

1-1-2001

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### Recommended Citation

PEŞTERELİ, HADİCE ELİF; ERDOĞAN, OKAN; KILIÇARSLAN, BAHAR; ÇOLAK, TANER; TERCAN, EVREN; AKAYDIN, MUSTAFA; and KARAVELİ, ŞEYDA (2001) "Immunohistochemical Expression of C-Erb-B2, Steroid Receptors and Cathepsin-D in Breast Carcinomas: A Clinicopathologic Trial," *Turkish Journal of Medical Sciences*: Vol. 31: No. 2, Article 7. Available at: <https://journals.tubitak.gov.tr/medical/vol31/iss2/7>

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## Immunohistochemical Expression of C-Erb-B2, Steroid Receptors and Cathepsin-D in Breast Carcinomas: A Clinicopathologic Trial

Received: August 18, 2000

**Abstract:** Aim: We investigated the role of c-erb-B2, hormone receptors and cathepsin D in breast carcinoma progression and their correlation with each other.

**Methods:** Forty-six breast carcinoma patients, operated on in the department of surgery and diagnosed in the pathology department, were classified according to their histopathological characteristics; the results of immunostaining with estrogen and progesterone receptors, c-erb-B2 and cathepsin-D were correlated with the stage and the disease-free survival of the patients.

**Results:** Nuclear positivity was observed in 23 (50 %) cases for estrogen receptor and in 16 (34.8 %) cases for progesterone receptor. There was membranous staining with c-erb-B2 in 37 (80.4 %) cases and

cytoplasmic staining with cathepsin-D in 44 (95.6 %) cases. Cathepsin-D positivity was significantly related to the axillary lymph node metastases ( $p < 0.001$ ). A positive correlation was observed between c-erb-B2 and cathepsin-D positivity and also between estrogen and progesterone receptors ( $r = 0.45$ ,  $p < 0.001$ ).

**Conclusion:** The results of this study revealed a high incidence of c-erb-B2 oncogene overexpression in breast carcinomas from Turkish mediterranean women. Cathepsin-D positivity of the tumor cells was correlated with the lymph node metastases. Only estrogen receptor positivity was correlated with the stage of the tumour ( $p = 0.02071$ ).

**Key Words:** Breast carcinoma, cathepsin-D, c-erb-B2

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### Introduction

In the last few years, in addition to research into well-known clinicopathological factors like patient age, tumour size, axillary node status and hormone receptors, there have been a lot of studies concerning the biological factors that affect tumour behavior and also some that have attempted to explain the carcinogenesis in breast carcinoma. A cell-surface growth receptor and protooncogene c-erb-B2 overexpression which is said to have an adverse effect on prognosis, and also influences the response to chemotherapy, is one of these factors (1). C-erb-B2 gene amplification has been observed in 10-40% of breast carcinomas (2). Cathepsin-D, an aspartyl protease, is one of the factors that determine the invasion and the metastatic capacity of the tumour. It also has a mitogenic effect, in addition to its estrogen induced proteolytic activity (3). With all these effects on tumour

behavior, it is thought that the positivity of cathepsin-D in tumour and stromal cells influences the disease free and overall survival of the patient. We investigated the relationship between these two markers (c-erb-B2 and cathepsin-D), hormone status and clinicopathologic findings; at the same time, we tried to determine whether there was any correlation between these factors. The significance of these factors was analysed by the Kaplan-Meier method.

### Materials and Methods

Forty-six patients who had undergone modified radical mastectomy at the surgery department and who had been diagnosed as having infiltrative breast carcinoma at the pathology department were included in this study. The ages of the patients ranged from 40 to 73 (mean 50.8) years old. On histopathological examination,

25 of the 46 patients were revealed as having infiltrative ductal tumours, 8 had infiltrative ductal and lobular tumours, 5 had infiltrative lobular tumours, 2 had medullary tumours, 2 had mucinous tumours, 2 had apocrine tumours, 1 signet ring cell tumours and there was 1 carcinoma with neuroendocrine differentiation. The tumours were greater than 2 cm in 32 (69.6 %) cases and smaller or equal to 2 cm in 14 (30.4 %). Staging was done according to UICC<sup>4</sup> and the clinicopathologic findings are summarized in Table 1. Immunohistochemical staining was performed on paraffin-embedded tissue sections fixed with buffered formalin. Sections of 4µm thickness were mounted on poly-L-lysine coated slides, deparaffinized and hydrated through graded alcohols to water. Optimum pretreatment and dilutions were determined by testing with both known positive and negative material. The slides were microwaved in citrate buffer for 10 min in an 800 W microwave oven for antigen retrieval, then allowed to cool for 20 min. The slides were stained with a Dako TechMate 500 automated immunostainer using the standard streptavidin-biotin method. The primary antibodies used are listed in Table 2. Following the endogenous peroxidase and protein blocking step, the slides were incubated with primary antibodies for 45 min. After brief washes, they were incubated in a cocktail of

Table 1. Clinicopathologic characteristics of the patients

Histopathologic type (n:46)		
Infiltrative ductal carcinoma	25	(54.3 %)
Infiltrative lobular carcinoma	5	(10.9 %)
Infiltrative ductal+ lobular	8	(17.4 %)
Medullary carcinoma	2	(4.3 %)
Apocrine carcinoma	2	(4.3%)
Mucinous carcinoma	2	(4.3 %)
Signet ring cell carcinoma	1	(2.2 %)
Carcinoma with neuroendocrine differentiation	1	(2.2 %)
Stage		
I	1	(2.17 %)
II A	15	(32.6 %)
II B	12	(26.1 %)
III A	16	(34.8 %)
III B	2	(4.3 %)
Size of the tumour		
< 2 cm	14	(30.4 %)
> 2 cm	32	(69.6 %)
Lymph node metastases		
Positive	34	(73.9 %)
Negative	12	(26.1 %)

Table 2. List of the antibodies used

Antibodies	Dilution	Source
Estrogen receptor	1:50	Dako
Progesterone receptor	1:10	Dako
C-erb-B2	1:50	Dako
Cathepsin-D	1:50	Dako

biotinylated anti-mouse IgG/IgM and anti-rabbit IgG for 30 min. The sections were then washed and incubated in streptavidin-biotin complex for 30 min, and reacted with diaminobenzidine and hydrogen peroxide in order to examine the end product. Mayer's haematoxylin was used as a counterstain. Immunohistochemical reactions were evaluated semiquantitatively for the percentage of the stained area and the intensity of the staining as follows: +/mild, ++/moderate, +++/ strong. Staining more than 5 % of specimen was regarded as positive for statistical analysis. Nuclear staining for hormone receptors, membranous for c-erb-B2 and cytoplasmic staining for cathepsin-D were all evaluated (Figures 1-3). Positive staining of stromal cells with Cathepsin-D was not observed.

**Statistical Analysis:**

Chi-square, Fisher's Chi-Square and Spearman correlation tests were used for statistical analysis. The follow-up period of the patients was between 2 and 40 months (mean 21 months) and the effect of each marker on the disease-free and overall survival was analysed by a Kaplan-Meier curve.

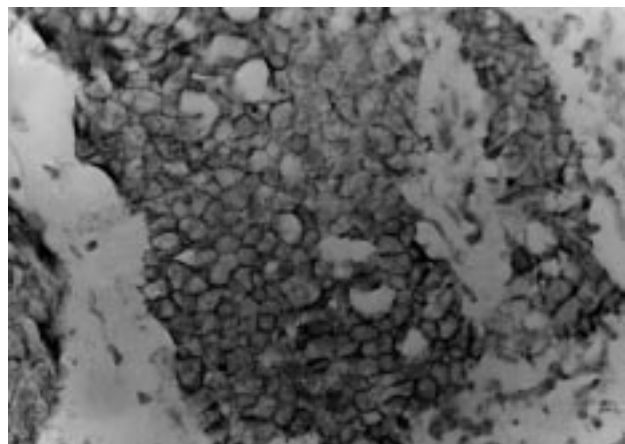


Figure 1. Membranous c-erb-B2 positivity in infiltrative ductal carcinoma, x400, DAB

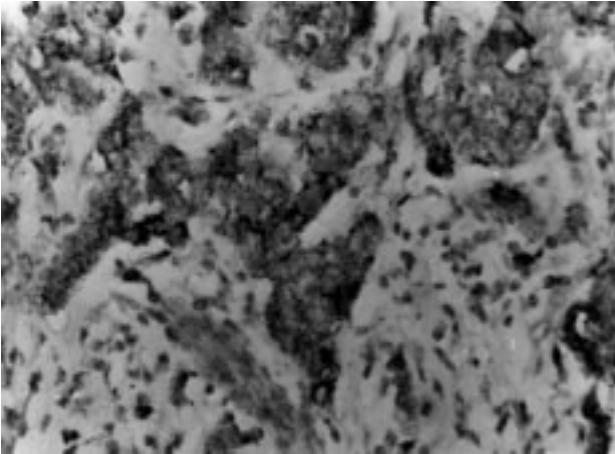


Figure 2. Cytoplasmic cathepsin-D positivity in infiltrative ductal carcinoma, x400, DAB

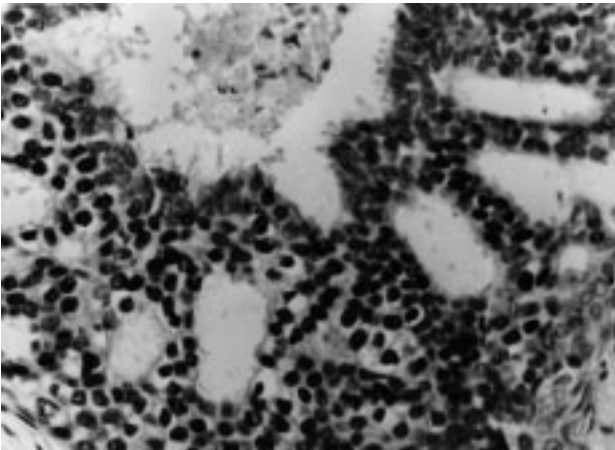


Figure 3a. Nuclear estrogen receptor positivity in infiltrative ductal carcinoma, x400, DAB

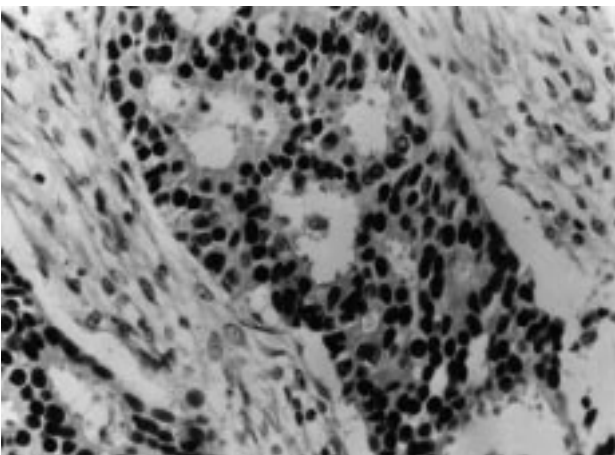


Figure 3b. Progesterone receptor positivity in infiltrative ductal carcinoma, x400, DAB

## Results

Most of the tumours (54.3 %) were infiltrative ductal carcinomas, and had lymph node metastases (76.1 %). Estrogen receptor was found to be positive in half of the carcinomas, whereas progesterone receptor was positive in 16 of them. Seventeen stage II and 5 stage-III cases were negative for estrogen receptor; for progesterone receptor, these values were 17 and 12 respectively. There was only a statistically significant difference between the estrogen receptor positivity of stage-II and stage-III carcinomas ( $p=0.02071$ ). We did not find any correlation between the progesterone receptor status and stage or the histopathologic type of carcinoma. In 12 cases, both estrogen and progesterone receptors were positive. A significant correlation between estrogen and progesterone receptor positivity was ( $r=0.53$ ,  $p=0.001$ ).

C-erb-B2 was positive in 37 (80.4 %) of the tumours. The positivity of c-erb-B2 was even higher, 83.3 % in stage-III carcinomas, but the differences in c-erb-B2 overexpression between the stages were not significant.

Cathepsin-D was positive in all the tumours except for two stage-II carcinomas. There were no correlations between cathepsin-D positivity and the stage or histopathological type. However, cathepsin-D was positive in all of the lymph node positive cases and the difference was statistically significant ( $p=0.01449$ ). The results are summarized in Tables 3,4 and 5.

A positive correlation was found between c-erb-B2 overexpression and cathepsin-D positivity ( $r=0.45$ ,  $p=0.008$ ).

Five patients had distant organ metastases (lung, bone), 1 in the 4<sup>th</sup> year, 2 in the 3<sup>rd</sup> year, 1 in the 2<sup>nd</sup> year and 1 in the 6<sup>th</sup> month of the postoperative period. All the carcinomas in these patients showed strong c-erb-B2 and cathepsin-D positivity (90 %, +++).

## Discussion

In this study, we did not find any relationship between hormone status, c-erb-B2 positivity, cathepsin-D positivity and the stage of the tumours. There was a significant difference only in the estrogen receptor positivity between the stages. In the present study, the number of the patients at each stage was small. Most of the tumours (32, 69.6 %) were T2/T3 and node positive (34, 73.9 %), and this may have affected the results. In most of the studies in the literature, including that of Van de Vijver et al., a positive correlation between neu-protein overexpression and tumour size was found (5). In our

	Stage II (n: 27)		Stage III (n: 18)
Estrogen receptor			
positive	10 (27.1 %)	p=0.02071	13 (72.2 %)
negative	17 (62.9 %)		5 (27.8 %)
progesterone receptor			
positive	10 (27.1 %)	p=0.79928	6 (33.3 %)
negative	17 (62.9 %)		12 (66.7 %)
C-erb-B2			
positive	22 (81.5 %)	p=1.00000	15 (83.3 %)
negative	5 (18.5 %)		3 (16.7 %)
Cathepsin-D			
positive	25 (92.6 %)	p=0.50909	18 (100 %)
negative	2 (7.4 %)		0 (0 %)

Table 3. Stages of the tumours and immunohistochemical results

	LN + (n:34)		LN - (n:12)
Estrogen receptor			
positive	18 (52.9 %)	p=0.50187	5 (41.7 %)
negative	16 (47.1 %)		7 (58.3 %)
Progesterone receptor			
positive	13 (38.2 %)	p=0.49770	3 (25 %)
negative	21 (61.8 %)		9 (75 %)
C-erb-B2			
positive	28 (82.4 %)	p=0.67773	9 (75 %)
negative	6 (17.6 %)		3 (25 %)
Cathepsin-D			
positive	34 (100 %)	p=0.01449	9 (75 %)
negative	0 (0 %)		3 (25 %)

Table 4. Lymph node (LN) status and immunohistochemical results

study, all of the T2/T3 tumours exhibited strong cell membrane positivity for c-erb-B2. Although the rate of c-erb-B2 positivity in our patients was much higher than that in most other studies, we thought that this may be due to the characteristics of the population that we were dealing with or due to the c-erb-B2 antibody that we used. To improve our hypothesis, we will study the same patients with more sophisticated techniques like PCR and FISH. Midulla et al., observed c-erb-B2 positivity in 89.4 % of 77 breast carcinoma patients (6).

Though cathepsin-D is an estrogen-induced protease, we did not discover any positive relationship between

these two markers using the immunohistochemical method. However, cathepsin-D was positive in all node positive tumours (34 out of 46 cases) and the difference between the lymph node positive and negative tumours was significant (p=0.01449). In the literature, while some investigators have mentioned the same relationship (7, 8, 9), others were unable to identify any correlation between node status and the cathepsin-D positivity of tumours (10, 11). We propose that cathepsin-D positivity of the tumour cells reveal aggressive tumour behavior.

The correlation between c-erb-B2 and cathepsin-D, which is known to affect prognosis adversely, also was

Table 5. Correlation of hormone status with c-erb-B2 and cathepsin-D

	Estrogen positive	receptor negative	Progesterone positive	receptor negative
C-erb-B2				
positive	19	19	14	23
negative	3	5	3	6
Cathepsin-D				
positive	22	22	16	28
negative	1	1	0	2

found by Schultz et al., (12). There have also been reports in the literature that reveal no correlation (13, 14).

The strong positivity of c-erb-B2 and cathepsin-D in tumours which metastasized and also in the tumours of patients who died of their illness supports studies showing a negative effect of these two markers on prognosis (5, 15, 16). However, in studies with multivariate analysis, there have been conflicting results with regard to the independent prognostic effect of these factors (1, 17, 18). In our study, we concluded that c-erb-B2 positivity in Turkish Mediterranean patients with breast carcinoma was extremely high. It could have a negative effect on tumour behaviour. Tumors with

cathepsin-D positivity might also exhibit aggressive behaviour, but to prove their role in tumour aggressiveness, the number of patients must be increased, and future studies should include at least ten years of follow-up.

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