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# Immunohistochemical determination and grading of CerbB-2 expression in breast cancer: correlation with interpectoral, apical nodal involvement and other prognostic factors

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**Abstract:** We aimed to investigate the correlation between quantitative CerbB-2 expressions with conventional prognostic factors, and distinct nodal involvement in patients with invasive breast carcinoma. One hundred fifty seven consecutive breast carcinoma patients were retrospectively analysed. Level I-II, Level III, and Rotter (Interpectoral) group lymph nodes were separately examined and recorded. For each patient estrogen receptor (ER), progesteron receptor (PR), CerbB-2, P53 status were defined using immunohistochemistry. Age, tumor localisation, menopausal status, grade and the presence of intraductal component were also recorded. CerbB-2 expression did not correlate with age, localisation and menopausal status. There was a reverse, but weak correlation with tumor size and CerbB-2 expression (p=0.034). In subgroup analysis of CerbB-2 positive cases, the magnitude of CerbB-2 positivity did not correlate with tumor size (p=0.551). In univariate analysis CerbB-2 expression did not correlate with nodal involvement in Level I-II, and Rotter. In subgroup analysis of patients with positive CerbB-2, positivity of CerbB-2 linearly increased with the number of positive lymph nodes in Level I-II, and this difference was significant (p=0,039). There was a significant correlation between CerbB-2 expression and Level III nodal metastases (p=0.005). But this correlation was not significant among CerbB-2 positive patients (p=0.82). P53, PR positivity and the presence of intraductal component did not differ according to oncogene expression. We detected a reverse correlation with ER positivity and CerbB-2 positivity (p=0.011). It is concluded that quantitative expression of CerbB2 positivity increases with nodal involvement in Level I-II axillary lymph nodes, and ER. Also, CerbB-2 positivity is more common among patients with Level III lymph node metastases.


*Keywords: CerbB-2, quantification, breast carcinoma, immunohistochemistry, prognostic factors*

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## 1 Introduction

Breast cancer comprises 30% of female malignancies, and is the third leading cause of death by cancer among women [1]. The heterogeneity of this malignancy leads clinicians to discover better prognostic parameters, and optimise the treatment options. Many prognostic factors including age at diagnosis, tumor size, lymph node status, histological type, stage and steroid receptors have been defined [2]. Beside these prognostic factors the roles of thymidine-labeling index, flow cytometry; proliferation markers like Ki-67, oncogenes, and tumor suppressor genes have also been stated [3]. The universally accepted prognostic markers are lymph node status, and tumor size. In the recent years, the workshop is based on CerbB-2 oncogene and P53 tumor suppressor gene.

CerbB-2 gene is an oncogene, which encodes a member of the four membered EGF receptor families, located at 17q21 chromosome. CerbB-2 encodes a 185-190 kDa weight transmembrane glycoprotein, a new member of tyrosine kinase family [4]. Amplification and/or over-expression of this gene play an important role in the pathogenesis of breast carcinoma, and occur in 20-40% of all breast cancers [5]. Both pre-clinical and clinical studies have shown the adverse effects of CerbB-2 expression in the outcome of patients with breast carcinoma [6, 7]. It also has been shown to have dismal effects on response to therapeutic strategies. Herceptine, an humanised monoclonal antibody, binding to the extracellular part of CerbB-2, has beneficial effects on patients with positive CerbB-2 expression [8, 9].

## 2 Statistical methods and Experimental Procedures

We have retrospectively analysed randomised 157 patients who have undergone modified radical mastectomy due to histologically proven infiltrative ductal carcinoma of the breast, between 2002 and 2005. Ethical committee approval was taken before the conduction of the study. All subjects had unilateral ductal carcinoma. Mean age of the patients was 50.8±12.65 (min 20, max 80). During the operation Interpectoral (Rotter) lymph nodes were separately dissected, and send for pathologic examination. In the same manner after the completion of dissection, Level I-II, and Level III groups were clipsed for distinct pathological diagnosis. Patients with positive lymph nodes (LN) were grouped due to the number of involved LN as follows: No positive LN, 1-3 positive LN, 4-9 positive LN, 10 and more positive LN.

Nodal involvements in Level I-II, Level III, and Interpectoral groups were considered in subgroups due to the number of the positive LN. In Level I-II 60 patients (38.2%) were metastases-free, whereas 57 (36.3%) had 1-3 positive LN, 21 (13.3%) had 4-9, and 19 (12.51) had 10 and more metastatic LN. There were 111 patients (70.7%) with uninvolved LN in Level III, 24 (15.2%) had 1-3 positive LN, 17 (10.8%) had 4-9, and 5 (3.1%) had 10 and more positive LN. Rotter LN were non-metastatic in 141 (89.8%), but 14 (8.9%) subjects had 1-3, and 2 (1.27%) had 4-9 metastatic LN located at the Interpectoral space.

Sixty-seven subjects (42.67%) were premenopausal, 67 were postmenopausal and the

remaining 23 (14.6%) were perimenopausal. The tumor sizes were T1 in 33 (21.01%), T2 in 71 (45.24%), and T3 in 53(33.75%) patients. Tumor grade was Grade 1 in 17 (10.8%), Grade 2 in 56 (35.6%), and Grade 3 in 84 (53.5%) patients. ER was positive in 110 (70.06%), PR in 100 (63.69%), and P53 in 79 (50.3%) patients. CerbB-2 expression was negative in 80 (50.95%), 1+ in 47 (29.93%), 2+ in 19 (12.1%), and 3+ in 11 (7.006%) cases.

Patient characteristics are shown in Table 1.

**Table 1** Patient characteristics.

| | | | | |
|---|---|---|---|---|
| Menopausal Status | Premenopausal 67 | Perimenopausal 23 | Postmenopausal 67 | |
| Tumor Size | T1(<2 cm) 33 (21.02%) | T2 (2-5 cm) 71 (45.22%) | T3 (>5 cm) ) 53 (33.75%) | |
| Tumor Grade | Grade 1 17 (10.82%) | Grade 2 56 (35.66%) | Grade 3 84 (53.51%) | |
| Estrogen Receptor | Negative 47 (29.93%) | Positive 110 (70.07%) | | |
| Progesterone Receptor | Negative 57 (36.31%) | Positive 100 (63.69%) | | |
| P53 | Negative 78 (49.68%) | Positive 79 (50.32%) | | |
| Level I-II LN Metastases | No positive LN 60 (38.21%) | 1-4 positive LN 57 (36.31%) | 4-9 positive LN 21 (13.37%) | 10 or more LN 19 (12.1%) |
| Level III LN Metastases | No positive LN 111 (70.7%) | 1-4 positive LN 24 (15.28%) | 4-9 positive LN 17 (10.82%) | 10 or more LN 5 (3.18%) |
| Rotter Metastases | No positive LN 141 (89.8%) | 1-4 positive LN 14 (8.91%) | 4-9 positive LN 2 (1.27%) | 10 or more LN - |
| Cerb-B2 | No 80 (50.95%) | 1+ 47 (29.93%) | 2+ 1 19 (12.1%) | 3+ 11 (7.006%) |
| Tumor Localisation | | | | |
| Upper Outer Q. 57 (36.3%) | Upper Inner Q. 34 (21.66%) | Lower Outer Q. 16 (10.2%) | Lower Inner Q. 23 (14.64%) | Central 27 (17.19%) |

Abbreviations: LN - Lymph Nodes, Q - Quadrant.

## 2.1 Experimental Procedures

The histological examination of the surgical specimens was performed on paraffin sections stained by Haematoxylin-Eosin. All tumors were invasive carcinomas. Histologic grading was assessed according to modified Bloom-Richardson criteria. Extensive intraductal component (invasive tumor in which 25% or more of the overall area involved by the invasive carcinoma is composed of DCIS), lymphovascular and neural invasion were examined. Immunohistochemical studies were performed following standard avidin-biotin-peroxidase techniques. Expressions of estrogen receptor (Neomarkers, Clone 105+6F11, mouse mon-

oclonal antibody, 1/50), progesterone receptor (Neomarkers, Clone hpRa2+hpRa3, mouse monoclonal antibody, 1/50), P53 (Neomarkers, Clone DO-7+BP53-12, mouse monoclonal antibody, 1/250), and CerbB-2 (Neomarkers, Clone e2-4001+365, mouse monoclonal antibody, 1/250) were determined. Estrogen and progesterone receptor immunoreactions were judged as positive when more than 10% of tumor cells revealed positive nuclear staining. This cut-off point was 5% for P53 immunoreactivity. CerbB-2 immunoreactivity was assessed by a barely perceptible partial membrane staining detected in more than 10% of tumor cells was regarded as 1+, a weak to moderate complete membrane staining observed in more than 10% of tumor cells was regarded as 2+, and string complete membrane staining as 3+. Cytoplasmic staining or membrane staining in less than 10% of the tumor cells was categorised as negative.

### 2.2 Statistical Methods

Statistical analyses were conducted using SPSS 10.0 for windows. The correlation between prognostic parameters and CerbB-2 positivity or negativity was analysed using Pearson Chi-Square test. Subgroup analysis CerbB-2 expression with correlating parameters were analysed by Pearson Chi-Square test for quantitative CerbB-2 expression. In cases of positive correlation with quantitative expression power analysis of the test was performed. Correlation between age and CerbB-2 expression was analyzed using t test. P values below 0.05 were considered significant.

## 3 Results

Mean age of patients in CerbB-2 positive group was 49.3±11.93, and 52.53±13.25 in negatives. CerbB-2 positivity did not correlate with age (t value=1.608, p=0.11). In a similar manner there were no significant difference in CerbB-2 expression due to localisation ($x^2$=2.431, p=0.657), and menopausal status ($x^2$=0.494, p=0.781). Percentage of CerbB-2 negative cases increased with tumor size. There was a reverse, but weak correlation with tumor size and CerbB-2 expression ($x^2$=6.772, p=0.034, power=%64). Though, in subgroup analysis of CerbB-2 positive cases, the magnitude of CerbB-2 positivity did not correlate with tumor size ($x^2$=3.039, p=0.551). Table 2 depicts menopausal status, tumor size and grade of patients arranged due to CerbB-2 expressions.

CerbB-2 oncogene positivity did not correlate with nodal involvement in Level I-II ($x^2$=5.539, p=0.136), and interpectoral nodes ($x^2$=3.264, p=0.196). But there was a significant correlation between CerbB-2 positivity and nodal metastases in Level III ($x^2$=12.67, p=0.005, power=%86). In subgroup analysis of patients with positive CerbB-2 status, positivity of CerbB-2 linearly increased with the number of positive lymph nodes in Level I-II, and this difference was significant ($x^2$=13.269, p=0,039, power=79%), but in Level III this difference was not significant among CerbB-2 positive patients ($x^2$=1.535, p=0.82). Interpectoral lymph node metastases were not also different among CerbB-2 positive patients. The correlation between lymph node metastases and CerbB-2 expres-

sion is listed in Table 3.

**Table 2** Menopausal status, tumor localization, size and grade of patients arranged due to CerbB–2 expressions.

| Prognostic Parameters | | CerbB-2 Expression | | | | Total | P |
|---|---|---|---|---|---|---|---|
| | | Negative | 1+ | 2+ | 3+ | | value |
| Menopausal Status | Premen. | 36 | 17 | 7 | 7 | 67 | |
| | Postmen. | 32 | 24 | 7 | 4 | 67 | 0.781 |
| | Perimen. | 12 | 6 | 5 | No | 23 | |
| Tumor Size | T1 | 12 | 15 | 5 | 1 | 33 | |
| | T2 | 34 | 22 | 8 | 7 | 71 | 0.034* |
| | T3 | 34 | 10 | 6 | 3 | 53 | |
| Tumor Grade | Grade 1 | 8 | 6 | 3 | No | 17 | |
| | Grade 2 | 31 | 15 | 7 | 3 | 56 | 0.27 |
| | Grade 3 | 41 | 26 | 9 | 8 | 84 | |
| Total | | 80 (50.95%) | 47 (29.93%) | 19 (12.1%) | 11 (7.006%) | 157 | |

Abbreviations: Premen - Premenopausal, Postmen - Postmenopausal, Perimen - Perimenopausal
* Rates of CerbB-2 negative cases increased with tumor size. There was a reverse, but weak correlation with tumor size and CerbB-2 expression ($x^2$=6.772, p=0.034). In subgroup analysis of CerbB-2 positive cases, the magnitude of CerbB-2 positivity did not correlate with tumor size ($x^2$=3.039, p=0.551).

**Table 3** Distribution of lymph node involvement according to CerbB-2 status.

| Nodal Involvement | | CerbB-2 Expression | | | | Total | P |
|---|---|---|---|---|---|---|---|
| | | None | 1+ | 2+ | 3+ | | value |
| Level I-II | No positive LN | 35 | 19 | 6 | No | 60 | |
| | 1-3 LN | 29 | 17 | 4 | 7 | 57 | 0.136* |
| | 4-9 | 6 | 5 | 7 | 3 | 21 | |
| | 10 and over | 10 | 6 | 2 | 1 | 19 | |
| Level III (Apex) | No positive LN | 59 | 31 | 14 | 7 | 111 | |
| | 1-3 LN | 13 | 7 | 3 | 1 | 24 | 0.005** |
| | 4-9 LN | 3 | 9 | 2 | 3 | 17 | |
| | 10 and over | 5 | No | No | No | 5 | |
| Rotter (Interpectoral) | No positive LN | 73 | 44 | 16 | 8 | 141 | |
| | 1-3 LN | 5 | 3 | 3 | 3 | 14 | 0.196*** |
| | 4-9 LN | 2 | No | No | No | 2 | |
| Total | | 80 (50.95%) | 47 (29.93%) | 19 (12.1%) | 11 (7.006%) | 157 | |

Abbreviations: LN - Lymph Nodes
*CerbB-2 positivity did not correlate with nodal involvement in Level I-II ($x^2$=5.539, p=0.136). In subgroup analysis of patients with positive CerbB-2 status, positivity of CerbB-2 significantly increased with the number of positive lymph nodes in Level I-II ($x^2$=13.269, p=0,039).
**There was a significant correlation between CerbB-2 positivity and nodal metastases in Level III ($x^2$=12.67, p=0.005), but this difference was not significant among CerbB-2 positive patients ($x^2$=1.535, p=0.82).
***Interpectoral nodal involvement did not have any effect on CerbB-2 positivity ($x^2$=3.264, p=0.196), and was not different among CerbB-2 positive patients ($x^2$=4.179, p=0.124).

CerbB-2 expression did not also correlate with the presence of intraductal component and tumor grade (p=0.973 and p=0.27, respectively). Neither PR, nor P53 expression correlated with CerbB-2 status ($x^2$=0.255, p=1.00, and $x^2$=2.305, p=0.129, respectively). ER status was not affected from CerbB-2 positivity ($x^2$=6.857, p=0.911). But in subjects with positive CerbB-2 expression we detected a reverse correlation with ER positivity and CerbB-2 positivity ($x^2$=9.007, p=0.011, power=77%). The reverse relation was confirmed by the correlation coefficient (r = 0.326). Distribution of patients due to tumoral parameters is listed in Table 4.

**Table 4** Distribution of patients due to tumoral parameters.

| Tumoral Parameters | | CerbB-2 Expression None | 1+ | 2+ | 3+ | Total | P value |
|---|---|---|---|---|---|---|---|
| Estrogen Receptor | Positive | 56 | 38 | 12 | 4 | 110 | 0.911* |
| | Negative | 24 | 9 | 7 | 7 | 47 | |
| Progesteron Receptor | Positive | 51 | 28 | 13 | 8 | 100 | 0.985 |
| | Negative | 29 | 19 | 6 | 3 | 57 | |
| P53 Status | Positive | 35 | 29 | 12 | 3 | 79 | 0.129 |
| | Negative | 45 | 18 | 7 | 8 | 78 | |
| Total | | 80 (50.95%) | 47 (29.93%) | 19 (12.1%) | 11 (7.006%) | 157 | |

* ER status did not correlate with CerbB-2 positivity ($x^2$=6.857, p=0.911). But in subjects with positive CerbB-2 expression, a reverse correlation with ER positivity and CerbB-2 positivity was detected ($x^2$=9.007, p=0.011).

## 4 Discussion

The impact of CerbB-2 amplification on axillary lymph node metastases and survival in breast carcinoma patients was first noticed by Slamon, and co-workers in 1987 [10]. In the following years the idea of accepting this oncogene as an independent prognostic parameter became evident, and was confirmed by others [11]. The correlation of amplification and/or over-expression of the oncogene with other prognostic parameters have been the subject to a numbers of studies, but the results are conflicting [12–14]. In the initial report by Slamon, CerbB-2 amplification was strongly correlated with lymph node involvement; though, tumor size, ER, PR, and age at diagnosis were not [10]. Reports by Tervahauta, and Tsutsui stated that expression of CerbB-2 was neither related to tumor size, nor nodal metastases [13, 15]. Another report of 539 invasive breast cancer patients stated that tumor size, lymphatic involvement, stage, and absence of steroid receptors were correlated with positive CerbB-2 expression [16]. Similarly, studies stating positive correlation of tumor size with CerbB-2 expression are also evident [14, 17].

The importance of Level III (apical=infraclavicular) metastases has been better understood by the introduction of AJCC (American Joint Committee on Cancer) 2003 breast cancer staging system [18]. Due to the new staging scheme, apical nodal involve-

ment is assessed as N3a, and staged as Stage IIIC. It should be noted that, involvement of infraclavicular lymph nodes is associated with a significantly worse disease-free and overall survival compared with non-metastatic patients (50% vs. 68% and 58% vs. 83%, respectively) [19]. In this manner, a portion of patients formerly staged due to the old classification scheme is transferred in to an advanced stage, thereby leading to the Stage Shift (Willy-Rogers) Phenomenon. This new approach increases the importance of Level III nodal metastases in breast cancer. The importance of Interpectoral nodal metastases has been investigated by a number of physicians. In a retrospective review by Komenaka, et al, it has been concluded that recurrence at the interpectoral nodes can be the initial site of surgical failure, and these nodes may represent the site of primary drainage in a percentage of patients. Therefore, work- up should include additional breast imaging and needle biopsy prior to operation [20]. Interpectoral nodal metastases occurring in cN0 breast cancer patients have also been confirmed by preoperative lymphoscintigraphy, but routes of interpectoral involvement has shown a distinct difference in drainage patterns between palpable and nonpalpable lesions [21]. In univariate analysis of our patients, CerbB-2 expression did not correlate with nodal involvement in Level I-II, and Rotter. In subgroup analysis of patients with positive CerbB-2 status, positivity of CerbB-2 linearly increased with the number of positive lymph nodes in Level I-II. There was a significant correlation between CerbB-2 expression and nodal metastases in Level III, but this difference was not significant among CerbB-2 positive patients.

Marx, et al investigated the role of CerbB-2 expression on prognostic parameters in 163 primary breast carcinoma cases. Despite from our results, he failed to demonstrate any influence of tumor size on oncogene status. But, he concluded that patients with 3 or more lymph node metastases express CerbB-2 more frequently than others [22]. In our series the percentage of CerbB-2 negative cases increased with tumor size. There was a reverse, but weak correlation with tumor size and CerbB-2 expression.

The influence of age at diagnosis on CerbB-2 status is also controversial. Despite the evidence of literature reporting a positive correlation of older age (greater than 50) on CerbB-2 status [23], Sjögren and Bebenek have stated that younger patients express CerbB-2 mutation more frequently [24, 25]. Quantification of CerbB-2 in our patients did not show any significant difference between age groups, and menopausal status

Histological grade, regardless of disease stage is related not only to recurrence, but also disease-free and overall survival. In a number of studies [14, 18, 26], a positive influence of histological grade on CerbB-2 expression was reported, but reverse is also evident [25, 27]. Even if the majority of our patients (53.9%) had Grade 3 tumors, CerbB-2 levels did not progress with advanced tumor grade.

The relationship between steroid receptors and CerbB-2 over-expression has been the mainstay of some researches, and a reversal correlation has been pointed out in many of them [15, 18, 23]. Some authors have advocated the theory that the ligands of CerbB-2, heregulin and gp-30, blockade the receptive activity of estrogen [28], while others stating the DNA mutation of estrogen receptors [29], or the co-incidence of HER-2/neu overexpression in patients with adverse prognosis [13]. Meanwhile, a number of authors

have failed to demonstrate a relationship between CerbB-2 expression and status of steroid receptors [27, 30]. In our study, CerbB-2 expression did no correlate with PR status, but we detected a reverse correlation with ER positivity and CerbB-2 positivity (p=0.011).

Overexpression of the mutant tumor suppressor gene P53 is related to dismal prognosis in breast carcinoma patients [25, 31]. Breast cancers expressing both of these markers, located on chromosome 17, loose a key mechanism in the control of cellular proliferation leading to activated malignant proliferation and aggressive neoplastic character [32, 33]. Co-incidence of both P53 and CerbB-2 overexpression has been concluded in some reports [13, 24, 27, 31], but the reverse is also true [34, 35]. Our results also indicate no correlation between P53 and CerbB-2 expression.

CerbB-2 positivity approximates %60-70 in patients with intraductal breast carcinoma [36]. The evidence of a load of literature regarding the correlation of CerbB-2 expression with intraductal component of invasive carcinomas is still lacking. In a few reports, it has been stated that patients with intraductal component express CerbB-2 more frequently [31, 36, 37]. In our study, CerbB-2 status did not correlate with intraductal component (p=0.973).

We detected a weak positive correlation of CerbB-2 expression with tumor size and Level III axillary nodal metastases, indicating a dismal outcome and Stage shift in patients with positive CerbB-2 expression. Furthermore, the reverse correlation of ER positivity with that of CerbB–2 confirms the resistance to endocrine treatment in CerbB–2 positive patients. The lack of randomized clinical trials on wider patient populations still exists, and the utility of such a method remains to be proven by further clinical studies. We think that this study might encourage investigators to concentrate on multicenter clinical trials, examining larger series of patients with breast cancer.

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