Advances in Brief

NH_2-terminal Truncated HER-2 Protein but not Full-Length Receptor Is Associated with Nodal Metastasis in Human Breast Cancer[1]

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Abstract

Background: **The full-length receptor p185HER-2 undergoes a metalloprotease-dependent cleavage producing a membrane-associated fragment (p95HER-2) in cultured breast cancer cells. P95HER-2 has potentially enhanced signaling activity, but its expression and role in human breast cancer is poorly characterized.**

Purpose: **The purpose of this project was to characterize the expression of p95HER-2 in primary breast cancers and nodal metastasis, and to study association with clinicopathological factors.**

Experimental Design: **P95HER-2 and p185HER-2 were examined in 337 primary breast tumors and 81 metastatic lymph nodes by Western blot analysis, and tested for associations with other clinicopathological factors.**

Results: **P95HER-2 was present in 20.9% of primary tumors from node-negative patients, in 29.1% from patients with one to three metastatic nodes, and in 36.7% from patients with four or more metastatic nodes ($P = 0.027$). Whereas p185HER-2 overexpression was unrelated to nodal disease ($P = 0.63$), the odds of lymph node metastasis were enhanced 2.9-fold by the presence of p95HER-2 (48.8% of node-negative *versus* 73.5% of node-positive patients; $P = 0.03$; odds ratio = 2.9). P95HER-2 was more frequent in metastatic lymph nodes than in primary tumors (45.7% *versus* 26.7%; $P = 0.0009$), whereas p185HER-2 overexpression was similar in both (22.3% *versus* 23.5%; $P = 0.933$). P95HER-2 did not significantly correlate with patient age, tumor size, stage, histotype, or hormone receptor status.**

Conclusions: **P95HER-2 in primary tumors was related to extent of lymph node involvement and was enhanced in nodal tissue suggesting an important role as a marker or cause in breast cancer metastasis. Examination of the prognostic value of p95HER-2 in breast cancer and its coexpression with metalloprotease activity seem warranted.**

Introduction

The HER (or ErbB) family of transmembrane tyrosine kinase receptors is composed of four members, HER1 to HER4 (1). HER-2, a ligand-less M_r 185,000 receptor encoded by the *neu* protooncogene, is overexpressed in 25–30% of human breast cancers, and predicts a significantly lower survival rate and a shorter time to relapse in patients with lymph-node positive disease (2). The significance of HER-2/*neu* in node-negative patients is controversial, and thus far, its clinical utility as a prognostic indicator is limited (2).

Recent evidence indicates that HER-2 amplifies the signal provided by other receptors of the ErbB family by heterodimerizing with them, playing an important role in the signaling network that drives epithelial cell proliferation (3). This fact, together with its extracellular accessibility and its overexpression in some human tumors, makes the HER-2 receptor an appropriate target for tumor-specific therapies. Several monoclonal antibodies directed against the HER-2 ECD[4] specifically inhibit the growth of tumor cell lines overexpressing HER-2. One of them, termed trastuzumab, has been humanized and is an effective therapeutic agent against HER-2-overexpressing breast cancers (4–7).

The HER-2 receptor p185HER-2 undergoes a slow but activable proteolytic shedding of its ECD from the surface of HER-2-overexpressing tumor cells in culture (8–10). The HER-2 ECD has been found in the serum of patients with advanced breast cancer where it correlates with recurrence (11) and with decreased responsiveness to endocrine therapy and chemotherapy (11–19). Serum ECD has also been reported to neutralize the activity of anti-HER-2 antibodies targeted to the ECD such as trastuzumab (7, 13).

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[4] The abbreviations used are: ECD, ectodomain; ER, estrogen receptor; PR, progesterone receptor; OR, odds ratio; CI, confidence interval; IHC, immunohistochemical; DFS, disease-free survival; OS, overall survival.

We have reported recently that proteolytic shedding of the HER-2 ECD from cultured tumor cells generates a M_r 95,000 membrane-associated fragment that has *in vitro* kinase activity and is tyrosine-phosphorylated (20, 21). This p95HER-2 fragment, which is missing its ECD, may have constitutive kinase activity and, thus, enhanced signaling potency in tumor cells. This view is suggested by studies showing that an engineered deletion of HER-2 ECD increases the tyrosine kinase activity and transforming efficiency of the resulting truncated protein (22, 23). Other lines of evidence support the concept that the ECD of tyrosine-kinase receptors constrains the kinase activity of their cytoplasmic region. First, several retroviral oncogenes code for receptors that lack most of the ECD resulting in production of constitutively active, membrane-bound receptor fragments (24). Second, mutant epidermal growth factor receptors with NH_2-terminal truncations, found in several human carcinomas, have enhanced oncogenic activity (25, 26). Third, ECD shedding of HER4 results in the formation of a membrane-truncated fragment that has tyrosine kinase activity, is tyrosine phosphorylated, and may act as a membrane-localized docking anchor for signaling molecules with SH2 domains (27).

Despite the potential importance of the truncated p95HER-2 in oncogenic signaling, its expression and significance in human breast cancer has not been extensively evaluated. Recently, we described the presence of the NH_2-terminally truncated, tyrosine phosphorylated p95HER-2 in a subset of primary human breast cancer tissues (20, 21). In our preliminary study, p95HER-2 appeared to be at higher levels in the primary tumors from patients with node-positive breast cancer (21).

Therefore, the study presented here was designed to comprehensively analyze the possible role of p95HER-2 compared with the full length receptor in lymph node metastases, which is the strongest risk factor for disease recurrence and poor outcome in breast cancer (2). To this end, we examined the expression of p95HER-2 and p185HER-2: (*a*) in metastatic lymph node tissue; and (*b*) in primary tumors from a well-characterized patient population stratified into groups that differed in the extent of lymph node involvement. The results of this study suggest a role for the truncated p95HER-2 distinct from the full length receptor in breast cancer metastasis.

Materials and Methods

Cells and Antibodies. The BT474 cell line was obtained from the American Type Culture Collection (Rockville, MD) and grown in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM of glutamine, and 10 μg/ml insulin (all from Life Technologies, Inc. Ltd., Paisley, United Kingdom). The 17-3-1 cells (NIH3T3 cells transfected with HER-2/*neu* cDNA) were provided by Applied Biotechnology Inc. (Cambridge, MA) and cultured in DMEM supplemented with 5% fetal bovine serum containing 0.4 mg/ml geneticin (G418; Life Technologies, Inc. Ltd.). The antibody against the COOH terminus of p185HER-2/*neu*, anti-neu(C), has been described previously (28). Secondary antibody, conjugated to horseradish peroxidase, was from Amersham Pharmacia Biotech (Little Chafont, United Kingdom) or from Bio-Rad (Hercules, CA).

Western Blot Analysis of p95 and p185 in Breast Cancer Tissues. Breast tissues used in this study were surgical resection specimens obtained at Oregon Health Sciences University Hospital (Portland, OR) following procedures approved by the Institutional Review Board Committee on Human Research, at Hospital Clínico Universitario of Valencia (Spain) and at Vall d′Hebron Hospital of Barcelona (Spain) following Institutional Guidelines. Breast tumor samples were analyzed as described previously (21). Several controls and extraction procedures were conducted previously to characterize p95 and to ensure that it was not generated as a degradation artifact (20, 21). About 0.1 g of tumor tissue, which had been fresh-frozen and stored at −80°C, was minced on dry ice, and suspended in buffer containing protease inhibitors (1% aprotinin, 2 mM of phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) and a phosphatase inhibitor (2 mM of sodium orthovanadate). After homogenization and fractionation, equal amounts of protein from each sample (20 μg) were resolved under denaturing and reducing conditions by SDS-PAGE in 8% gels. Each gel also contained 3 μg of protein from extracts of 17-3-1 and 4-aminophenylmercuric acetate-treated BT474 cells (20) to mark the migration of the bands and to provide a standard for the entire study. Electrophoretic transfer to nitrocellulose membranes (Bio-Rad) was followed by immunoblotting with the anti-neu(C) antibody. Finally, membranes were incubated with an antirabbit secondary antibody conjugated to horseradish peroxidase, which was detected by chemiluminescence (Pierce, Rockford, IL) and exposure to film.

HER-2 Protein Detection. The samples were arbitrarily scored as p185HER-2 overexpressers if the p185HER-2 signal could be detected by 1-min exposure of the film and if the signal was greater than or equal to the p185 level in 3 μg of 17-3-1 cells or BT-474. Specimens were scored as positive for p95HER-2 if this band was detected after a 15-min exposure of the membrane to the film. The levels of p95HER-2 in tumor samples were usually lower than those of p185HER-2. Because of the high titer of the primary antibody, there were rarely any background bands.

Clinical Data. A computer database, coded to protect the identity of the individual, was compiled containing clinical information on each patient including age, nodal status, histotype, and size of the primary tumor, age of the patient, stage of disease at diagnosis, ER and PR levels. The type of tumor of the specimens were as follows: 74.4% infiltrating ductal, 6.5% infiltrating lobular, 6.0% nonspecific adenocarcinoma, 3.5% mixed ductal/lobular, 2% medullary, and 7.5% other (mucinous, papillary, inflammatory, and so forth).

Statistical Analysis. Associations between variables were determined by χ^2 analysis using StatView by SaS Institute Inc. Strength of association for study comparisons were calculated as OR and 95% CI using standard formulas. All of the *P*s reported are two-sided.

Results

p185 and p95HER-2 Levels in Primary Breast Tumor Tissue. Tumor tissues (n = 337) collected in three different hospitals from the United States (Oregon Health Sciences University Hospital) and Spain (Hospital Clínico of Valencia and

Table 1 p95HER-2 and p185HER-2 expression in 337 primary breast cancers[a]

p185 expression	% p95 positive
Negative (95)	2.1
Low (82)	19.5
Moderate (85)	31.8
Overexpression (75)	60.0

[a] Breast cancer tissues were examined by Western blot analysis for p95HER-2 and p185HER-2 as described in "Materials and Methods." Compared to p185 negative tissue, p95HER-2 was significantly higher in tissues with low ($P < 0.001$; OR = 11.27; CI = 2.51–50.7) or moderate p185 levels ($P < 0.00001$; OR = 21.65; CI = 4.96–94.46). p95 levels in tissue with p185 overexpression was significantly higher than in tissue with moderate p185 ($P < 0.001$; OR = 3.22; CI = 1.68–6.17) or than in p185-negative tissues ($P < 0.00001$; OR = 69.75; CI = 15.96–304.87).

Table 2 Relationship between p95HER-2 expression and p185HER-2 overexpression with extent of lymph node involvement[a]

Nodal status	% p95 positive[b]	% p185 overexpression[c]
Negative (172)	20.9	23.8
1–3 node (86)	29.1	18.6
4 or more nodes (79)	36.7	22.8

[a] Primary breast cancer tissue from 337 patients was examined by Western blot and grouped according to the number of lymph nodes positive for breast cancer.

[b] Overall, p95HER-2 associated with increased extent of node involvement ($P = 0.027$) and was significantly more frequent in patients with four or more positive nodes compared with those that were node negative ($P = 0.013$; OR = 2.19; CI = 1.22–3.94).

[c] p185HER-2 overexpression did not correlate with extent of lymph node involvement ($P = 0.630$) and was not significantly different in patients with four or more metastatic nodes *versus* node negative ($P = 0.982$; OR = 0.94; CI = 0.5–1.77).

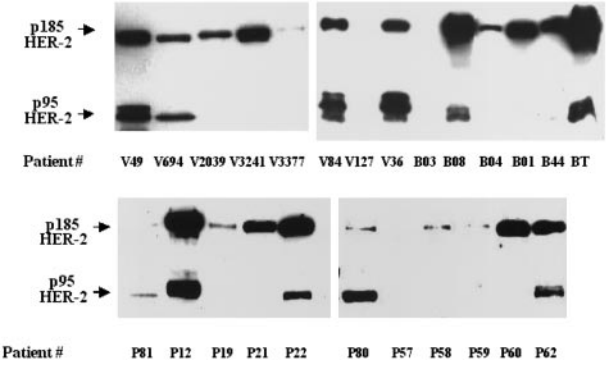

Fig. 1 Western blotting analysis of 24 breast cancer tissues. Tissues were extracted and subjected to Western blotting with anti-HER-2 as described in "Materials and Methods." Of the 337 tumors analyzed, only 24 of them are presented, representative of samples expressing no HER-2 forms (V3377, V127, B03, P57), only p185 HER-2 (V2039, V3241, B04, B01, B44, P19, P21, P58, P59, P60), or p185 and p95 HER-2 (V49, V649, V84, V36, B08, P81, P12, P22, P80, P62). Samples are also representative of tumors expressing no p185 HER-2 (V3377, V127, B03, P57), low or moderate (P81, P19, P80, P58, P59, V694, V2039, V84, V36, B04), or high p185HER-2 levels (V49, V3241, B08, B01, B44, P12, P21, P22, P60 and P62). In most tumors expressing both HER-2 forms, the amount of p95 was lower or comparable with p185 (as in V49, V649, V84, B08, P12, P22, P62); only in some cases was the truncated fragment present at higher levels than the full-length receptor (as in V36 or P80). A control lane is also represented (*BT*) containing 3 μg of protein from an APMA-induced (20) BT-474 culture. Exposure time was 15′.

Vall d'Hebron Hospital of Barcelona) were examined for HER-2/*neu* proteins by Western analysis using an antibody that recognizes the receptor COOH terminus (28). As reported previously in numerous studies, there were distinct expression patterns of p185 in the breast cancer samples (Table 1; Fig. 1). Analysis of 40 tumor samples revealed that the tumors with high p185 levels, determined by Western blot analysis, showed moderate or intense complete membrane IHC staining in >10% of tumor cells. This suggested that a similar group of HER-2-overexpressing samples were identified by Western blot analysis and by immunohistochemistry.

Tumors were also heterogeneous for p95HER-2 expression. Western analyses revealed that 26.7% of the primary breast cancer samples expressed p95. To examine the association of p95 with p185HER-2, the samples were stratified into those that were negative for p185HER-2 or those that expressed low, moderate, or high levels of the full-length receptor. The truncated fragment was apparent in only 2.1% of the p185-negative tumors, in 19.5% of low, in 31.8% of moderate, and in 60% of tumors that were highly positive for p185HER-2 ($P < 0.001$; see Fig. 1 and Table 1). Therefore, p95 expression strongly correlated with p185 expression in tumor tissue.

Relationship of p95 and p185 Levels in Primary Tumor with the Extent of Lymph Node Involvement. In a preliminary study, p95HER-2 expression was significantly higher in node-positive *versus* node-negative samples (21). In the current study, we grouped the samples into three categories according to the number of metastatic lymph nodes detected in patients: zero, one to three, or four or more nodes affected. The frequency of p185 HER-2 overexpression in primary tumor in these three groups was 23.8%, 18.6%, and 22.8%, respectively (Table 2). Correlation analysis showed that p185HER-2 overexpression in primary tumors did not associate with lymph node status either when the three groups were considered ($P = 0.630$) or when patients that had four or more affected nodes were compared with those that were node-negative ($P = 0.982$; OR = 0.94; CI = 0.5–1.77).

The truncated p95 was detected in 20.9%, 29.1%, and 36.7% of patients having zero, one to three, and four or more nodes affected, respectively (Table 2). Overall, the expression of the truncated fragment was significantly associated with increased extent of lymph node involvement ($P = 0.027$). The proportion of primary tumors positive for p95HER-2 was significantly higher in patients that had four or more positive nodes compared with those that were node-negative ($P = 0.013$; OR = 2.19; CI = 1.22–3.94).

Relationship of p95 Positivity and Lymph Node Status in the p185HER-2-overexpressing Group. Whereas ~60% of the tumors that overexpressed p185HER-2 were also positive for p95 (Table 1), p185 overexpression did not correlate with the extent of lymph-node metastasis (Table 2). Therefore, we assessed whether the presence or absence of p95 in the p185 HER-2-overexpressing tumors ($n = 78$) affected the relationship with lymph node status (Table 3). In this group, we found

Table 3 Relationship between p95HER-2 expression and lymph node status in the group that overexpresses p185HER-2[a]

Nodal status	% p95 positive	% p95 negative
Negative (41)	48.8	51.2
Positive (34)	73.5	26.5

[a] Primary breast cancer tissues (75) that overexpressed p185HER-2 (see Table 1) were arranged into p95-positive and p95-negative groups and assessed for lymph node status. The p185-overexpressing samples that were p95-positive were more frequently from patients with metastasis to the lymph nodes, whereas those that were p95-negative were more frequently from patients that did not have metastasis ($P = 0.03$ by Fisher's exact test; OR = 2.9; CI = 1.1–7.75).

Table 4 Relationship between p95HER-2 expression and p185HER-2 overexpression with other prognostic factors[a]

	% p95 positive[b]	% p185 overexpression[b]
ER		
Negative (116)	27.6	24.1
Positive (221)	26.2	21.2
PR		
Negative (144)	22.2	23.6
Positive (193)	30.1	21.2
Age		
0–59 (176)	26.7	22.7
≥60 (161)	26.7	21.7
Stage		
I (85)	18.8	21.2
II (141)	29.1	26.2
III–IV (88)	26.1	19.3
Histotype		
Infiltrating ductal (251)	28.2	22.1
Other (86)	19.1	19.1
Tumor size ($n = 218$)[c]		
<3 cm (144)	28.5	18.8
≥3 cm (74)	31.1	21.6

[a] Primary breast cancer tissues (337) were examined by Western analysis.

[b] There was no significant association between p185HER-2 overexpression with any of the above factors ($P > 0.1$). There was no significant association between p95 with any of the above factors. $P \geq 0.1$ for all factors except histotype ($P = 0.08$).

that 48.8% of the node-negative patients expressed p95, whereas the truncated fragment was present in 73.5% of node-positive patients. Therefore, p185HER-2 overexpression in the absence of p95 correlated with node-negative disease, whereas in the presence of p95, there was an association with lymph node-positive disease ($P = 0.03$; OR = 2.92; CI = 1.10–7.75).

Relationship between p95, p185, and Other Prognostic Factors in Primary Breast Cancer. We analyzed the possible association of p95 and p185 expression levels with several clinical parameters (Table 4) known to be risk factors (2). There was no significant association of either p95 or p185 overexpression with ER or PR status ($P < 0.1$). Moreover, p95 and p185 were at similar levels in tumors from patients that were less than or greater than age 60 years ($P < 0.1$). Stratification of patients into groups based on stage of the disease revealed no significant difference in p95 or p185 in these different groups ($P \geq 0.1$). Moreover, neither of the two HER-2 forms were significantly elevated in large (>3 cm) tumors ($P > 0.1$). Finally, when the samples were grouped into infiltrating ductal *versus* other histological subtypes, there was no significant difference in p185 overexpression ($P > 0.1$) nor p95 expression in the two groups ($P = 0.08$).

Table 5 p95HER-2 expression and p185HER-2 overexpression in primary breast cancer and in metastatic nodes[a]

	% p95 positive[b]	% p185 overexpression[c]
Primary breast (337)	26.7	22.3
Metastatic nodes (81)	45.7	23.5

[a] Primary tumor and metastatic tissue came from different patients.

[b] p95HER-2 was significantly higher in metastatic lymph node tissue than in primary breast cancer ($P = 0.0009$; OR = 2.31; CI = 1.4–3.8).

[c] p185HER-2 overexpression was not significantly different in metastatic lymph node tissue *versus* primary breast cancer ($P = 0.933$; OR = 1.07; CI = 0.6–1.90).

Expression of p95 and p185HER-2 in Metastatic Nodes. To additionally evaluate the involvement of p95 and p185HER-2 in metastasis, we examined whether the expression of either of the HER-2 forms was altered in breast cancer tissue obtained from metastatic lymph nodes. The truncated fragment was present in 45.7% of metastatic nodes compared with 26.7% of samples from primary tumors ($P = 0.0009$; OR = 2.31; CI = 1.4–3.8; Table 5). In contrast, p185 overexpression was not significantly different in metastatic node tissue relative to primary tumor, 22.3% *versus* 23.5% ($P = 0.933$; OR = 1.07; CI = 0.6–1.9). This is the first time that an alternative form of the HER-2 receptor has been found to be elevated in metastatic tissue.

Discussion

In this report, we describe the expression of full-length (p185) and NH_2-terminal truncated (p95) HER-2 receptor by Western blot analysis in a panel of 337 primary breast cancer samples and in 81 samples from metastatic nodes. Breast tumors were found to show different expression patterns for the two HER-2 receptor forms. An important conclusion of our study is that the significance of p95 and p185HER-2 in regard to breast cancer metastasis to the lymph nodes is different. Thus, whereas p95HER-2 expression was significantly more frequent in tissue from metastatic nodes, and its presence in primary tumor correlated with the extent of lymph node metastasis; p185HER-2 overexpression was equally probable in primary *versus* metastatic tissue and in node-negative *versus* node-positive patients.

Whereas almost all of the samples (98%) that contained p95 showed detectable levels of p185HER-2, some tumors expressed p185 but not p95 (see Fig. 1 and Table 1), suggesting that the level of truncated receptor is regulated. Our previous studies have demonstrated that p95HER-2 is generated by proteolytic shedding of the receptor ECD in cultured breast carcinoma cells (20, 21). A similar processing event may be responsible for generation of p95 in breast tumor tissue, because the shed ECD has also been found in the serum of patients with HER-2-positive breast cancer (16–19). However, the possibility remains that p95 or the ECD in serum could be the product of alternative HER-2 transcripts (29). Some additional evidence supports the interpretation that p95 in tumors is generated by

shedding. First, the truncated HER-2 in tumors exactly comigrates in gels with the p95HER-2 generated by proteolytic cleavage in cultured cells. Second, an alternative HER-2 transcript that expresses a product resembling p95 has not been found. The protease responsible for the HER-2 shedding from breast carcinoma cells has not yet been identified but is likely a metalloprotease (20, 21, 30). Identification of this sheddase will be important to understand the role it might play in regulation of HER-2 cleavage and generation of the p95 fragment.

Western analysis is currently the only method that can discriminate between the two HER-2 protein forms. IHC analysis, most commonly used for clinical detection of HER-2 protein expression, cannot discern the presence of p95HER-2. Western blotting, in contrast to IHC, involves analysis of block tissue and is, therefore, incapable of discriminating the specific cells in the tumor tissue that express the HER-2 protein. Because p95 and p185HER-2 are detected only in carcinoma epithelial cells (20), those tissue samples highly contaminated with stroma would tend to show low levels of both HER-2 protein forms. When we analyzed 40 tumors by IHC, the results were coincident with those obtained by Western blotting for p185HER-2 overexpression, suggesting that false negatives are not present in a significant proportion.

We found p185HER-2 overexpression in 22.3% of primary breast tumors examined by Western analysis (Table 5), which is within the range of 20–35% reported in most studies using a variety of methods of detection (2, 31). Lymph node metastases overexpressed the full-length receptor to a similar extent (23.5%). This is in agreement with a recent study (32) that evaluated p185HER-2 in primary and metastatic tissue from 196 breast cancer patients. The receptor status of primary tumors was found to be maintained in the majority of their metastases. Therefore, a conclusion of this study (32) was that loss of p185HER-2 expression in metastatic tissue was not likely responsible for failure to respond to trastuzumab therapy in a significant fraction of HER-2-overexpressing patients. We report here that, in contrast to the full length receptor, p95HER-2 is more frequently expressed in lymph node metastatic tissue (45.7%) than in primary tumors (26.7%). If the truncated kinase p95 has a role in metastasis, as our study suggests, its presence may identify patients that are unlikely to respond to trastuzumab. Small molecule tyrosine kinase inhibitors may prove to be effective against p95-positive breast cancer.

The presence of axillary lymph node metastasis as well as tumor size are the most important prognostic factors for DFS and OS in women with breast carcinoma (33). Detection of positive nodes affects more treatment decisions than any other single factor (2). According to our results, neither p185HER-2 overexpression nor moderate expression in the primary tumor significantly correlated with lymph node metastasis (see Tables 2 and 3). The numerous studies addressing whether HER-2 overexpression is associated with node status have yielded conflicting results. Most of the more recent reports, some of them involving >800 patients (34–36), have not found a significant association of p185HER-2 overexpression assessed by IHC (35–38) or HER-2/*neu* gene amplification (34, 39) with lymph node involvement. Only a few authors have reported a significant correlation between these two parameters (40, 41). Our results, showing that only the truncated p95 HER-2 associates with node involvement, may help clarify why previous studies, which did not discern the presence of p95, failed to find consistent correlations between HER-2 expression and lymph node metastasis.

A possible explanation for the association of p95 but not p185HER-2 with metastasis is that the truncated fragment has enhanced biological potency. *In vitro* studies have demonstrated that engineered deletion of the ECD increases the kinase activity and transforming efficiency of the resulting HER-2 truncated protein (22, 23). Moreover, p95 HER-2, generated by proteolytic shedding, is tyrosine phosphorylated and has kinase activity (20, 21), two properties associated with signaling activity. On the basis of our findings we propose that overexpression of the full-length HER-2 receptor may promote the initial events of abnormal proliferation in the primary tumor but, in the absence of p95, these tumors are less likely to metastasize. An alternative explanation is that p95 could mark tumors that express a particular protease that is relevant for metastasis, although based on the above-mentioned biochemical properties of p95, we consider this second possibility rather improbable.

Neither the presence of p95 in primary breast tumor nor the overexpression of p185HER-2 correlated with other prognostic markers in this study such as stage of the disease, tumor size, histological subtype, or hormone receptor status (Table 4). The lack of correlation with tumor size may explain why there was no association between p95HER-2 and stage, which is based on tumor size as well as presence of metastatic nodes. Several studies have failed to find an association between HER-2 overexpression and stage or tumor size (39, 41–43), whereas only a few authors have reported a significant correlation between these two parameters (35, 38). Interestingly, in all of the cases where only node-negative patients were analyzed, p185HER-2 did not correlate with tumor size (44–46). Noncoincident results also exist regarding the association of HER-2 overexpression and tumor stage (39, 40, 47), or ER and PR negativity (34, 38, 39, 42, 45, 48). Finally, in the limited number of studies where histotype of the tumor was determined, it did not relate with HER-2 overexpression (34, 46).

Of the new cases of breast cancer that occur in the United States annually, about two-thirds have node-negative disease (49). Approximately 30% of patients diagnosed with node-negative breast cancer will relapse within 10 years after surgery and, therefore, may benefit from adjuvant systemic therapy (50). Current prognostic indicators, such as histomorphological and clinical factors, are not sufficiently sensitive to identify this subgroup of patients, and there is no singular predictor of outcome for node-negative disease. Therefore, it remains a challenge to identify new markers with predictive value to differentiate the node-negative patients at the greatest risk of relapse. Although it seems to be widely accepted that *c-erbB-2* amplification and overexpression in tumor cells is associated with a worse DFS and OS in node-positive patients, the prognostic value in node-negative patients is still controversial (2). Some studies have not found an association between HER-2 amplification or overexpression and DFS or OS in node-negative breast cancer patients (35, 42, 50, 51), whereas others have described a correlation with DFS (33, 44, 46) or with OS and DFS (40, 45, 49). In some reports, the significance has been restricted to subsets of lymph node-negative patients (52, 53) additionally emphasizing the limited prognostic value of

HER-2. These discrepancies have been attributed to methodological differences (reviewed in Ref. 31) or insufficient statistical power (2, 45). According to our results, p95HER-2 in the primary tumor indicates a 2.9-fold increased chance of metastasis, which is the single strongest predictor of poor outcome, in HER-2-overexpressing patients. Therefore, p95HER-2, which was detected in node-negative patients, could be of value in predicting outcome for this group. It will be important to conduct follow-up studies to determine the value of p95HER-2 in identifying node-negative breast cancer patients at high risk of developing recurrent disease.

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References

1. Groenen, L. C., Nice, E. C., and Burgess, A. W. Structure-function relationships for the EGF/TGF-α family of mitogens. Growth Factors., *11:* 235–257, 1994.

2. Henderson, I. C., and Patek, A. J. The relationship between prognostic and predictive factor in the management of breast cancer. Breast Cancer Res. Treat., *52:* 261–268, 1998.

3. Klapper, L. N., Kirschbaum, M. H., Sela, M., and Yarden, Y. Biochemical and clinical implications of the ErbB/HER signaling network of growth factor receptors. Adv. Cancer Res., *77:* 25–79, 2000.

4. Carter, P., Presta, L., Gorman, C. M., Ridgway, J. B., Henner, D., Wong, W. L., Rowland, A. M., Kotts, C., Carver, M. E., and Shepard, H. M. Humanization of an anti-p185HER-2 antibody for human cancer therapy. Proc. Natl. Acad. Sci. USA, *89:* 4285–4289, 1992.

5. Fendly, B. M., Winget, M., Hudziak, R. M., Lipari, M. T., Napier, M. A., and Ullrich, A. Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER-2/neu gene product. Cancer Res., *50:* 1550–1558, 1990.

6. Pegram, M. D., Lipton, A., Hayes, D. F., Weber, B. L., Baselga, J. M., Tripathy, D., Baly, D., Baughman, S. A., Twaddell, T., Glaspy, J. A., and Slamon, D. J. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER-2/neu monoclonal antibody plus cisplatin in patients with HER-2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. J. Clin. Oncol., *16:* 2659–2671, 1998.

7. Baselga, J., Tripathy, D., Mendelsohn, J., Baughman, S., Benz, C. C., Dantis, L., Sklarin, N. T., Seidman, A. D., Hudis, C. A., Moore, J., Rosen, P. P., Twaddell, T., Henderson, I. C., and Norton, L. Phase II study of weekly intravenous recombinant humanized anti-p185^{HER-2} monoclonal antibody in patients with HER-2/neu-overexpressing metastatic breast cancer. J. Clin. Oncol., *14:* 737–744, 1996.

8. Lin, Y. L., and Clinton, G. M. A soluble protein related to the HER-2 proto-oncogene product is released from human breast carcinoma cells. Oncogene, *6:* 639–643, 1991.

9. Pupa, S. M., Menard, S., Morelli, D., Pozzi, B., De Palo, G., and Colnaghi, M. The extracellular domain of the c-erbB-2 oncoprotein is released from tumor cells by proteolytic cleavage. Oncogene, *8:* 2917–2923, 1993.

10. Zabrecky, J. R., Lam, T., McKenzie, S. J., and Carney, W. The extracellular domain of p185/neu is released from the surface of human breast carcinoma cells, SK-BR-3. J. Biol. Chem., *266:* 1716–1720, 1991.

11. Brandt-Rauf, P. W. The c-erb transmembrane growth factor receptors as serum biomarkers in human cancer studies. Mutat. Res., *333:* 203–208, 1995.

12. Molina, R., Zanon, J. J., Filella, X., Farrus, B., Munoz, R., Latre, M. L., Pahisa, J., Velasco, M., Fernandez, P., Estape, J., and Bassesta, A. M. Utility of c-erbB-2 in tissue and in serum in the early diagnosis of recurrence in breast cancer patients: comparison with carcinoembryonic antigen and CA. Br. J. Cancer, *4:* 1126–1131, 1996.

13. Brodowicz, T., Wiltschke, C., Budinsky, A. C., Krainer, M., Steger, G. G., and Zielinski, C. C. Soluble HER-2/neu neutralizes biologic effects of anti-HER-2/neu antibody on breast cancer cells *in vitro*. Int. J. Cancer., *73:* 875–879, 1997.

14. Kandl, H., Seymour, L., and Bezwoda, W. R. Soluble c-erbB-2 fragment in serum correlates with disease state and predicts for shortened survival in patients with early-stage and advanced breast cancer. Br. J. Cancer., *70:* 739–742, 1994.

15. Fehm, T., Maimonis, P., Katalinic, A., and Jager, W. H. The prognostic significance of c-erbB-2 serum protein in metastatic breast cancer. Oncology, *55:* 33–38, 1998.

16. Leitzel, K., Teramoto, Y., Konrad, K., Chinchilli, V. M., Volas, G., Grossberg, H., Harvey, H., Demers, L., and Lipton, A. Elevated serum c-erbB-2 antigen levels and decreased response to hormonal therapy of breast cancer. J. Clin. Oncol., *13:* 1129–1235, 1995.

17. Yamauchi, H., O'Neill, A., Gelman, R., Carney, W., Tenney, D. Y., Hosch, S., and Hayes, D. Prediction of response to antiestrogen therapy in advanced breast cancer patients by pretreatment circulating levels of extracellular domain of the HER-2/c-neu protein. J. Clin. Oncol., *15:* 18–25, 1996.

18. Colomer, R., Montero, S., Lluch, A., Ojeda, B., Barnadas, A., Casado, A., Massutí, A., Cortés-Funes, H., and Lloveras, B. Circulating HER-2 extracellular domain and resistance to chemotherapy in advanced breast cancer. Clin. Cancer Res., *6:* 2356–2362, 2000.

19. Harris, L. N., Trock, B., Berris, M., Esteva-Lorenzo, F., and Paik, S. The role of ERBB2 extracellular domain in predicting response to chemotherapy in breast cancer patients. Proc. Am. Soc. Clin. Oncol., *15:* A96, 1996.

20. Molina, M. A., Codony-Servat, J., Albanell, J., Rojo, F., Arribas, J., and Baselga, J. Trastuzumab (Herceptin), a humanized anti-HER-2 receptor monoclonal antibody, inhibits basal and activated HER-2 ectodomain cleavage in breast cancer cells. Cancer Res., in press, 2001.

21. Christianson, T. A., Doherty, J. K., Lin, Y. J., Ramsey, E. E., Holmes, R., Keenan, E. J., and Clinton, G. M. NH_2-terminally truncated HER-2/neu protein: relationship with shedding of the extracellular domain and with prognostic factors in breast cancer. Cancer Res., *15:* 5123–5129, 1998.

22. DiFiore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R., and Aaronson, S. A. erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science (Wash. DC), *237:* 178–182, 1987.

23. Segatto, O., King, C. R., Pierce, J. H., Di Fiore, P. P., and Aaronson, S. A. Different structural alterations upregulate *in vitro* tyrosine kinase activity and transforming potency of the *erbB-2* gene. Mol. Cell. Biol., *8:* 5570–5574, 1988.

24. Rodrigues, G. A., and Park, M. Oncogenic activation of tyrosine kinases. Curr. Opin. Genet. Dev., *4:* 15–24, 1994.

25. Moscatello, D. K., Montgomery, R. B., Sundareshan, P., McDanel, H., Wong, M. Y., and Wong, A. J. Transformation and altered signal transduction by a naturally occurring mutant EGF receptor. Oncogene, *13:* 85–96, 1996.

26. Moscatello, D. K., Holgado-Maduraga, M., Godwin, A. K., Ramirez, G., Gunn, G., Zoltick, P. W., Biegel, J. A., Hayes, R. L., and Wong, A. J. Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. Cancer Res., *55:* 5536–5539, 1995.

27. Vecchi, M., and Carpenter, G. Constitutive proteolysis of the ErbB-4 receptor tyrosine kinase by a unique, sequential mechanism. J. Cell. Biol., *139:* 995–1003, 1997.

28. Lin, Y. J., Li, S., and Clinton, G. M. Insulin and epidermal growth factor stimulate phosphorylation of p185HER-2/neu in the breast carcinoma line, BT474. Mol. Cell. Endocrinol., *69:* 111–119, 1990.

29. Scott, G. K., Robles, R., Park, J. W., Montgomery, P. A., Daniel, J., Holmes, W. E., Lee, J., Keller, G. A., Li, W., Fendly, B. M., Wood, W. I., Shepard, M., and Benz, C. C. A truncated intracellular HER-2/neu receptor produced by alternative RNA processing affects growth of human carcinoma cells. Mol. Cell. Biol., *13:* 2247–2257, 1993.

30. Codony-Servat, J., Albanell, J., Lopez-Talavera, J. C., Arribas, J., and Baselga, J. Cleavage of the HER-2 ectodomain is a pervanadate activable process that is inhibited by the tissue inhibitor of metalloproteases TIMP-1 in breast cancer cells. Cancer Res., *59:* 1196–1201, 1999.

31. Fitzgibbons, P. L., Page, D. L., Weaver, D., Thor, A. D., Allred, D. C., Clark, G. M., Ruby, S. G., O'Malley, F., Simpson, J. F., Connolly, J. L., Hayes, D. F., Edge, S. B., Lichter, A., and Schnitt, S. J. Prognostic factors in breast cancer. College of American Pathologists consensus statement 1999. Arch. Pathol. Lab. Med., *124:* 966–978, 2000.

32. Simon, R., Nocito, A., Hubsher, T., Bucher, C., Torhorst, J., Scharml, P., Bubendorf, L., Mihatsch, M. M., Moch, H., Wilber, K., Schotzau, A, Kononen, J., and Sauter, G. Patterns of HER-2/neu amplification and overexpression in primary and metastatic breast cancer. J. Natl. Cancer. Inst., *93:* 1141–1146, 2001.

33. Harris, J. R., Lippman, M. E., Veronesi, U., and Willet, W. Breast cancer. N. Engl. J. Med., *327:* 473–480, 1992.

34. Torregrosa, D., Bolufer, P., Lluch, A., Lopez, J. A., Barragan, E., Ruiz, A., Guillem, V., Munarriz, B., and Garcia-Conde, J. Prognostic significance of *c-erbB-2/neu* amplification and epidermal growth factor receptor (EGFR) in primary breast cancer and their relation to estradiol receptor (ER) status. Clin. Chim. Acta., *262:* 99–119, 1997.

35. Thor, A. D., Liu, S., Edgerton, S., Moore, D., Kasowitz, K. M., Benz, C. C., Stern, D. F., and DiGiovanna, M. P. Activation (tyrosine phosphorylation) of ErbB-2 (HER-2/neu). A study of incidence and correlation with outcome in breast cancer. J. Clin. Oncol., *18:* 3230–3239, 2000.

36. Barth, A., Craig, P. H., and Silverstein, M. J. Predictors of axillary lymph node metastases in patients with T1 breast carcinoma. Cancer (Phila.), *79:* 1918–1922, 1997.

37. Mittra, I., Redkar, A. A., and Badwe, R. A. Prognosis of breast cancer: evidence for interaction between c-erbB-2 overexpression and number of involved axillary lymph nodes. J. Surg. Oncol., *60:* 106–111, 1995.

38. Beckmann, M. W., Niederacher, D., Massenkeil, G., Tutschek, B., Beckmann, A., Schenko, G., Schnurch, H-G., and Bender, H. G. Expression analyses of epidermal growth factor receptor and HER-2/neu: no advantage of prediction in recurrence or survival in breast cancer patients. Oncology, *53:* 441–447, 1996.

39. Scorilas, A., Yotis, J., Stravolemos, K., Gouriotis, D., Keramopoulos, A., Ampela, K., Talieri, M., and Trangas, T. c-*erb*B-2 overexpression may be used as an independent prognostic factor for breast cancer patients. Anticancer Res., *15:* 1543–1548, 1995.

40. Depowski, P. L., Brien, T. P., Sheehan, C. E., Stylos, S., Johnson, R. L., and Ross, J. S. Prognostic significance of $p34^{cdc2}$ cyclin-dependent kinase and MIB1 overexpression, and HER-2/*neu* gene amplification detected by fluorescence *in situ* hybridization in breast cancer. Am. J. Clin. Pathol., *112:* 459–469, 1999.

41. Tetu, B., and Brisson, J. Prognostic significance of HER-2/neu oncoprotein expression in node-positive breast cancer. Cancer (Phila.), *73:* 2359–2365, 1994.

42. Tandon, A. K., Clark, G. M., Chamness, G. C., Ullrich, A., and McGuire, W. L. HER-2/neu oncogene protein and prognosis in breast cancer. J. Clin. Oncol., *7:* 1120–1128, 1989.

43. Singleton, T. P., and Strickler, J. G. Clinical and pathologic significance of the *c-erbB-2 (HER-2/neu)* oncogene. Pathol. Annu., *27:* 165–198, 1992.

44. Paterson, M. C., Dietrich, K. D., Danyluk, J., Paterson, A. H., Lees, A. W., Jamil, N., Hanson, J., Jenkins, H., Krause, B. E., McBlain, W. A., Slamon, D. J., and Fourney, R. M. Correlation between c-erbB-2 amplification and risk of recurrent disease in node-negative breast cancer. Cancer Res., *51:* 556–567, 1991.

45. Press, M. F., Bernstein, L., Thomas, P. A., Meisner, L. F., Zhou, J. Y., Ma, Y., Hung, G., Robinson, R. A., Harris, C., El-Naggar, A. S., Slamon, D. J., Phillips, R. N., Ross, J. S., Wolman, S. R., and Flom, K. J. 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.

46. Andrulis, I. L., Bull, S. B., Blackstein, M. E., Sutherland, D., Mak, C., Sidlofsky, S., Pritzker, K. P., Hartwick, R. W., Hann, W., Lickley, L., Wilkinson, R., Qizilbash, A., Ambus, U., Lipa, M., Weizel, H., Katz, A., Baida, M., Mariz, S., Stoik, G., Dacamara, P., Strongitharm, D., Geddie, W., and McCready, D. *neu/erbB-2* amplification identifies a poor-prognosis group of women with node-negative breast cancer. J. Clin. Oncol., *16:* 1340–1349, 1998.

47. Agrup, M., Stal, O., Olsen, K, and Wingren, S. C-erbB-2 overexpression and survival in early onset breast cancer. Breast Cancer Res. Treat., *63:* 23–29, 2000.

48. Carlomagno, C., Perrone, F., Gallo, C., DeLaurentiis, M., Lauria, R., Morabito, A., Pettinato, G., Panico, L., D'Antonio, A., Bianco, A. R., and De Placido, S. c-erbB-2 overexpression decreases the benefit of adjuvant tamoxifen in early-stage breast cancer without axillary lymph node metastases. J. Clin. Oncol., *14:* 2702–2708, 1996.

49. O'Malley, F. P., Saad, Z., Kerkvliet, N., Doig, G., Stitt, L., Ainsworth, P., Hundal, H., Chambers, A. F., Turnbull, D. I., and Bramwell, V. The predictive power of semiquantitative immunohistochemical assessment of p53 and c-*erb*B-2 in lymph node-negative breast cancer. Hum. Pathol., *27:* 955–963, 1996.

50. Harbeck, N., Dettmar, P., Thomssen, C., Berger, U., Ulm, K., Kates, R., Hofler, H., Janicke, F., Graeff, H., and Schmitt, M. Risk-group discrimination in node-negative breast cancer using invasion and proliferation markers: 6-year median follow-up. Br. J. Cancer., *80:* 419–426, 1999.

51. Reed, W., Hannisdal, E., Boehler, P. J., Gundersen, S., Host, H., and Marthin, J. The prognostic value of p53 and c-*erb*B-2 immunostaining is overrated for patients with lymph node negative breast carcinoma. Cancer (Phila.), *88:* 804–813, 2000.

52. Paik, S., Hazan, R., Fisher, E. R., Sass, R. E., Fisher, B., Redmond, C., Schlessinger, J., Lippman, M. E., and King, C. R. 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:* 103–112, 1990.

53. Allred, D. C., Clark, G. M., Tandon, A. K., Molina, R., Tormey, D. C., Osborne, C. K., Gilchrist, K. W., Mansour, E. G., Abeloff, M., Eudey, L., and McGuire, W. L. HER-2/neu in breast cancer: prognostic significance of overexpression influenced by the presence of *in situ* carcinoma. J. Clin. Oncol., *10:* 1049–1056, 1992.

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