

EXPERIMENTAL / LABORATORY STUDIES

**A Novel Method of Reporting c-erbB-2 Overexpression:
Correlation with Grade But Not with Other Prognostic
Parameters in Breast Cancer***

Mustafa SAMUR¹, Şeyda KARAVELİ², Hakan BOZCUK¹, Elif PESTERELİ², Mustafa ÖZDOĞAN¹,
Mustafa YILDIZ¹, Mehmet ARTAC¹, Burhan SAVAŞ¹

¹Department of Medical Oncology, Faculty of Medicine, Akdeniz University, Antalya - Turkey

²Department of Pathology, Faculty of Medicine, Akdeniz University, Antalya - Turkey

Received: May 02, 2003

Abstract: Overexpression of erbB-2 protein is a marker of clinically aggressive behavior in breast cancer patients. In general erbB-2 status is reported on a scale from negative to 3+, depending on staining intensity, if more than 10% of the tumor cells are stained. However, the original definition does not take into account the percentage of stained cells in detail, since all samples score in the same way if they are above the cut-off, established at 10%. In this study, we evaluated the erbB-2 overexpression by immunohistochemistry in 169 patients and evaluated any correlation with the other well known prognostic parameters. We also tested whether there was a difference between the original definition of c-erbB-2 positivity (2+ or 3+) and a novel system, the total c-erbB-2 score, which takes into account both the percentage, and intensity of staining as a continuous variable. The total c-erbB-2 score was correlated with the grade (P = 0.006) but not with other parameters. When 2+ and 3+ levels of staining together were regarded as positive, a similar correlation with grade was demonstrated. However, when only 3+ cases were considered, there was no significant correlation. In conclusion reporting c-erbB-2 as a continuous variable may make more sense than treating a cut-off value as positive.

Key Words: c-erbB-2, HER-2/neu, breast cancer, grade

Introduction

The HER-2/neu gene encodes a tyrosine kinase receptor, c-erbB-2, which is one of the members of the epidermal growth factor receptor family. Although the exact mechanism is not totally clear, it is believed that the binding of a specific ligand to this receptor triggers multiple pathways of intracellular signal transduction, leading to increased tumorigenicity, invasion and metastasis (1-2). Although the data are inconclusive, overexpression of c-erbB-2 protein appears as a poor prognostic factor in breast cancer and causes decreased sensitivity to hormonal treatment and to CMF-based chemotherapy and relatively higher sensitivity to

anthracyclines (3-6). Correlation with other poor prognostic factors also confirms that overexpression of c-erbB-2 protein is a marker of clinically aggressive behavior (7).

c-erbB-2 protein overexpression can be detected with different methods. Fluorescence in situ hybridization (FISH) is the most sensitive of these methods. However, it is not readily available in every clinic. Although less sensitive than FISH, immunohistochemical (IHC) assays are widely used due to their availability and simplicity.

When reporting results, the degree of erbB-2 protein overexpression measured by IHC is scored according to the intensity of membrane staining and the percentage of

* Abstract of the manuscript was presented as a poster in National Cancer Congress in Turkey.

tumor cells stained. A 2+ score denotes a weak to moderate complete membrane staining in more than 10% of the tumor cells, whereas for a 3+ score strong complete staining is needed in more than 10% of the tumor cells. It is not known if there is a real difference between 10% and 100% staining from a clinical point of view. Studies on c-erbB-2 do not address the percentage of positive staining in detail. In this regard, it is difficult to compare different studies, because of different definitions of positivity.

In this study, we evaluated c-erbB-2 overexpression by IHC and looked for correlations with grade and other well known prognostic parameters. We also evaluated whether there was a difference between the original definition of erBB-2 positivity (2+ or 3+) and a novel system, which regards both the percentage and intensity of staining as a continuous variable.

Materials and Methods

We retrospectively evaluated 169 previously operated breast cancer patients admitted to the Medical Oncology Department of Akdeniz University Medical School for adjuvant chemotherapy between April 1998 and September 2002. Information on age, menopausal status, histological diagnosis, tumor diameter and axillary lymph node status was obtained from the medical records. Tumors were graded according to the modified Bloom and Richardson method proposed by Elston and Ellis (8).

Immunohistochemical Methods

Immunohistochemical staining was performed on buffered formalin fixed, paraffin embedded tissue sections. Sections 4 mm thick 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 known positive and negative material. The slides were microwaved with citrate buffer, pH = 6.6, for 10 min in an 800 W microwave oven for antigen retrieval, then allowed to cool for 20 min. The slides were stained on Dako TechMate 500 automated immunostainer with the standard streptavidin-biotin method. Following a hydrogen peroxide block of endogenous peroxide and incubation with proteinase K for 60 min, the slides were incubated with primary antibodies, c-erbB2 (Clone e2-4001+3b5, 1:100 dilution, Neomarkers, CA, USA),

estrogen receptor (ER) (Clone 1D5, 1:50 dilution, Dako, Denmark) and progesterone receptor (PR) (Clone hPRa2+hPRa3, Neomarkers, CA, USA) for 60 min, followed by brief buffer washes. The slides were then treated with a Universal LSAB2 kit (K0675, Dako, Denmark) and reacted with diaminobenzidine and hydrogen peroxide to visualize the end product. Mayer's hematoxylin was used as a counter stain. Negative control sections were incubated in PBS in place of the primary antibody.

The magnitude of c-erbB-2 expression

c-erbB-2 was first defined along the original recommendation of 2+ or 3+ (a 2+ score denotes a weak to moderate complete membrane staining in more than 10% of the tumor cells, whereas for a 3+ score, strong complete staining is needed in more than 10% of the tumor cells). c-erbB-2 expression was also defined as a continuous variable on a 300 (3 x 100) scale, basically as a ratio, the denominator always being 300, and the nominator being the product of the percentage of cell staining (from 0 to 100) and the intensity of staining (from 0+ to 3+), where 0 = no staining, 1 = faint membrane staining, 2 = weak to moderate complete membrane staining and 3 = strong complete membrane staining. Cytoplasmic staining is considered non-specific and negative. For example strong complete staining in 50% of cells gave a score of $3 \times 50/300 = 150/300 = 0.5$ whereas weak-moderate staining in 30% gave the result $2 \times 30/300 = 60/300 = 0.2$. We analyzed both of the reporting methods of c-erbB-2 and searched for correlations with the other classical prognostic factors. Hormone receptor status was also evaluated as the ER and the PR score as continuous variables, according to staining intensity (weak, moderate and strong), and stained percentage of cells as described above for c-erbB-2.

Statistical Analysis

The correlation of c-erbB-2 expression as a continuous variable with grade and other continuous clinical parameters was evaluated by the Spearman correlation coefficient. c-erbB-2 overexpression in the form of a categorical variable (3+ vs. other or 2-3+ vs. other) in relation to the continuous variables in question was evaluated by Mann-Whitney U test, and by the X^2 test if in relation to categorical variables. A P value of <0.05 was considered statistically significant. SPSS 10.0 software was used for the analysis.

Results

We included 169 breast cancer patients aged between 26 and 70 (median 44) in our study. Of our patients, 67% were premenopausal. Two-thirds of our patients had at least 1 positive axillary lymph node, and 57% of the samples were ER positive, 42% were c-erbB-2/3+ and most of them were either grade 2 (55.4%) or 3 (38.9%). Details of tumor characteristics are given in Table 1.

When evaluated as a continuous variable, the total c-erbB-2 score was positively correlated only with grade (P = 0.006). (Table 2 and Figure). On the other hand, when the original definition of 3+ staining only was accepted as positive and the others as negative, as categorical variables, correlation with grade was not statistically significant ($X^2 = 3.568$, $df = 1$, $P = 0.059$). When the combination of 2+ and 3+ staining tumor samples was treated as positive, correlation with grade was again apparent ($X^2 = 7.512$, $df = 1$, $P = 0.006$) (Table 2). There was a strong correlation between ER and PR scores and between tumor size and hormone receptors, as detailed in Table 2.

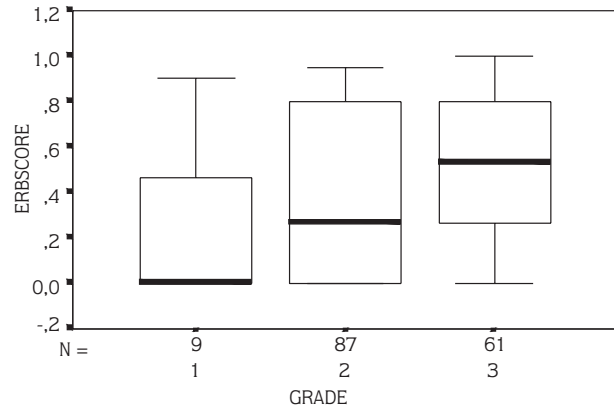


Figure. c-erbB-2 score with respect to grade.

Discussion

HER2/neu is amplified in approximately 30% of breast tumors, causing receptor overexpression, and is generally regarded as a poor prognostic factor (9-10). In our study a higher percentage of patients overexpressed erbB-2 (42%, 3+) compared to the literature. This may be due to the patient properties of our study-group representing, an advanced stage. Nearly 65% of our patients had axillary lymph node metastasis. Another explanation may be the different disease profile between eastern and western countries. Moreover, this discrepancy might be due to the antibody used in our study which is a different one to those the ones used in trastuzumab trials (11).

Many studies have investigated the correlation of c-erbB-2 protein overexpression with the other prognosticators, as well as its role as a predictor of clinical outcome with different types of therapies. However, its value as a prognostic and predictive factor is still not conclusively established. Different testing methods, heterogeneous and small patient populations and retrospective analyses cause major problems in comparing different studies. The method of reporting results of c-erbB-2 positivity might also have caused an inconsistency in the interpretation of the results. In most studies that use immunohistochemical methods c-erbB-2 protein expression was reported as positive or negative as a categorical variable, and the degree of positivity (percentage of stained cells and intensity together) was not taken into account. In some reports, >1% staining was accepted as positive (6), whereas in others a 10% cut-off was chosen as originally defined (12), or even 50% of tumor cells were required to be stained positively

Table 1. Patient characteristics.

	N (%)	Mean (min-max)	Median
Age		45 (26-70)	44
Premenopausal	113 (66.8)		
Postmenopausal	56 (33.1)		
ER+	97 (57.4)		
PR+	80 (47.3)		
ER score		0.26 (0-0.95)	
PR score		0.17 (0-0.90)	
ErbB-2, 2+	28 (16.5)		
ErbB-2, 3+	71 (42.0)		
ErbB-2 score		0.41 (0-1.00)	0.40
Grade			
1	9 (5.7)		
2	87 (55.4)		
3	61 (38.9)		
Tumor size (T)			
1	35 (21.1)		
2	104 (62.6)		
3	27 (16.3)		
Axillary LAP (N)			
0	58 (34.9)		
1-3	57 (34.4)		
>3	51 (30.7)		

Table 2. Correlations between c-erbB-2 score and other clinical and pathologic variables.

Correlation coef. P value	c-erbB-2	PR- score	ER- score	Grade score	Axillary LAP (n)	Tumor size
PR-score	.086 .269					
ER-score	.109 .159	.461 .000*				
Grade	.220 .006*	-.023 .774	-.040 .618			
Axillary LAP	-.064 .413	-.058 .461	-.010 .900	-.034 .675		
Tumor size	-.060 .446	-.198 .011*	-.286 .000*	.135 .094	.148 .060	
Age	.068 .378	-.099 .202	.107 .167	.005 .955	-.063 .417	-.145 .063

* Correlation is significant at the .05 level (2-tailed).

to be regarded as positive (3-4). As a result, 2%, 11% and 100% complete strong staining of tumor cells were all deemed to be erbB-2 positive in the above mentioned studies, although there is no scientific proof that these are all the same. It is possible that both the magnitude of positivity and the percentage of tumor cells staining positive may have an impact. Such a relation has already been demonstrated for estrogen receptor. The Early Breast Cancer Trialists' Collaborative Group overview showed that tamoxifen has greater benefits in women with strongly positive ER levels, than in those with moderate levels (13).

Addressing the potential prognostic significance of HER-2 gene amplification, Slamon et al. demonstrated that the numbers of gene copies are important (14). Patients who had 5 or more gene copies had a shorter relapse-free survival than those who had 2 or more copies and those with no gene amplification (37%, 56% and 60%, respectively). This frequently cited study is the first report of HER-2 gene amplification as a prognostic factor. We also think that this study is important because it demonstrates a survival difference with regard to the degree of amplification.

In this study, we define a novel method of reporting c-erbB-2 results. In our patients we evaluated c-erbB-2

both as a categorical variable (2+ or 3+ or negative as defined originally) and as a total c-erbB-2 score calculated by intensity multiplied by the percentage of cells stained divided by 300. We analyzed the potential utility of these 2 different approaches. As a result, in both methods, c-erbB-2 was correlated with grade. These results are compatible with those of most others, showing that c-erbB-2 positivity is related to more aggressive tumor behavior in relation to grade (15-17). However, in our patients relation with grade was not significant when 3+ cases only were accepted as positive. On the other hand, it was significant when the erbB-2 score was used as a continuous variable and when both 2+ and 3+ cases were treated as positive. This finding is an indication that the percentage of tumor cells stained positive may be of importance. There may be a prognostic and predictive difference between 10% staining and 100% staining. One recent study showed that there is an association between hormonal receptors and Her-2/neu protein when both were quantitatively measured with enzyme-linked immunosorbent assay and evaluated as continuous variables (18). It therefore may be justified to report the results as a c-erbB-2 score when the IHC method is used and to make further evaluations in homogenously treated patient populations to establish whether this score has a

prognostic and predictive impact. It would be also worthwhile evaluating whether different percentages of strong complete staining predict a response to trastuzumab.

We did not find any relation between hormone receptor status and c-erbB2 overexpression, nor any relation between axillary lymph node status, age and menopause. In the literature, a positive correlation and a lack of correlation were both reported in this regard (19-25). The reason for this discrepancy may be the different methods of detecting c-erbB-2 overexpression and hormone receptor status. Another reason may be the different definitions of c-erbB2 and ER/PR positivity, as mentioned above.

In conclusion, different methodologies in both measuring and reporting c-erbB-2 positivity still pose a problem and consensus on a universally accepted standard is urgently needed. Such a consensus may help

to analyze data from various centers more homogeneously. We think that when IHC methods are used to evaluate c-erbB-2 expression, studies reporting c-erbB-2 expression as a continuous variable, instead of reporting as positive/negative with different cut-off values, will be more suitable for exploring both the prognostic and predictive values of c-erbB-2, and will make it easier to compare different studies or to evaluate them in a meta-analysis. In short, we believe our results justify further evaluation of our novel method of c-erbB-2 reporting in a clinical setting.

Corresponding author:

Mustafa SAMUR

Akdeniz University, Faculty of Medicine

Internal Medicine Dep. Division of Medical Oncology

Antalya - Turkey

e-mail: msamur@akdeniz.edu.tr

References

1. Neve RM, Lane HA, Hynes NE. The role of overexpressed Her2 in transformation. *Annals Oncol* 12 (suppl. 1): 9-13, 2001.
2. Bozcuk H, Uslu G, Pestereli E, et al. Predictors of distant metastasis at presentation in breast cancer: A study also evaluating associations among common biologic indicators. *Breast Cancer Res Tr* 68: 239-248, 2001.
3. Thor AD, Berry DA, Budman DR, et al. erbB-2, p53, and the efficacy of adjuvant therapy in lymph node positive breast cancer. *J Natl Cancer Inst* 90: 1346-1360, 1998.
4. Paik S, Bryant J, Park C et al. erbB-2 and response to doxorubicin in patients with axillary lymph node-positive hormone receptor-negative breast cancer. *J Natl Cancer Inst* 90: 1361-1370, 1998.
5. Yamauchi H, O'Neill A, Gelman R, et al. Prediction of response to antiestrogen therapy in advanced breast cancer patients by pretreatment circulating levels or extracellular domain of the Her-2/c-neu protein. *J Clin Oncol* 15: 2518-2525, 1997.
6. Elledge RM, Green S, Ciocca D, et al. HER-2 expression and response to tamoxifen in estrogen receptor-positive breast cancer: a Southwest Oncology Group Study. *Clin Cancer Res* 4: 7-12, 1998.
7. Berger MS, Locher GW, Saurer S, et al. Correlation of c-erbB-2 gene amplification and protein expression of human breast carcinoma with nodal status and nuclear grading. *Cancer Res* 48: 1238-1243, 1988.
8. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 19: 403-10, 1991.
9. Press MF, Pike MC, Chazin VR, et al. Her-2/neu expression in node-negative breast cancer: Direct tissue quantitation by computerized image analysis and association with overexpression with increased risk of recurrent disease. *Cancer Res* 53: 4960-4970, 1993.
10. Press MF, Bernstein L, Thomas PA, et al. HER-2/neu gene amplification characterized by fluorescence in situ hybridization: poor prognosis in node-negative breast carcinomas. *J Clin Oncol* 15: 2894-2904, 1997.
11. Idris N, Carothers Carraway CA, Carraway KL. Differential localization of ErbB2 in different tissues of the rat female reproductive tract: implications for the use of specific antibodies for ErbB2 analysis. *J Cell Physiol* 189: 162-70, 2001.
12. Menard S, Valagussa P, Pilotti S, et al. Response to cyclophosphamide, methotrexate, and fluorouracil in lymph node-positive breast cancer according to HER2 overexpression and other tumor biologic variables. *J Clin Oncol* 19: 329-35, 2001.
13. Tamoxifen for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. *Lancet* 351(9114): 1451-67, 1998.
14. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 235: 177-82, 1987.
15. Tsuda H, Hirohashi S, Shimosato Y, et al. Correlation between histologic grade of malignancy and copy number of c-erbB-2 gene in breast carcinoma. A retrospective analysis of 176 cases. *Cancer* 65: 1794-80, 1990.

16. Hoff ER, Tubbs RR, Myles JL, et al. HER2/neu amplification in breast cancer: stratification by tumor type and grade. *Am J Clin Pathol* 117: 916-21, 2002.
17. Paik S, Hazan R, Fisher ER, et al. Pathologic findings from the National Surgical Adjuvant Breast and Bowel Project: prognostic significance of erbB-2 protein overexpression in primary breast cancer. *J Clin Oncol* 8: 103-12, 1990.
18. Konecny G, Pauletti G, Pegram M, et al. Quantitative association between HER-2/neu and steroid hormone receptors in hormone receptor-positive primary breast cancer. *J Natl Cancer Inst* 15: 142-53, 2003.
19. Ariazi EA, Clark GM, Mertz JE. Estrogen-related receptor alpha and estrogen-related receptor gamma associate with unfavorable and favorable biomarkers, respectively, in human breast cancer. *Cancer Res* 62: 6510-8, 2002.
20. Roux-Dosseto M, Romain S, Dussault N, et al. Correlation of erbB-2 gene amplification with low levels of estrogen and/or progesterone receptors in primary breast cancer: do erbB-2 products delineate hormone-independent tumors? *Biomed Pharmacother* 43: 641-9, 1989.
21. Bouras T, Southey MC, Venter DJ. Overexpression of the steroid receptor coactivator AIB1 in breast cancer correlates with the absence of estrogen and progesterone receptors and positivity for p53 and HER2/neu. *Cancer Res* 61: 903-7, 2001.
22. Keshgegian AA. ErbB-2 oncoprotein overexpression in breast carcinoma: inverse correlation with biochemically- and immunohistochemically-determined hormone receptors. *Breast Cancer Res Treat* 35: 201-10, 1995.
23. Querzoli P, Marchetti E, Fabris G, et al. Immunohistochemical expression of c-erbB-2 in human breast cancer by monoclonal antibody: correlation with lymph node and ER status. *Tumori* 76: 461-4, 1990.
24. Bohn U, Aguiar J, Bilbao C et al. Prognostic value of the quantitative measurement of the oncoprotein p185(Her-2/neu) in a group of patients with breast cancer and positive node involvement. *Int J Cancer* 101: 539-44, 2002.
25. Sutterlin MW, Haller A, Gassel AM, et al. The correlation of c-erbB-2 oncoprotein and established prognostic factors in human breast cancer. *Anticancer Res* 20: 5083-8, 2000.