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Immunohistochemical Study of c-*erb*B-2 Expression in Primary Breast Cancer

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SUMMARY **An immunohistochemical (IHC) study of the c-erbB-2 protein was performed in paraffin-embedded tissues from 506 primary breast carcinomas. An overexpression of c-*erb*B-2 was detected in 32% of the tumors and was correlated with a negative estrogen receptor status, increasing tumor size as well as axillary lymph node involvement. The five-year disease free survival was analyzed in 183 patients who have been followed for at least five years. No statistically significant association of c-erbB-2 status with survival was shown. However, longer survival in women over 50 years compared to under 50 years of age was detected among the c-erbB-2 positive patients. In the multivariate Cox's regression analysis, lymph node and vascular invasions were independent prognostic indicators among these patients. But c-erbB-2 status and other factors did not predict the relapse of breast cancer. However, these data may not negate the benefit of c-erbB-2 detected by IHC for identification of patients who have a poor prognosis and require more aggressive adjuvant therapy. Further studies in a larger group of patients with longer follow-up time may provide more valid information.**

Oncogene overexpression is one significant genetic alteration that has been correlated with poor survival rate in a number of tumor types. The c-erbB-2 transmembrane protein has been shown to be a member of the type 1 family of growth factor receptor, which includes epidermal growth factor receptors.[1] It is assumed to play a role in controlling cellular growth and amplification of the c-*erb*B-2 gene has been demonstrated in breast carcinoma.[2-4] The correlation between c-*erb*B-2 oncogene amplification and overexpression of c-erbB-2 membrane staining was shown in several studies.[2,5-7] It has been suggested that the c-erbB-2 protein may have an important role in the pathogenesis of human breast cancer. This gene is overexpressed in approximately 10-43% of the primary breast cancers, detected by immunohistochemistry (IHC) on frozen and formalin-fixed paraffin-embedded tissues.[8-12]

Studies of the overall survival and disease-free survival by many authors show a similar pattern of earlier recurrence and death in c-erbB-2 positive tumors[10,13-16] compared to c-erbB-2 negative ones.[11,17] Moreover, several reports demonstrated an association of c-*erb*B-2 overexpression with negative therapy response such as resistance of a tumor treated with hormonal therapy alone.[17-20]

This study investigated the presence of c-*erb*B-2 overexpression by IHC in primary breast cancer specimens and its association to other prognostic factors as well as its impact on survival in relation to adjuvant therapy.

MATERIALS AND METHODS

Patients and tissues

Surgical breast cancer specimens were obtained from 506 pri-

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mary breast cancer patients admitted at Siriraj Hospital during 1992 to 2000 and stored at –80°C until use. Clinical and surgical data could not be obtained from all patients due to incomplete records. Demographic characteristics of patients were as follows: ages ranged from 24 to 89 years (mean: 50.8 years); according to TNM classification,[21] 50 cases were classified as stage I, 366 cases as stage II, 60 cases as stage III, 5 cases as stage IV, and 6 cases as unknown stage. Histological types were 88% invasive ductal carcinoma, 2.6% non-invasive intraductal carcinoma, 1.6% mucinous carcinoma and less than 4% of other types. The postoperative follow-up period ranged from 6 to 195 months (median: 48 months). The follow-up period of only 183 patients was equal to or longer than five years.

Measurements of estrogen and progesterone receptors

ER was measured by commercial enzyme-immunoassay kit (ER-EIA, Abbott Laboratories) on cytosol fraction. Tumors were considered as ER-positive if a value of over 15 fmol/mg protein was obtained. PR was determined by radioreceptor assay using controlled-pored glass bead separation technique.[22] Samples with a PR value higher than 10 fmol/mg protein were defined as PR-positive tumors.

Immunohistochemical assay of c-erbB-2 protein

Paraffin-embedded breast cancer tissues were cut into 3-4 μm thick slices, heat-fixed to the slide at 60°C for 1 hour and air-dried overnight at room temperature. The sections were deparaffinized with xylene, rehydrated with alcohol and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol solution. The unspecific bindings were washed of by rinsing the slide 4 times in Tris-phosphate buffered saline (PBS) followed by a 20 to 30-minute incubation in 3% normal swine serum (DAKO, Denmark) diluted in PBS. The slides were incubated with 1:400 PBS diluted primary antibodies (polyclonal rabbit anti-human c-erbB-2, DAKO) for 30 minutes at room temperature. Subsequently the slides were immersed in 3% normal swine serum for 3 minutes before and after adding 1:200 dilution of biotinylated donkey anti-rabbit immunoglobulin (DAKO). Streptavidin-biotin horseradish peroxidase complex at 1:500 dilution was added and left for 30 minutes before incubating the slide with 0.1% diaminobenzidine tetrahydrochloride in PBS containing 0.02% hydrogen peroxide as a chromogen. After 10 minutes, the slides were counter-stained with haematoxylin for 30 seconds, dehydrated in alcohol, cleared in xylene and mounted in permount.

Only membrane staining was scored; - for no staining, + for < 10% staining and ++ for > 10% staining. A known positive and a negative control slide were included in each batch. For the negative control section the primary antibody was replaced by PBS.

Statistical methods

The Mann-Whitney U test or chi-square with Fisher exact test were applied when appropriate to evaluate a significant difference between variables. Multivariate analysis was performed by Cox's proportional hazard regression for detection of relative risk. Disease free survival (DFS) time was taken as the time from surgical treatment until evidence for local recurrence or metastatic disease. The significant level was set at p value < 0.05. Statistical calculations were performed using Statview PC 4.5.

RESULTS

C-erbB-2 positive staining was found in 163 patients (32%). The relationship between c-erbB-2 status and established prognostic features is shown in Table1. Tumors with larger diameters or ER-negative tumors had a statistically higher c-*erb*B-2 expression compared with smaller or ER-positive tumors. PR alone did not significantly relate to c-erbB-2 status but combined ER- and PR- negative tumors were found to have the highest c-*erb*B-2 overexpression. The concentrations of ER and PR in different menopausal stage and c-erbB-2 status are shown in Fig 1. C-erbB-2 positive tumors had lesser ER than c-erbB-2 negative tumors both in pre- and post-menopausal stages (p = 0.0359 and p = 0.0104, respectively).

Age, menopausal stages, lymph node status (-/+), pathological stages or histological types of breast cancer had no relationship to c-*erb*B-2 expression. However, when lymph nodes were grouped in different ranges, the more positive nodes, the more c-erbB-2 staining was detected (Table 2).

From all patients studied, 15 cases had a local recurrence and 34 cases had distant metastasis. An association between number of positive lymph nodes and c-erbB-2 status in relapsed and non-relapsed patients is shown in Fig. 2. C-erbB-2 positive patients with relapse had significantly higher number of positive nodes than the non-relapsed group (p = 0.0053). Although c-erbB-2 status did not relate to vascular invasion, a positive associa-

Table 1 Clinical characteristics in relation to c-erbB-2 status

Characteristics	N	% c-erbB-2 membrane staining			Chi-square *p* value
		-	+	++	
Age ≤ 50 years	246	51.3	45.2	44.0	0.3502
> 50 y	255	48.7	54.8	56.0	
Menopausal status					
Pre menopause	251	52.1	45.2	47.0	0.4694
Post menopause	249	47.9	54.8	53.0	
Tumor diameter					
≤ 20 mm	88	20.3	23.7	9.4	0.0293
> 20 mm	387	79.7	76.3	90.6	
Axillary lymph node status					
Negative	203	43.6	43.5	33.0	0.1636
Positive	286	56.4	56.5	67.0	
Pathological stage					
0-1	50	12.3	9.8	4.1	0.0668
2	365	76.0	77.1	74.0	
3	60	11.1	9.8	18.8	
4	1	0	0	1.0	
Unknown	6	0.6	3.3	2.1	
Histology					
Noninvasive intraductal	13	2.4	1.6	4.0	0.2875
Invasive ductal	413	83.1	85.5	84.8	
Invasive ductal with predominance intraductal	20	3.6	6.5	4.0	
Invasive lobular	9	2.7	0	0	
Mucinous	8	2.1	1.6	0	
Apocrine	11	1.8	1.6	4.0	
Medullary	5	1.2	1.6	0	
Papillary	6	1.8	0	0	
Paget disease	5	0.6	0	3.0	
Others	3	0.6	1.6	0	
ER status					
Negative	235	39.4	61.3	61.4	< 0.0001
Positive	271	60.6	38.7	38.6	
PR status					
Negative	413	79.5	87.1	87.1	0.1133
Positive	91	20.5	12.9	12.9	
ERPR status					
- -	224	37.2	61.3	58.4	0.0005
- +	11	2.4	0	3.0	
+ -	189	42.2	25.8	28.7	
+ +	80	18.2	12.9	9.9	

Table 2 C-erbB-2 status in relation to lymph node invasion

Number of positive nodes	C-erbB-2 status	
	% negative	% positive
0	70.9	29.1
1-3	62.7	37.3
4-10	74.5	25.5
>10	55.2	44.8

$p = 0.035$, N = 487

Table 3 Multivariate analysis of relative risk for relapse of disease within 5 years in relation to established prognostic factor of breast cancer and adjuvant therapy (N = 95)

Parameter	Relative risk	95% Confidence interval	*p* value
Age (<50 y)	1.14	0.28-4.57	0.8552
Menopausal status (pre)	1.32	0.33-5.38	0.6940
Tumor diameter (< 20mm)	0.72	0.07-7.31	0.7850
Axillary node positive	1.07	1.03-1.11	0.0010
ER (-)	1.73	0.60-5.00	0.3089
PR (-)	3.36	0.39-29.28	0.2724
Vascular invasion (-)	0.16	0.04-0.64	0.0099
Lymphatic invasion (-)	0.58	0.20-1.69	0.3186
C-erbB-2 status (-)	1.65	0.55-4.91	0.3681
Adjuvant chemotherapy (-)	0.66	0.21-2.05	0.9561
Adjuvant endocrine therapy (-)	0.97	0.33-2.83	0.4753

tion between lymph nodes and vascular invasions was detected ($p < 0.0001$).

No statistical difference in 5-year DFS between c-erbB-2 negative and positive status was detected among 183 patients (Fig. 3). However, patients aged under 50 years with positive c-erbB-2 had a shorter DFS than those over 50 years of age (Fig. 4).

Multivariate analysis by Cox's proportional hazard model was performed to identify whether c-erbB-2 or any other factor had an independent prognostic significance (Table 3). C-*erb*B-2 overexpression was not a predictive factor for relapse of the disease. Invasion of lymph nodes and blood vessels indicated greater risk of relapse within 5 years. Fig. 5 shows the Kaplan-Meier plots of 5-year DFS for lymph nodes and vascular invasions among 183 breast cancer patients.

DISCUSSION

Immunohistochemical staining of paraffin embedded tissue has been the predominant method to localize the site of c-erbB-2 protein expression at the cellular level. It provides a more feasible, good sensitivity and specificity while requiring less tissue. We have noted that some tumors had focal areas of strong c-erbB-2 staining suggesting a greater heterogeneity of these tumors. Variability in tumor sampling may mislead the results of other methods such as gene amplification for detection of c-*erb*B-2. However, different antibodies used in IHC and a lack of an agreed scoring system may produce a variation in interpretation of results between different studies.

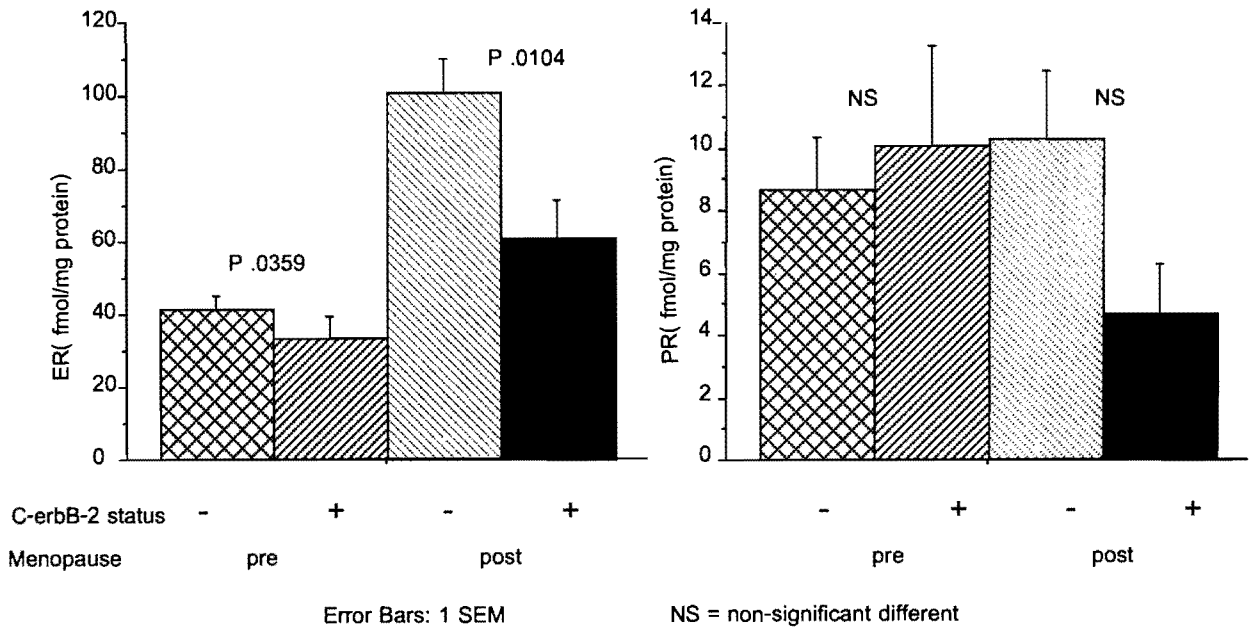

Fig. 1 Concentrations of ER (left) and PR (Right) separated by c-erbB-2 and menopausal status.

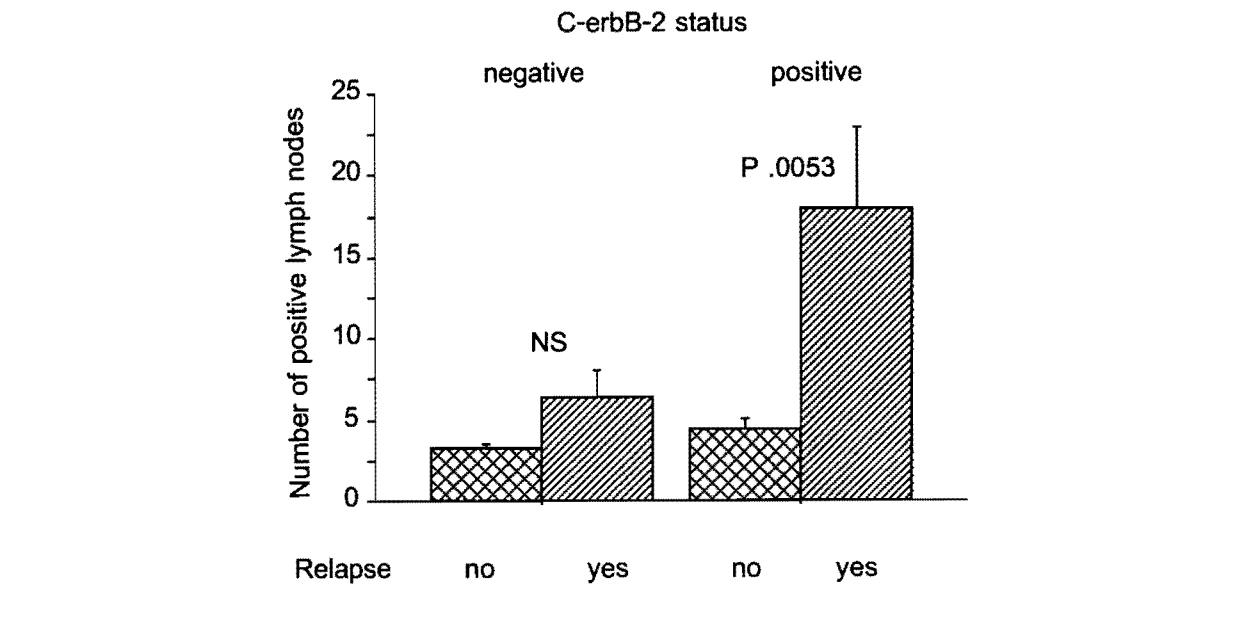

Fig. 2 Number of positive axillary lymph nodes in relation to c-erbB-2 status in relapsed and non-relapsed breast cancer patients.

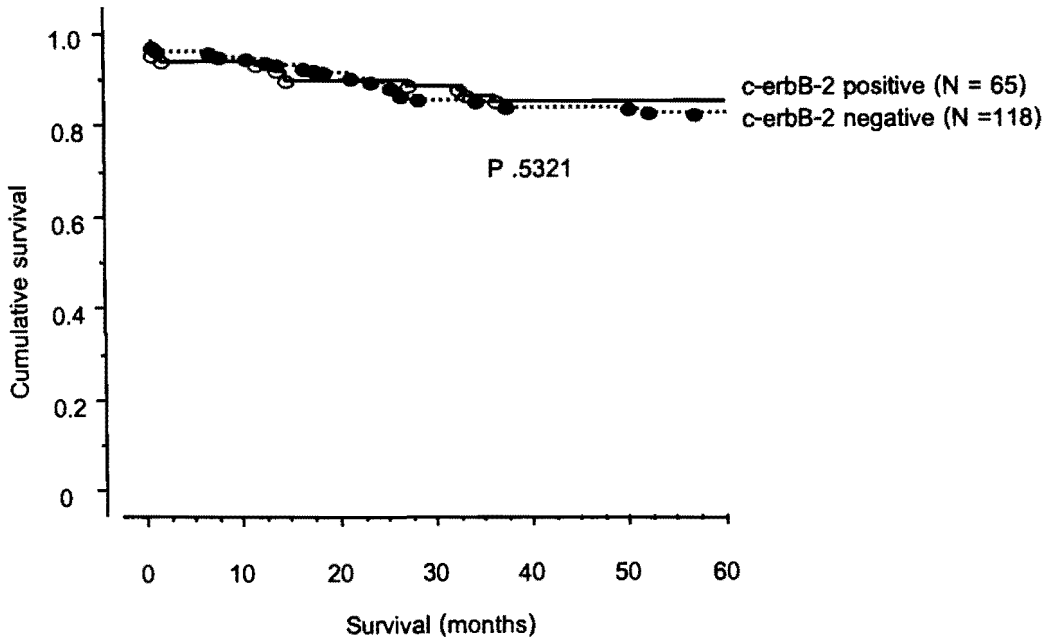

Fig. 3 Kaplan-Meier plot of 5- year disease-free survival for c-erbB-2 overexpression.

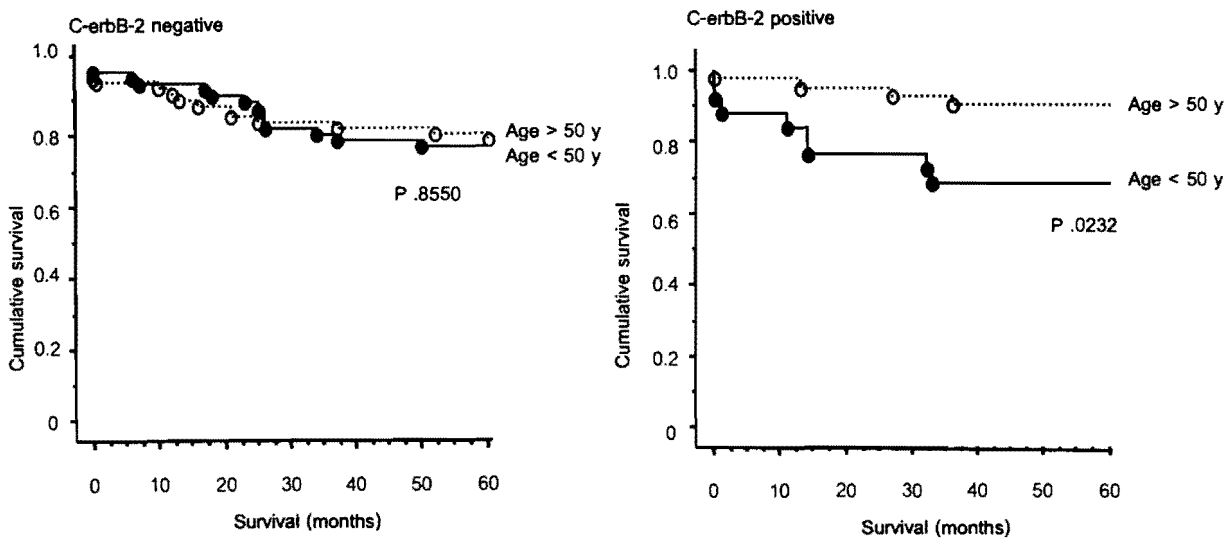

Fig. 4 Kaplan-Meier plot of 5- year disease-free survival for breast cancer patients aged under and over 50 years in relation to c-erbB-2 status.

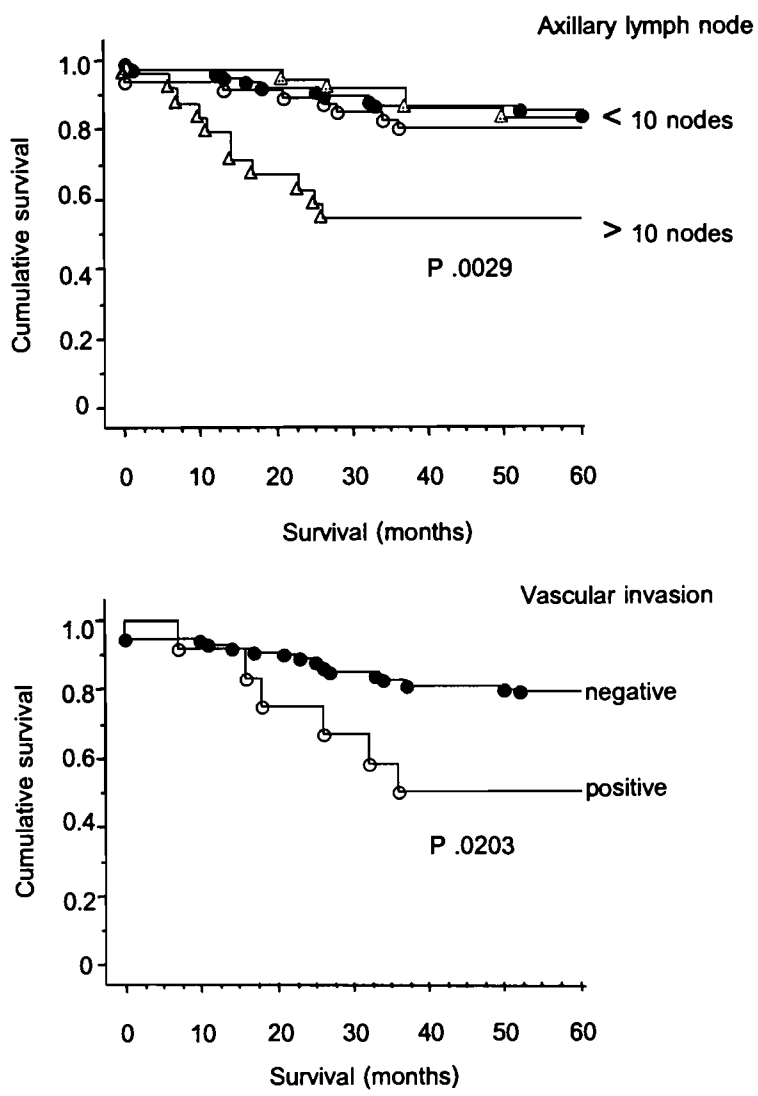

Fig. 5 Kaplan-Meier plot of 5- year disease-free survival for different status of axillary lymph node and vascular invasions.

C-*erb*B-2 overexpression was found in 32% of Thai women with primary breast cancer. This result is similar to the values reported by previous groups.[2,6,23-26] Higher expression of c-*erb*B-2 in tumors with negative ER and PR, larger size, or a higher number of lymph node invasion found in the present study indicates that increased malignancy of breast cancer is associated with c-*erb*B-2 overexpression. Similar findings have been previously shown.[3,9,27] It has been reported that overexpression of c-*erb*B-2 down regulated ER expression and activity in the experimental studies.[28,29] This reduced the benefit of endocrine therapy in c-erbB-2 positive breast cancers.[12,26] There is a possibility that these patients would be better treated with chemotherapy.

The most common histological type of breast carcinoma was the invasive ductal type as in other reports. Moreover, our findings are compatible with previous studies that immuno-staining for c-erbB-2 was mainly seen in a subgroup of invasive ductal tumors (85%) but undetected (0%) in subgroups of the invasive lobular type.[30-32] Other types of breast carcinoma known to have a good prognosis such as the papillary, medullary or mucinous types had less than 1% of c-erbB-2 staining.

Overexpression of c-*erb*B-2 has been a consistent feature of mammary Paget disease and ductal carcinoma *in situ*.[32-33] We report 60% c-*erb*B-2 overexpression in our patients with Paget disease of the breast. It has been suggested that perturbations of c-*erb*B-2 oncogene are among the earliest and most common genetic lesions in human breast cancer.[32] Intraductal spread is a special histologic feature observed in patients with invasive breast carcinoma, and it is considered to be an important risk factor for local recurrence in breast-conserving therapy.[34] Overexpression of c-erbB-2 protein was found more often in the group that was positive for intraductal spread than in the group that was negative.[35] Our results seem to support these findings though statistical significance could not be achieved. Both invasive and non-invasive intraductal spread had a higher c-erbB-2 staining than groups without intraductal spread (5% *vs* 3.6% and 3.1% *vs* 2.4%, respectively).

In 183 patients who have been followed up for at least 5 years, c-*erb*B-2 overexpression had no impact on DFS. This result agrees with several reports[11,20,33,36,37] but is contrary to other studies in dif-

ferent groups of breast cancer patients.[13,14,17,25,38] However, a significant relationship between c-*erb*B-2 overexpression, age at diagnosis and DFS is shown in the present study. The survival rate for c-erbB-2 positive patients under 50 years was worse than that of the older patients. This difference was not detected in c-erbB-2 negative group. Decreased frequency of c-erbB-2 positive cases diagnosed at an advanced age may explain the better survival of older patients as reported elsewhere.[17]

Multivariate analysis of prognostic variables of breast cancer including c-erbB-2 status in our study reveals no benefit of c-erbB-2 protein as a predictor of survival. This may cause by insufficient number of relapsed patients in the study. Significant prognostic factors of DFS in these patients were lymph node and vascular invasions of tumors.

The cancer cells may spread via lymphatic or hematogenous route as well as direct contact of cells on organ surface. Vascular invasion is a critical step for a metastatic tumor. It has been suggested that c-erbB-2 is related to hematogenous spread of breast cancer cells.[8,39] Our data could not confirm this suggestion since insignificant association between c-erbB-2 status and vascular invasion was obtained. But there is evidence for increased lymph node invasion in relapsed patients who were c-erbB-2 positive in our study (Fig. 2). This suggests that c-*erb*B-2 overexpression may have some linkage to lymph node metastasis. Concordance of vascular invasion and positive lymph node was also shown in the present study as in previous report.[35]

Controversy reports concerning the relationship between c-*erb*B-2 overexpression and responses to therapy have been published.[12,20,26,30,40] From limited data, we did not detect any different response either to endocrine or to chemotherapy between patients with and without c-*erb*B-2 overexpression.

In conclusion, the present study does not reveal a significant role of c-*erb*B-2 overexpression determined by immunohistochemistry in the primary breast cancer as a prognostic factor for disease free survival or reducing the benefits of adjuvant therapies. This may simply be due to not enough data to obtain a statistical significance. However, the positive relationship between c-*erb*B-2 overexpression and other poor prognostic parameters such as; ER-negative tumor, large tumor size as well as increased lymph node invasion suggests that c-erbB-2 protein may play some important role in the invasive progression of human breast cancer and also its resistance to therapy. It is possible that immunohistochemistry for routine examination of c-erbB-2 protein in combination with other prognostic indicators may provide more information for identification of breast cancer patients with a bad prognosis.

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