Expression of p53, c-erbB-2 and Cathepsin D in Relation to Hormone Receptors in Primary Breast Cancer

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Abstract : **The immunohistochemical expression of p53, c-erbB-2, and cathepsin D oncogene proteins was examined in 494 primary breast carcinomas. This study was aimed to investigate an association of expression of these three proteins with other variables known to be related to poor prognosis as well as with 5-year disease free survival (DFS). P53, c-erbB-2, and cathepsin D alone or in combination were negatively correlated with the presence of estrogen and progesterone receptors in breast cancer tissues. Alteration of these oncogenes rendering an expression of the proteins might affect the synthesis of steroid receptor proteins during the course of breast cancers. However, their significance as predictors of 5- year DFS was not achieved in this group of patients. Lymph node invasion was the only independent indicator for recurrent or metastatic breast carcinoma.**

เรื่องย่อ : ความสัมพันธ์ของพี 53, ซีเอิร์บบีสอง, คาเทปซิน ดี และตัวรับฮอร์โมนในมะเร็งเต้านมชนิดปฐมภูมิ

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ได้ตรวจวัดออองโคยีนโปรตีน พี 53, ซีเอิร์บบีสอง, และคาเทปซิน ดี ในมะเร็งเต้านมชนิดปฐมภูมิจากผู้ป่วยสตรี 494 ราย การศึกษาครั้งนี้มีจุดมุ่งหมายเพื่อหาความสัมพันธ์ระหว่างโปรตีนทั้ง 3 ชนิดในเนื้อมะเร็งกับปัจจัยอื่นๆ ซึ่งบ่งชี้ความรุนแรงของโรค รวมทั้งความสัมพันธ์กับระยะปลอดโรคในช่วง 5 ปีของการติดตามผู้ป่วย พบว่าโปรตีน พี 53, ซีเอิร์บบีสอง และคาเทปซิน ดี ที่ตรวจพบไม่ว่าเพียงลำพัง หรือพบร่วมกันเป็นคู่ มีความสัมพันธ์ตรงกันข้ามกับตัวรับเอสโตรเจนและตัวรับโปรเจสเตอโรนในเนื้อมะเร็ง โปรตีนที่เกิดจากการเปลี่ยนแปลงของยีนมะเร็งทั้ง 3 ชนิด อาจรบกวนการสร้างตัวรับสตีรอยด์ทั้งสองชนิดในการดำเนินโรค อย่างไรก็ตามไม่พบความสัมพันธ์ที่มีนัยสำคัญระหว่างโปรตีนที่ศึกษาชนิดใดกับระยะปลอดโรค 5 ปี การลุกลามของเซลล์มะเร็งไปยังต่อมน้ำเหลืองเป็นปัจจัยอิสระเพียงชนิดเดียวที่สามารถบ่งชี้การกลับเป็นซ้ำหรือการกระจายของโรคมะเร็งเต้านมในระยะ 5 ปีได้

INTRODUCTION

Breast cancer is the second most common malignancy among Thai women. About 40-50 % of breast cancer patients will eventually die from their disease. Adjuvant therapy after initial surgery though improves patient's survival, its cytotoxic and adverse effects also exist. Information about tumor size, histologic type, nuclear grade, axillary lymph node involvement, as well as estrogen and progesterone receptors status have been used as the predictive factors of prognosis and treatment outcome in breast cancer. Recently, the role of oncogene proteins in the pathogenesis and progression of breast carcinoma has been widely studied, since they may provide useful information concerning the course of the disease and may find a solution to treatment.

The p53 gene is located on the short arm of chromosome 17. Mutations in exon 5-9 of the p53 gene have been reported in different human tumors.[1] Such mutations favor the expression of a more stable protein that accumulates in the nucleus.[2] It has been suggested that p53 protein expression is present in the early stages of human breast cancer[3] and overexpression of p53 is an indicator of tumor progression and poor prognosis.[2,4,5]

The proto-oncogene, c-erbB-2 is also detected on chromosome 17 at q21. It encodes a protein that is generally derived from gene amplification.[6] Amplification of the c-erbB-2 gene has been observed in about 29% of primary breast cancers in Thai women.[7] However, there is controversy concerning c-erbB-2 protein overexpression in human breast carcinoma in relation to its ability to predict survival and responsiveness to treatment.[8-9]

Cathepsin D (CD), an estrogen-induced lysosomal enzyme involved in intracellular proteolysis, is overexpressed and secreted in most breast cancer cells[10] as well as in stromal cells.[11] It has been originally proposed that the presence of cathepsin D indicates poor prognosis of breast carcinoma[12-14] but additional studies disagree with the previous results.[15-17]

The conflicting data about the association of these three oncogene proteins with the course of breast cancer requires further study. Immunohistochemistry (IHC) provides the opportunity to study the histomorphology together with specific localization of cell and tissue antigens. We have used the IHC assay to localize the protein p53, c-erbB-2, and cathepsin D in primary breast cancer in Thai women. The expressions of these protein markers have been correlated with other prognostic indicators, as well as with the patient's survival.

MATERIALS AND METHODS

Patients and Tumor Tissues

Breast cancer specimens were collected from 494 women admitted to the Department of Surgery, Faculty of Medicine Siriraj Hospital from 1992 to 2000. Tumor tissues were kept at -80°C before fixing in 10% fomalin with 2% $ZnSO_4$, dehydrated with absolute alcohol, cleared with xylene and

embedded in paraffin. The TNM classification of pathological stage[18] was used to classify the breast cancer patients. Estrogen receptor (ER) and progesterone receptor (PR) were measured as described previously.[19,20] Only 479 out of 494 cases were followed up for the duration of the study. The follow-up time ranged from 6-195 months (median 48 months).

Immunohistochemistry

Procedures: Fomalin-fixed, paraffin-embedded tissue sections 3-4 mm thick were stained for each protein by a modified avidin-biotin-complex method.[21] After deparaffinization with xylene, the tissue slides were dehydrated with absolute alcohol and treated with 0.3% hydrogen peroxide in methanol for 20-30 min to inhibit an endogenous peroxidase activity in tissue. The quality of staining was improved by immersing the slides in 10 mM citrate buffer, pH 6.0 and heating in a microwave oven for 5-10 min. The slides were then rinsed in Tris-phosphate buffered saline (PBS) a few times before incubation with 3% normal swine serum (NSS, DAKO) in PBS for 20-30 min at room temperature. An appropriate dilution in 2-3% NSS of individual primary antibody (DAKO polyclonal rabbit anti-human c-erbB-2 at 1:400 dilution or anti-cathepsin D at 1:600 dilution or anti-p53-DO-7 monoclonal mouse antibody at 1:800 dilution) was applied to the slides and left for 30 min before washing the slides in PBS and re-incubated in 2-3% NSS for 20-30 min.

The second antibody (DAKO, 1:200 biotinylated donkey anti-rabbit immunoglobulin for c-erbB-2 and cathepsin D staining or 1:200 biotinylated rabbit anti-mouse immunoglobulin for p53 staining) was applied to an appropriate slide. After 30 min, the slides were washed with PBS and NSS respectively. The streptavidin biotin horse radish peroxidase complex (1:500) was applied to slides and left for 30 min before adding 0.1% to diaminobenzidine tetrahydrochloride in 0.02% hydrogen peroxide as a chromogen. Five to ten min later, haematoxylin staining was performed, followed by dehydrating in absolute alcohol, clearing in xylene and mounting in permount.

The positive and negative control slides for each oncogene protein were included in every assay batch.

Scoring: Five hundred tumor cells on each slide were counted for immuno-reactivity. The c-erbB-2 cytoplasmic staining or no membrane staining was scored as negative, + for < 10% membrane staining and ++ for >10% membrane staining.

Less than 5% of p53 nuclear staining or cytoplasmic staining only was scored negative. Nuclear staining between 5-20% was scored +, and ++ for > 20% stained.

For cathepsin D, <10% of cytoplasmic staining was scored negative, + for > 10-25% stained, and ++ for > 25% stained.

One hundred and twenty breast cancer tissue specimens were simultaneously assayed for CD protein expression and in cytosol as described elsewhere.[22]

Statistical Analysis

Quantitative variables were compared using Mann-Whitney U or Kruskal-Wallis test. Chi-square was used to evaluate significance of correlation. Cox regression test and Kaplan Meier method with log rank comparison were used for survival analysis. A probability level of < 0.05 was the criterion of significance. Calculations were performed using Statview PC version 4.5.

RESULTS

The positivities of p53, c-erbB-2 and cathepsin D by IHC assay were 24.1% (119/494), 32.6% (161/494) and 35.4% (175/494) respectively. The distribution of these three oncogene proteins' immunoreactivity and the staining pattern of positive scores are shown in Table 1 and Figure 1-3. Positive and negative agreements between each pair of protein markers are tabulated in Table 2. Expression of pairs of proteins was significantly correlated to each other. Statistical agreement between the two methods used for CD measurement was not detected and the positive/negative agreement was only 50%.

Clinicopathological characteristics of 494 patients according to p53, c-erbB-2 and cathepsin D positivity status are summarized in Table 3. Higher expression of p53 and c-erbB-2 was seen in ER- or PR-negative tumors compared with ER- or PR-positive ones and the highest percentage expression was found in both ERPR-negative breast cancer.

However, ER-positive tumors overexpressed cathepsin D immunoreactivity more than ER-negative tumors. The Mann-Whitney U test confirmed the negative relationship between steroid receptors and p53 or c-erbB-2 as well as the positive association between PR and cathepsin D but did not support the positive relationship between ER and cathepsin D (Figure 4). However, when the two proteins were considered together, the significant negative association between ER, PR or ERPR and positivity of the protein pair was shown (Table 4).

Concerning other poor prognostic indicators of breast cancer, a positive relationship was shown between c-erbB-2 status and the number of lymph nodes invaded ($p = .04$). No significant association of proteins studied with any other factor determining a bad prognosis was detected (Table 3).

Cox univariate analysis among the 281 patients shows that the number of positive lymph nodes and lymphatic invasion were significant indicators for 5-year disease free survival (DFS) in these patients (Table 5, Figure 5). However, when multivariate analysis that included all possible poor prognostic factors and treatment was performed, the number of positive axillary lymph node was the only independent predictor of survival in breast cancer.

DISCUSSION

In the present study, p53 expression indicating mutation of the gene occurred in 24% of breast tissue samples. This is within the range 16-52% reported by several authors.[23] Differences in the antibodies used and methodological conditions as well as scoring criteria may explain the variation of p53 expression in different studies. It has been suggested that wild type p53 regulates ER expression and is associated with ER positivity.[24] Conversely, mutant p53 gene that renders the protein dysfunctional and thereby impairs ER expression is associated with ER negativity.[24] Significant negative correlation between mutant protein p53 and ER or PR was observed among our patients in which in 67% of p53 positive tumors both ER and PR could not be detected. A similar relationship between ER and p53 expression has been reported before[2,5,8,24,25] but is opposite to other findings.[26,27] The association of p53 expression with other poor prognostic factors in breast cancer was not detected in this study as in others.[2,24,28,29] However, a positive relationship between p53 expression and large tumor size,[26,30] lymph node invasion[27] or histological grade[25,30] has been reported by several authors.

Overexpression of the c-erbB-2 protein was observed in 32.6% by IHC in this study. It has been suggested that breast cancer prognosis is related to a gradual loss of estrogen requirement for tumor growth, until the neoplasm becomes estrogen independent. This escape from hormonal control may be associated with c-erbB-2 overexpression and is probably related to a particularly invasive potential.[31] Thus, an association of c-erbB-2 overexpression with increased axillary node involvement and ER-negative status found in this study which corresponded to previous findings[26, 32] might be resulted from the metastatic capability of c-erbB-2 positive tumor.

Estrogen-regulated proteins such as cathepsin D may be better prognostic indicators than ER alone, since their presence denotes a functioning ER.[12] In this study, cathepsin D positivity was higher in ER-positive tumor compared to ER-negative tumor. Only 40% of cathepsin D positive tumor had positive ER. This is similar to a previous report[33] of 50% ER positivity in CD positive female breast cancers. Both data indicate that some cathepsin D positive breast tumors tissues are not regulated by estrogen. Cathepsin D expression in the present study was 35.4%, which is close to the value 36.4% positive CD staining in tumor epithelial cells reported by Donoghue's group.[34] These authors as well as others [12,34,35] found that not only breast cancer cells, but also stromal cells and macrophages stained positive for CD immunoreactivity. We also found a discrepancy between CD protein expression in tumor cells detected by IHC and CD concentrations in tissue cytosol measured by enzyme immunoassay, the latter of which might include CD from several cell sources. Since CD is expressed in many different cell types, this may partly explain the higher percentage of CD expression (40-60%) published previously.[17,35-37]

Significant correlation between two oncogene proteins was detected and the positive and negative agreement of each pair were not much

Table 1. Comparison of antibodies, staining patterns and per cent immunoreactivity scores of p53, c-erbB-2, and cathepsin D.

Source	Antiserum Type	Dilution	Pattern of Reactivity Scored	% Immunoreactivity -	+	++
p53 DAKO*	Monoclonal	1:800	Nucleus	75.9	13.2	10.9
c-erbB-2 DAKO*	Polyclonal	1:400	Membrane	67.4	12.6	20.0
Cathepsin D DAKO*	Polyclonal	1:600	Cytoplasm	64.4	20.4	15.2

*DAKO Corporation, California

Table 2. Positive and negative agreements between protein pairs.

	% Negative Agreement	% Positive Agreement	% Total Agreement
p53/c-erbB-2*	55.3	11.9	67.2
c-erbB-2/cathepsin D*	48.8	16.8	65.6
p53/cathepsin D*	51.1	10.9	62.0

*Significant association between two markers at p value < .01

different (Table 2). A combination of p53 and c-erbB-2 gives the highest agreement (67.2%) in our patients, which is similar to 68.5% reported by Albanell's group.[38] Moreover, overexpression of each protein pair was associated with negativity of ER and PR (Table 4). The negative relationship between steroid receptor status and expression of protein markers suggests the poor prognostic prediction of these proteins alone or in combination on outcomes of breast cancer patients.

Univariate and multivariate Cox survival analyses did not show a significant relationship of any studied protein markers on 5-year DFS. Our results agree with most investigators[8,12,15,16,25,30,34,35,38] but disagree with some studies.[5,29,36] Expression of p53, c-erbB-2 and cathepsin D may not have strong predictive value for relapse. However, the expression of these protein markers may predict the patient who will not response to chemo- or hormone therapy. Axillary lymph node and lymphatic invasion were two predictive factors for 5-year DFS in our patients in univariate analysis, but in multivariate analysis only lymph node invasion was shown to be a significant survival indictor among breast cancer patients.

Table 3. Relationship between p53, c-erbB-2, and cathepsin D overexpression and prognostic factors of breast cancer patients (N = 494).

	N	p53 Number (%) positive	P value	c-erbB-2 Number (%) positive	P value	Cathepsin D Number (%) positive	P value
Age							
≤ 50 y	242	55 (22.7)	.47	71 (29.3)	.11	87 (36.0)	.79
> 50 y	247	63 (25.5)		89 (36.0)		86 (34.8)	
Menopausal status							
Premenopause	246	53 (21.5)	.17	74 (30.1)	.20	87 (35.4)	.97
Postmenopause	242	65 (26.9)		86 (35.5)		86 (35.5)	
Tumor size							
≤ 20 mm	85	14 (16.5)	.07	23 (27.1)	.19	25 (29.4)	.21
> 20 mm	378	97 (25.7)		130 (34.4)		138 (36.5)	
Nodal status							
Negative	200	48 (24.0)	.09	59 (29.5)	**.04**	73 (36.5)	.66
1-3	129	31 (24.0)		48 (37.2)		42 (32.6)	
4-10	91	28 (30.8)		24 (26.4)		36 (39.6)	
> 10	57	7 (12.3)		26 (45.6)		18 (31.6)	
Pathological stage							
1	45	8 (17.8)	.57	9 (20.0)	.06	15 (33.3)	.31
2	354	83 (23.4)		116 (32.8)		123 (34.7)	
3	59	19 (32.2)		24 (40.7)		20 (33.9)	
4	1	0 (0)		1 (100.0)		1 (100.0)	
Histological type of invasive tumors							
Invasive ductal	422	101 (23.9)	.08	143 (33.9)	.18	150 (35.5)	.50
Invasive lobular	9	2 (22.2)		0 (0)		3 (33.3)	
ER status							
Negative	233	82 (35.2)	**<.0001**	99 (42.5)	**<.0001**	71 (30.5)	**.03**
Positive	261	37 (14.2)		62 (23.8)		104 (39.9)	
PR status							
Negative	405	106 (26.2)	**.03**	141 (34.8)	**.03**	138 (34.1)	.13
Positive	87	13 (14.9)		20 (23.0)		37 (42.5)	
ERPR status							
- -	223	80 (35.9)	**<.0001**	97 (43.5)	**<.0001**	69 (30.9)	.07
- +	10	2 (20.0)		2 (20.0)		2 (20.0)	
+ -	182	26 (14.3)		44 (24.2)		69 (37.9)	
+ +	77	11 (14.3)		18 (23.4)		35 (45.4)	

Table 4. The association between overexpression of pairs of protein markers and hormone receptor status.

	N	p53 + c-erbB-2+ Number (%)	P value	c-erbB-2 + Cathepsin D + Number (%)	P value	p53 + Cathepsin D + Number (%)	P value
ER status							
Negative	233	44 (18.9)	<.0001	48 (20.6)	<.0001	31 (13.3)	<.0001
Positive	261	15 (5.7)		35 (13.4)		23 (8.8)	
PR status							
Negative	405	52 (12.8)	.02	70 (17.3)	.047	47 (11.6)	.03
Positive	87	7 (8.0)		13 (14.9)		7 (8.0)	
ERPR status							
- -	223	42 (18.8)	<.0001	47 (21.1)	<.0001	30 (13.4)	<.0001
- +	10	2 (20.0)		1 (10.0)		1 (10.0)	
+ -	182	10 (5.5)		23 (12.6)		17 (9.3)	
+ +	77	5 (6.5)		12 (15.6)		6 (7.8)	

Table 5. Cox univariate and multivariate analyses with 5 - year disease free survival (N=281).

	Univariated			Multivariated		
	Relative risk	Range	P value	Relative risk	Range	P value
Number of positive lymph nodes	1.07	1.04-1.09	<.0001	1.08	1.02-1.15	.005
Lymphatic invasion (-)	0.42	0.21-0.86	.018	0.61	0.21-1.78	.363
ER (-)	1.18	0.65-2.15	.585	0.85	0.22-3.35	.815
PR (-)	1.73	0.68-4.40	.249	4.29	0.30-61.82	.284
p53 (-)	1.18	0.57-2.46	.660	0.75	0.19-2.97	.679
c-erbB-2 (-)	0.96	0.51-1.79	.886	1.05	0.30-3.68	.941
Cathepsin D (-)	0.71	0.39-1.31	.275	0.59	0.16-2.21	.430

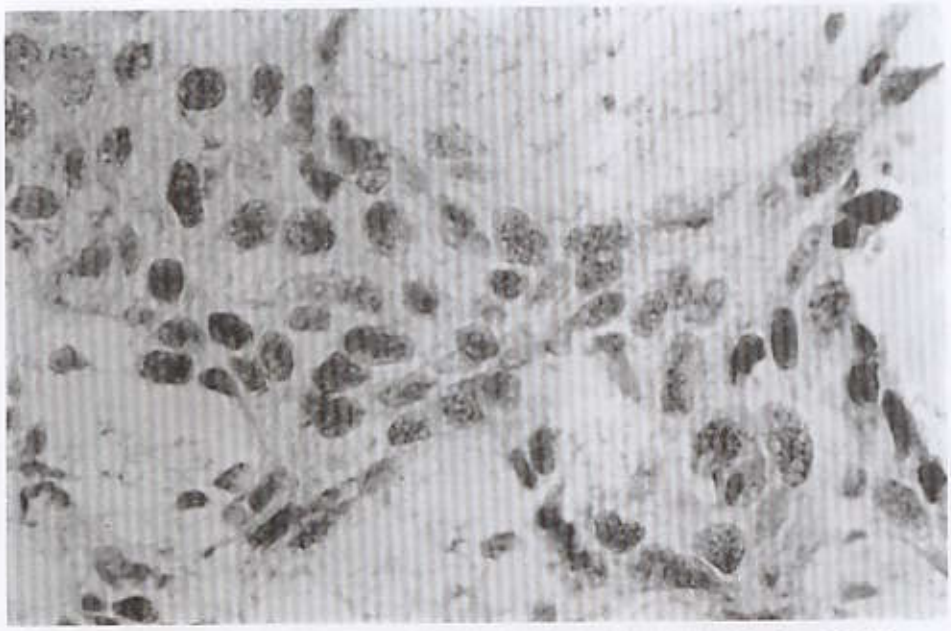

Figure 1. Nuclear IHC staining of p53 protein (original magnification X 400).

Figure 2. Membrane IHC staining of c-erbB-2 protein (original magnification X 400).

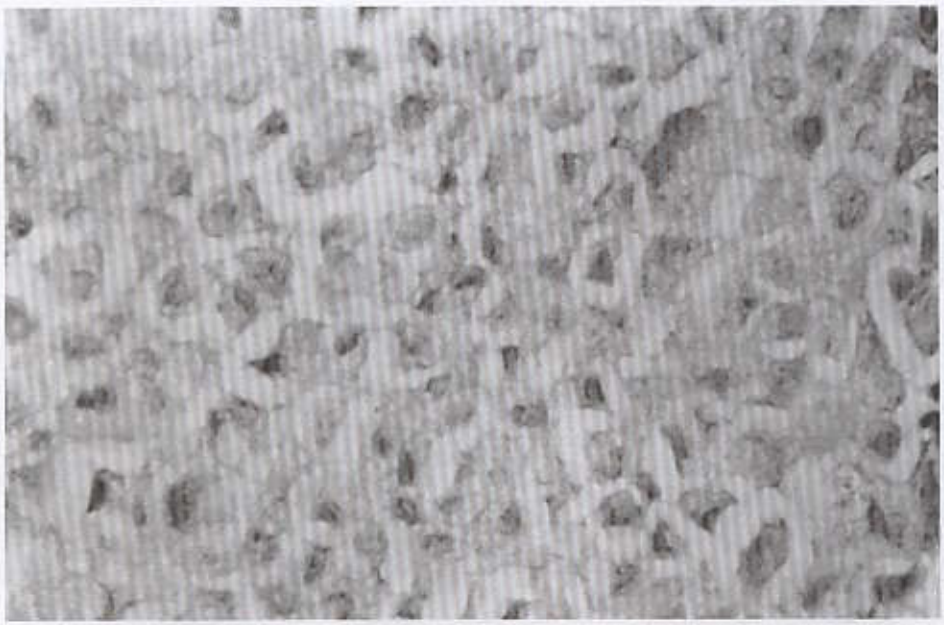

Figure 3. Cytoplasmic IHC staining of cathepsin-D protein (original magnification X 400).

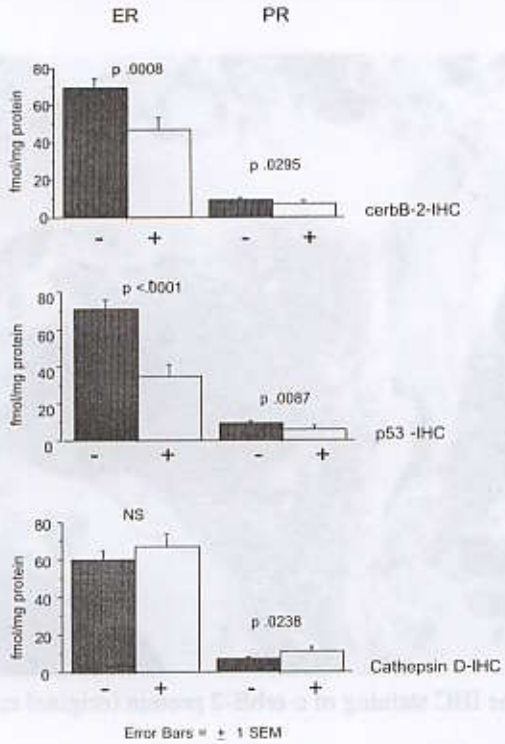

Figure 4. Concentrations of ER and PR in relation to c-erbB-2, p53, and cathepsin D expression.

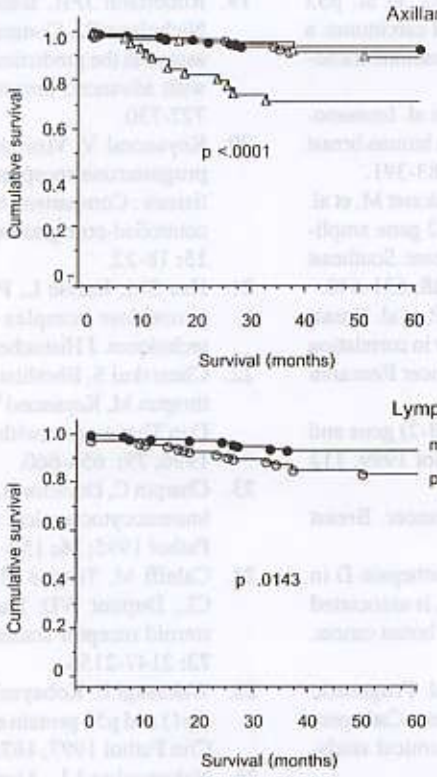

Figure 5. Kaplan-Meier plot of 5- year disease free survival for lymph node and lymphatic invasions.

CONCLUSION

Overexpression of oncogene proteins; p53, c-erbB-2 and cathepsin D measured by the IHC method revealed good agreement between each protein pair and was related to ER and PR negativity. This suggests some relevance of these oncogenes during the course of breast carcinoma. Though these protein markers had no predictive value on 5-year DFS in the present study, we cannot rule out the benefit of detecting one or combined protein markers among p53, c-erbB-2 and CD for identification of high-risk patients at the treatment stage.

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