histoscore <100 (-)	56	36.60%
Progesterone receptors		
Histoscore ≥100 (+)	68	44.44%
Histoscore <100 (-)	85	55.56%
Ki-67		
Positive cells >10%	61	39.87%
Positive cells ≤10%	92	60.13%
c-erbB-2		
Positive	47	30.72%
Negative	106	69.28%
p53		
Positive	67	43.79%
Negative	86	56.21%

p53

The stain was spread uniformly throughout the nucleus (Fig. 5). Stains for p53 antibody were found in 57 tumors (43.79%) (Table 1). The histoscore was between 0 and 8 (X=1.66 and SD=2.76).

The analysis of distribution by histological type (Table 2) highlighted the absence of any preference for p53 positivity and/or negativity in the case of ductal carcinoma, negativity in lobular carcinoma and strong positivity in medullary carcinoma.

It has already been noted that positivity for p53

Table 2. Association between p53 and histopathological characteristics.

	p53		
	Negative	Positive	p
Histological type			
Invasive ductal	68 (58.1%)	49 (41.9%)	
Lobular	10 (76.9%)	3 (23.1%)	
Medullar	2 (20.0%)	8 (80.0%)	
Others	6	7	
Differentiation grade			
I	42 (62.7%)	25 (37.3%)	
II	34 (57.6%)	25 (42.4%)	0.0048
III	10 (37.0%)	17 (63.0%)	
Lymph-node status			
Negative	28 (48.3%)	30 (51.7%)	N.S.
Positive	36 (60.0%)	24 (40.0%)	

increased as the degree of differentiation rose, and vice versa (Table 2).

The statistical analysis confirmed this relation (p=0.0048).

No association was found between the presence or absence of lymph-node metastasis and p53 expression (Table 2).

However, a comparison of HR and p53 (Table 3)

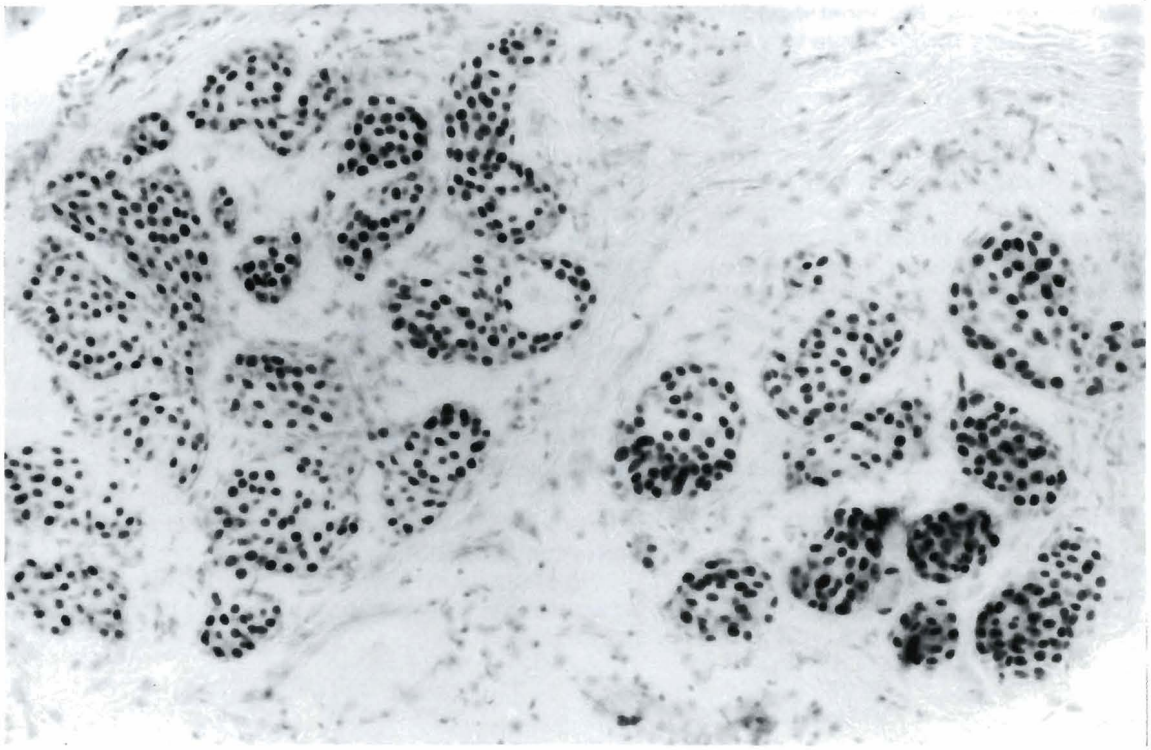

Fig. 1. Infiltrating lobular carcinoma. In situ lobular component. Estrogen receptors. Intensity of 3 in 94% of the cells (histoscore= 376). PAP, x 250

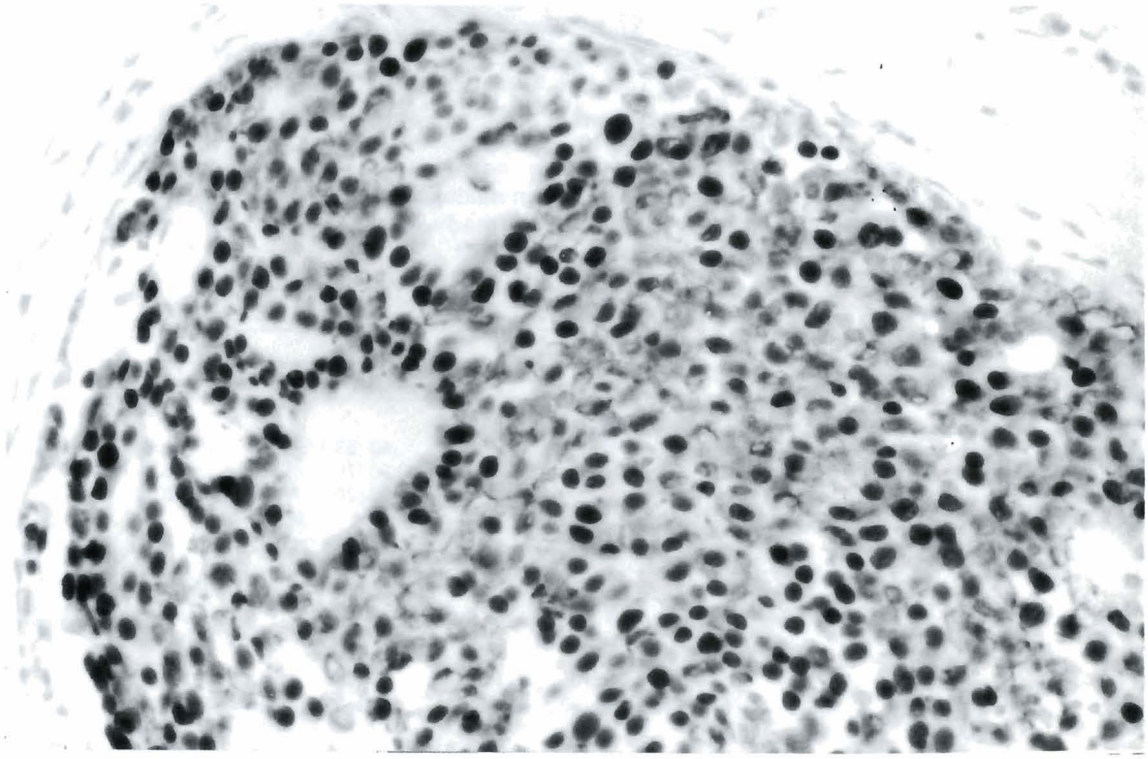

Fig. 2. Infiltrating ductal carcinoma. Intraductal component. Progesterone receptors. Intensity of 3 in 72% of the cells (histoscore= 288). PAP, x 400

showed that a higher histoscore was related to diminished immunoreactivity with p53 in both ER and PR, and vice versa. This inverse relationship was statistically significant (p=0.0052 and P=0.0215, respectively).

We then looked for a relationship between Ki-67 and p53, and found that as the number of cells positive for Ki-67 increased, the p53 score also rose (p=0.0035)

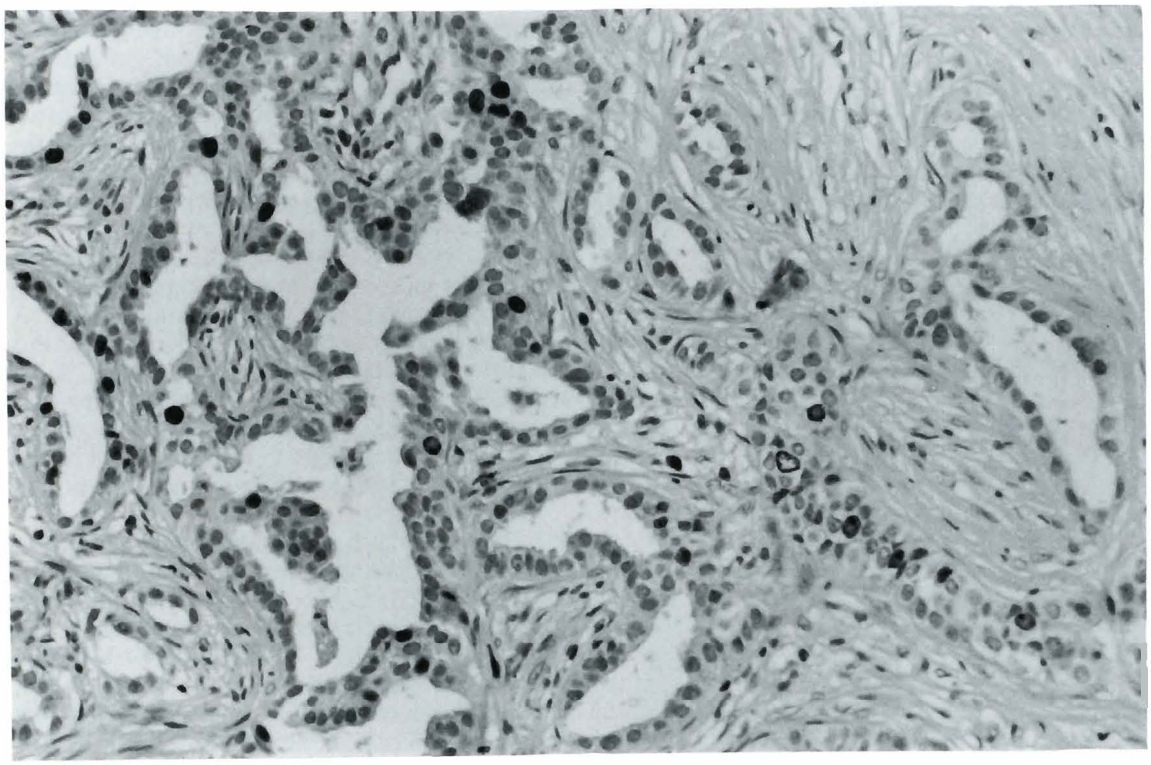

Fig. 3. Infiltrating ductal carcinoma. Ki-67. Low proliferation rate (7%). StreptABC, x 250

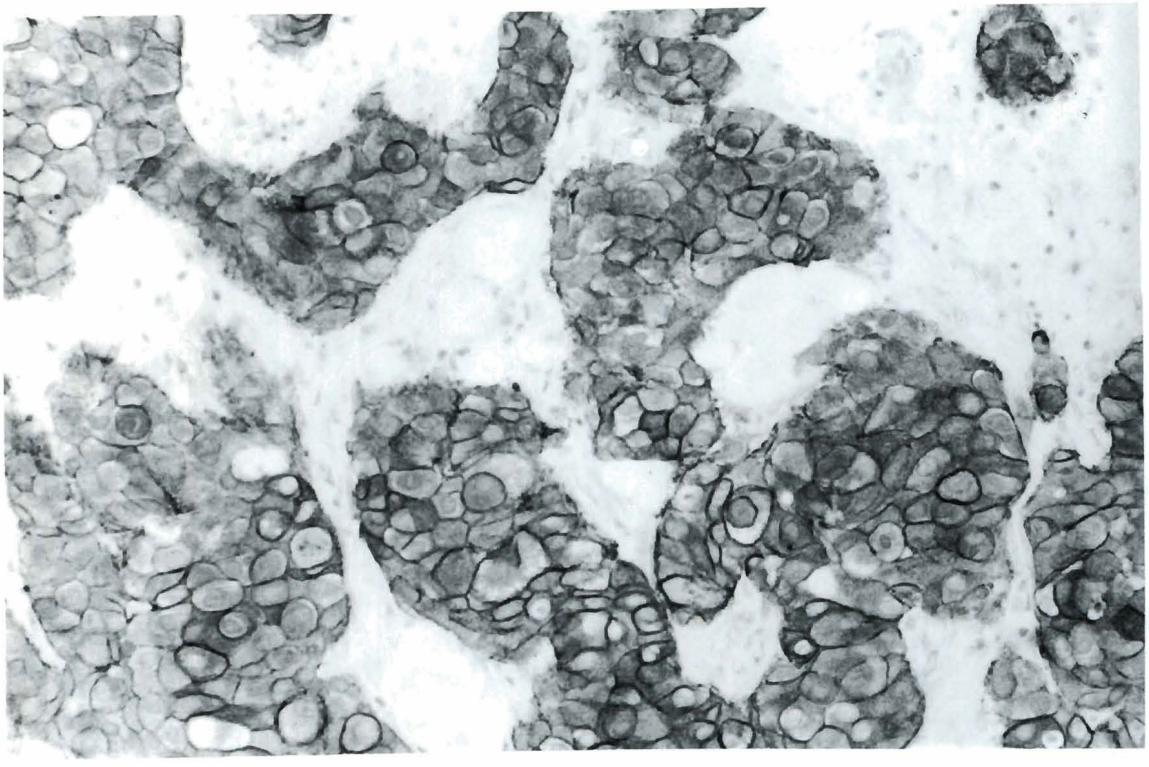

Fig. 4. Infiltrating ductal carcinoma. c-erbB-2. High positive rate (2) in cell cytoplasmic membrane (histoscore= 8). ABC, x 400

(Table 3).

No statistically significant relationship was found for the expression of c-erbB-2 upon comparing it with p53 expression (Table 3).

Discussion

At present the most important prognostic factor for breast cancer is still lymph-node status (Fisher et al., 1968; McGuire, 1987). Nevertheless, numerous attempts have been made to find other parameters that will aid in predicting the clinical outcome more accurately and in selecting the most appropriate therapy for each case (McGuire, 1987; Blamey, 1989).

There has been a systematic tendency to consider the histological grade as one of the factors to bear in mind in the clinical course of breast-cancer patients. In our study, as in previous works (Allred et al., 1993; Martinazzi et al., 1993; Sirvent et al., 1994), we observed a direct relationship between this parameter and others likewise indicative of poor prognosis (high cell-proliferation fraction as determined by Ki-67 and expression of the c-erbB-2 oncogene), and an inverse relationship with others considered to indicate a good prognosis (HR) and better therapeutic response.

Recent studies have shown that high proliferative activity in breast cancer, as determined by Ki-67, is indicative of poor prognosis (Bacus et al., 1989). A positive relationship has been documented between this factor and histological and nuclear grade (Bacus et al., 1989; Barbareschi et al., 1992), mitotic index and overexpression of c-erbB-2 (Sirvent et al., 1994); while a negative relation has been shown to exist with ER and PR (Leonardi et al., 1992), which our study has also shown.

Ki-67 is not only a good indicator of tumor proliferation, but is also an excellent relapse parameter (Veronese et al., 1993). Cases with a positive rate over 10% have a high incidence of relapse, and when the rate goes above 15%, death is the common outcome. However, patients whose rate is less than 4% do not usually relapse (Sahin et al., 1991). It seems feasible that the determination of cell proliferation (Ki-67) may be

Table 3. Association between p53 and RH, ki-67 and c-erbB-2.

	p53		
	Negative	Positive	p
Estrogen recptors			
Negative	22 (39.3%)	34 (60.7%)	0.0052
Positive	64 (66.0%)	33 (34.0%)	
Progesterone receptors			
Negative	45 (52.9%)	40 (47.1%)	0.0215
Positive	41 (60.3%)	27 (39.7%)	
ki-67			
≤10%	55 (55.8%)	37 (40.2%)	0.0035
>10%	31 (50.8%)	30 (49.2%)	
c-erbB-2			
Negative	62 (58.5%)	44 (41.5%)	0.0970
Positive	24 (51.1%)	23 (48.9%)	

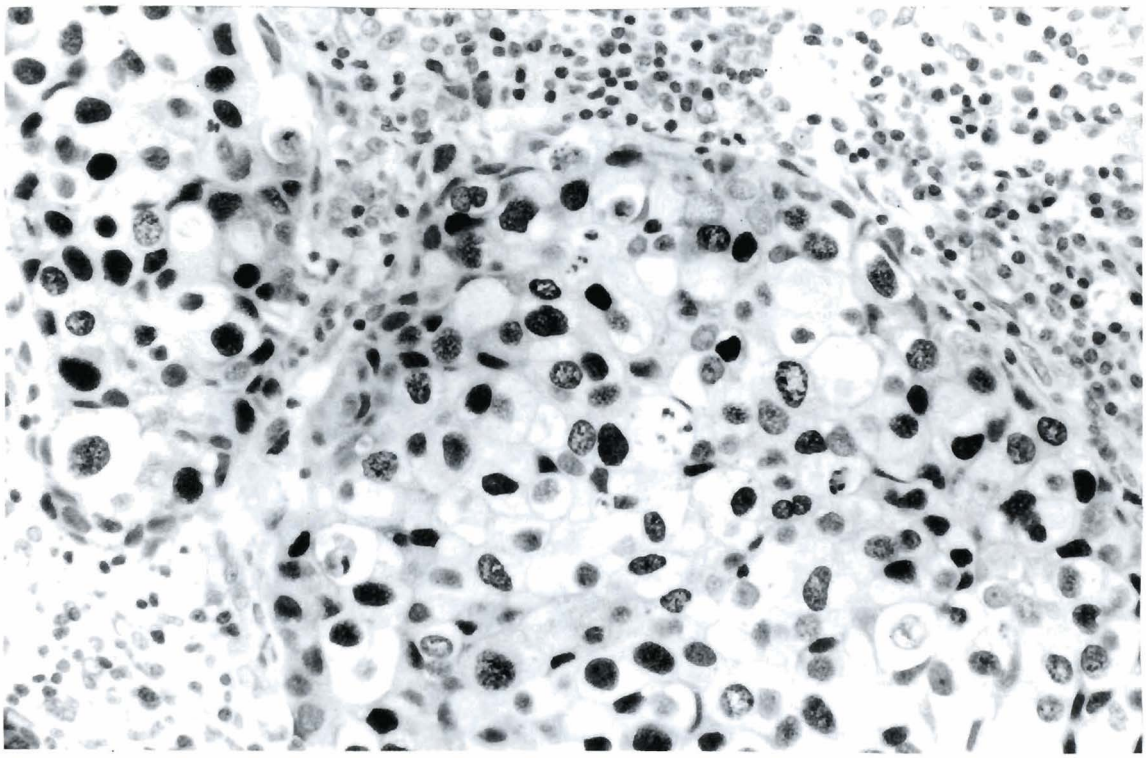

Fig. 5. Infiltrating ductal carcinoma. p53. High positive rate (2) in most cells (87%) (histoscore= 8). StreptABC, x 400

useful in predicting the likelihood of recurrence in the short term (Bacus et al., 1989). However, until recently, determination of the cell-proliferation fraction by Ki-67 could only be done with frozen tissue, which made it impossible to do retrospective studies when this material was not available. We now have a rabbit PAb which reacts in the Western Blot with bands identical to those of the Ki-67 MAb of 395 and 345 kilodaltons and, furthermore, has the advantage that it can be used with wax-embedded archival material that has been previously treated with two doses of 10 mM citrate buffer at pH 6 over a period of 5 minutes (Key et al., 1993).

The number of cases positive for c-erbB-2 varies according to the series and histological types, ranging from 25 to 30% (Schimmelpening et al., 1992). Its positivity has been correlated with poor prognosis, particularly in node-negative patients (Dykins et al., 1991). It has also been correlated with histological grade (Corbett et al., 1990; Rilke et al., 1991) and high cell-proliferation figures (Ki-67) and a diminished response to endocrine therapy (Nicholson et al., 1993).

The p53 gene appears to play a prime role in controlling cell proliferation and apopthosis, and in DNA repair (Oren, 1992; Yonish-Rouach et al., 1993). The genetic changes most commonly found in breast cancer are alterations in the p53 tumor-suppressor gene, with an incidence ranging from 15 to 50% in different series. These variations can be explained by the quality of the tissue used (frozen, fixed, stored, for a long time, etc), the number and type of antibody used, and also the interpretation of the results; it is well known that some positive cells may be taken as negative, which often happens (Bosari et al., 1992; Hurlimann et al., 1994). It may also depend on the number of cases of each histological type in a given series, since the accumulation of p53 protein is more common in high-grade ductal carcinoma and medullary carcinoma (Domagala et al., 1993; Martinazzi et al., 1993).

The p53 alteration may also reflect a greater degree of tumor progression and a higher proliferation rate, as well as a greater probability of micrometastases (Elledge et al., 1993). Mutation and the overexpression of p53 protein are directly related to histological grade (Isola et al., 1992; Bhargava et al., 1994; Hurlimann et al., 1994) and cell-proliferation fraction (Yamaguchi et al., 1992; Martinazzi et al., 1993); and they are inversely related to estrogen and progesterone receptors (Elledge et al., 1993; Martinazzi et al., 1993; Bhargava et al., 1994; Hurlimann et al., 1994). Cases positive for p53 and c-erbB-2 could be interpreted as those which have lost a mechanism for controlling the inhibition of cell proliferation and have gained an activator for malignancy potential (Barbareschi et al., 1992).

It has been found that the immunohistochemical expression of p53 is an independent prognostic marker on survival curves and in disease-free interval (Thor et al., 1992; Silvestrini et al., 1993). It has been seen that p53 is a very important independent indicator of early relapse of the disease, followed by tumor size and a high percentage of cells in S phase (Allred et al., 1993).

In another series the prognostic importance of p53 was second in significance only to lymph nodes (Barnes et al., 1993).

However, not all studies have reached the same conclusions. Bosari et al. (1992) looked at a group of 124 node-negative patients and found a correlation between p53 and other prognostic factors. But p53 expression was not shown to be an independent prognostic factor in disease-free interval or ten-year survival.

To be certainty, it will be necessary to unify the criteria for interpreting results, their significance, the use of different antibodies, tissue storage, etc (Battifora, 1994).

It is also possible that the p53 protein plays an important role in the progression of malignant human tumors (Porter et al., 1992). There are studies which suggest that p53 mutations occur late, such as in cancer of the colon. However, in breast cancer, immunohistochemical positivity is found in up to 25% of in situ carcinomas, which suggests that they may occur in early stages of the disease, before it becomes infiltrating. The staining patterns of metastatic lymph nodes are usually similar to those of primary tumors; only very rarely does a positive stain for p53 occur in a node when the tumor is negative (Poller et al., 1993; Bhargava et al., 1994).

In light of the lack of survival curves and disease-free interval data in the patient series, and bearing in mind that the prognosis of breast cancer depends on not just one factor but the conjugation of several (Bosari et al., 1992), greater tumor agressiveness could be postulated in those cases presenting a high degree of differentiation, negative HR, a high cell-proliferation rate (Ki-67) and positivity for c-erbB-2 and p53.

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