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Expression of erbB/HER Receptors, Heregulin and P38 in Primary Breast Cancer using Quantitative Immunohistochemistry

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The purpose of this study was to investigate the frequency of expression of the erbB/HER family of growth factor receptors, their ligand heregulin, and the two different signaling pathways p38 and mitogen-activated protein kinase (MAPK), as well as the status of HER-2 phosphorylation in tumor specimens from patients with primary breast cancer. The level of expression of these proteins was measured by quantitative immunohistochemistry combined with microscope-based image analysis in paraffin-embedded breast cancer tissue from 35 patients. The frequency of expression was: EGFR (51%), HER-2 (54%), P-HER-2 (48%), HER-3 (48%), HER-4 (57%), heregulin (48%), p38 (17%), MAPK (48%). There was evidence of associations among the coexpression of heregulin, EGFR, HER-2, and HER-3. Also, there was evidence of a positive asso-

ciation between P-MAPK and HER-4. HER-3 was expressed at high levels in patients younger than 50 years of age. There was a trend for expression of higher levels of HER-4 in tumors larger than 2 cm. The expression of EGFR, HER-2, heregulin, p38 and MAPK was independent of age, tumor size, number of lymph nodes involved or hormone receptor status. The HER family of growth factor receptors appear to be regulated independently in invasive breast cancer. Assessing the expression of multiple tumor markers by quantitative immunohistochemistry is feasible. Further research is needed to determine the prognostic and predictive roles of the various associations between HER receptors, their ligands and signal transduction molecules in patients with early-stage breast cancer. (Pathology Oncology Research Vol 7, No 3, 171-177, 2001)

Keywords: Tumor markers, biological; proto-oncogene proteins; immunohistochemistry; ligands; breast neoplasm

Introduction

The epidermal growth factor (EGF) family of tyrosine kinase receptors and ligands play an important role in the pathogenesis of breast cancer.¹⁻³ Overexpression of either the EGF receptor (EGFR) or the human epidermal growth factor receptor 2 (HER-2) have been associated with a poor prognosis in patients who have early-stage

breast cancer.⁴⁻⁶ Patients who develop tumors in which HER-2 is overexpressed and who are treated with adjuvant doxorubicin-containing chemotherapy have an improved survival compared with those patients treated with non-doxorubicin-containing chemotherapy.⁷⁻⁹ The impact of HER-2 overexpression on tumor response to alkylating agents,¹⁰ taxanes,¹¹ and tamoxifen^{12,13} remains controversial. There are limited data available regarding the frequency of expression of HER-3¹⁴⁻¹⁶ and HER-4¹⁷ in breast cancer patients, and in fact whether expression of either influences prognosis or response to therapy is unknown.

Receptor phosphorylation and dimerization activates intracytoplasmic signal transduction pathways that result in increased cell proliferation, invasion, and metastatic capacity (*Figure 1*). Receptors from the EGFR family undergo phosphorylation by ligand binding and subse-

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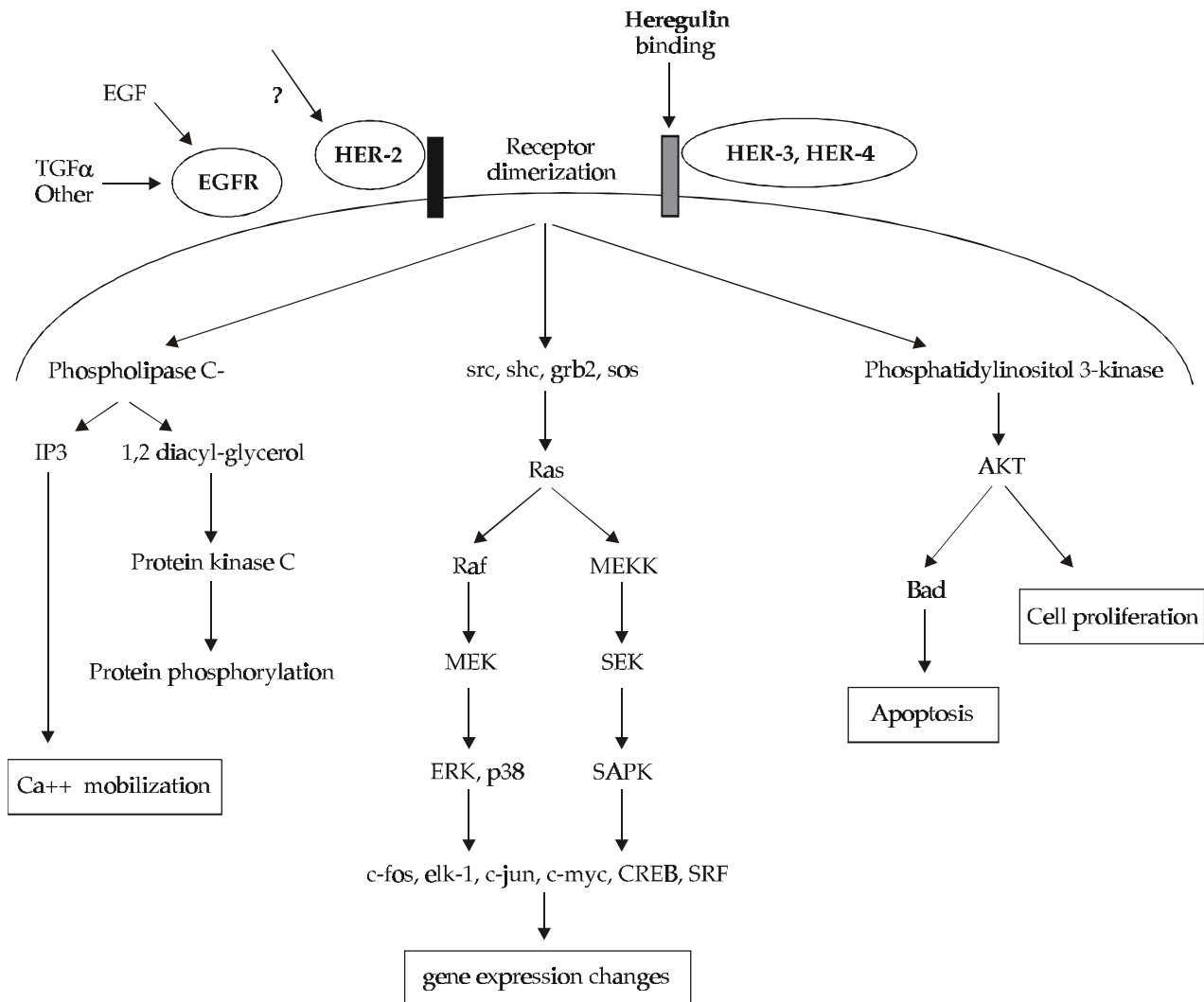


Figure 1. HER signaling pathways

quently form homodimers and heterodimers with one another (e.g., HER-2/HER-3, HER-2/HER-4, HER-2/HER-2). There are 15 ligands of the EGF family that bind to these receptors. However, none of the known EGF family proteins bind HER-2. Ligands can also activate receptors that they do not bind directly, through transmodulation. As an example, EGF can activate HER-2 through the formation of the EGFR/HER-2 heterodimers. Heregulin, also known as neuregulin or *neu* differentiation factor (NDF), is the most potent ligand for HER-3 and HER-4 receptors and can transactivate HER-2. A well-characterized intracellular signal transduction pathway in breast cancer progression involves activation of the mitogen-activated protein kinase (MAPK), c-JUN NH₂-terminal protein kinase, and p38.¹⁸

The optimal testing methodology for members of the HER family in breast cancer specimens is not well defined. In this study we explored the expression levels of

the HER receptors, their ligand heregulin and signal transduction molecules in invasive carcinomas of the breast using quantitative immunohistochemistry.

Materials and Methods

Patient Samples

Specimens were obtained from 35 patients with stage II breast cancer who were involved in a clinical trial of adjuvant chemotherapy conducted at The University of Texas M.D. Anderson Cancer Center. The patients underwent a mastectomy or breast-conserving surgery prior to administration of chemotherapy. The primary tumors were fixed using formalin and embedded in paraffin. Pathologic confirmation of breast cancer was obtained using hematoxylin-eosin stain on slides prepared from each block. Five-micrometer sections were prepared from each block for immunohistochemical studies.

Immunohistochemistry

Antibodies to EGFR (HER-1) and HER-2 were purchased from Dako Corporation (Carpinteria, Ca). Antibodies to HER-3, HER-4, and Heregulin were purchased from Santa Cruz Biotechnology (Santa Cruz, Ca). Antibody to phosphorylated p38 (P-p38) was purchased from New England Bio Labs (Beverly, MA). Antibody to phosphorylated HER-2 (P-HER-2) was a gift from Dr. Michael DiGiovanna of Yale University (New Haven, Ct). Phosphorylated MAPK (P-MAPK) was a gift from Dr. Rony Seger of the Weizmann Institute (Rehovot, Israel). All antibodies used were monoclonals except for the HER-2, HER-4 and heregulin, which were polyclonals.

Immunohistochemical staining was performed using either the peroxidase (P-p38 and MAPK) or the alkaline phosphatase (EGFR, HER-2, HER-3, HER-4, heregulin, P-HER-2) methodologies. 3,3'-Diaminobenzidine (Dako Corp. Carpinteria, Ca) was the chromogen employed for the peroxidase antibodies while CAS RED (Quantitative Diagnostics Lab, Elmhurst, IL) was the chromogen used for the alkaline phosphatase antibodies. Briefly, sections for the various antibodies were deparaffinized and hydrated to water in the usual manner. Sections for HER-2, P-p38 and MAPK were antigen retrieved by using citrate buffer (pH 6) in a microwave. The sections for the peroxidase antibodies (P-p38 and MAPK) were quenched by 3% Hydrogen peroxide/Methanol and then all sections were blocked by 10% goat Serum/0.1% Triton-X. EGFR (1:200 dilution), HER-2 (1:100 dilution), HER-3 (1:80 dilution), HER-4 (1:100 dilution), heregulin (1:50 dilution), P-HER-2 (1:20 dilution), P-p38 (1:100 dilution), and MAPK (1:200 dilution) primary antibodies were applied to their respective sections. Biotinylated goat-anti mouse (1:200 dilution) was the linking antibody employed for the monoclonal antibodies and biotinylated goat-anti rabbit (1:300 dilution) was the link for the polyclonals. Strep-ABC (Dako) was employed as the label for the peroxidase antibodies and Strep-alkaline phosphatase (1:300 dilution) for the alkaline phosphatase antibodies. DAB and CAS RED were used as the chromogens to visualize the bound primary antibodies. The sections were washed well with TBS between each step. Peroxidase antibodies were counterstained with ethyl green (Sigma Chemical St. Louis, MO) and CAS Dna (QDL) stain was the counterstain employed for the alkaline phosphatase antibodies.

Image analysis was performed as described.¹⁹ Briefly, quantitation was performed on the CAS 200 Image Analyzer (Becton Dickinson Corp., San Jose, CA). This is a microscope-based image analyser equipped with a two-color imaging channel system. One color channel finds the total area of the tissue. The other channel is specifically matched to have maximum absorption for the chromogen used to recognize the specific amount of antigen. Quanti-

tative results are reported in arbitrary units of optical density corresponding to the staining which is indicative of the amount of antigen on the tissues. As the amount of the specific antigen is increased, so would the immunohistochemistry staining indicating increased levels of specific antigens.

Statistical Analysis

The patient demographic, clinicopathologic, and laboratory variables analyzed included patient's age at the time of enrollment in the study, tumor size, number of positive nodes, estrogen- and progesterone-receptor status, and expression of EGFR, HER-2, HER-3, HER-4, heregulin, P-p38, P-MAPK, and P-HER-2. Associations among the values of these markers were evaluated using rank-correlation coefficients. Differences in patient characteristics between groups were evaluated using Fisher's exact test for categorical variables or Wilcoxon's rank sum test for continuous variables.²⁰ The sample size of 35 patients was selected because this was the largest number for which there were resources to complete the marker studies.

Results

The levels of protein markers were measured in tumor tissue from 35 evaluable patients. The ranges were recorded, and the median values were calculated. *Table 1* shows the percentage of patients whose tumors expressed high levels of HER proteins, heregulin, p38 and MAPK. Correlation coefficients were calculated for all marker measurements (*Table 2*). Pairings for which there was significant evidence ($p < 0.05$) of a linear correlation different from zero are indicated in bold type. There was evidence of associations among the coexpression of heregulin, EGFR, HER-2, and

Table 1. Summary of tumor marker distributions*

Tumor marker	Median (OD)	Range	Overexpression n (%)
EGFR	0.07	0.01–0.73	18 (51)
HER-2	1.60	0.04–6.10	19 (54)
HER-3	0.85	0.01–2.80	17 (48)
HER-4	0.08	0.01–0.89	20 (57)
Heregulin	0.24	0.01–1.08	17 (48)
P-p38	0	0–0.64	6 (17)
P-MAPK	17	0–73	17 (48)
P-HER-2	0.14	0–0.5	17 (48)

*The marker levels were determined by quantitative immunohistochemistry and image analysis. The values are depicted in arbitrary units of optical density (OD) which correspond to the amount of staining of the various proteins. The median OD value was chosen as cutpoint to discriminate between high and low levels for each tumor marker.

Table 2. Correlation Coefficients

EGFR	1.00	0.25	0.45^a	0.14	0.57	0.33	0.01	0.04	0.18	0.02	0.00
HER2	1.00	0.60	0.16	0.10	0.31	0.02	0.08	0.04	0.07	0.26	
HER3			1.00	0.21	0.43	0.26	0.05	0.13	0.21	0.09	0.22
HER4			1.00	0.07	0.09	0.44	0.27	0.07	0.45	0.01	
Heregulin				1.00	0.04	0.17	0.02	0.08	0.14	0.02	
P-p38						1.00	0.08	0.05			
P-MAPK						1.00	0.04				
P-HER2						1.00					

^a Items in bold represent correlation coefficients for pairings for which there was significant evidence of a linear correlation different from zero.

HER-3. Also, there was evidence of a positive association between P-MAPK and HER-4. For each marker, patients were divided according to whether that marker was expressed in the tumor at levels above or below the median value. The expression of HER proteins, heregulin, p38 and MAPK was correlated with other known prognostic factors. These included age, tumor size, number of involved axillary lymph nodes and hormone receptor status.

HER-2 staining was characterized predominantly by membranous pattern with faint cytoplasmic component (*Figure 2*). In contrast, HER-3 staining was predominantly cytoplasmic (*Figure 3*). The staining pattern was homogeneous throughout the tumor with both invasive and non invasive components showing positivity. HER-3 was overexpressed in 75% of patients older than 50 years and in 25% of patients of patients younger than 50 years of age ($p < 0.01$). There was no correlation between the levels of HER-3 expression and tumor size. Fifty eight percent of patients whose tumors overexpressed HER-3 had tumors smaller than 2 cm, while 42% of HER-3 overexpressing tumors were greater than 2 cm in maximum diameter. The number of involved axillary lymph nodes was equally distributed in patients whose primary tumors expressed high

and low levels of HER-3. Among patients with HER-3 overexpressing tumors, 75% had 1-3 positive lymph nodes, 8% had 4-9 positive lymph nodes and 17% had 2 positive lymph nodes. For patients with low HER-3 level expression, 61% had 1-3 positive lymph nodes; 39% had 4-9 positive lymph nodes; and no patients had more than 10 involved axillary lymph nodes. There was no correlation between HER-3 expression and expression of the estrogen receptor or the progesterone receptor.

HER-4 immunostaining demonstrated diffuse cytoplasmic positivity (*Figure 4*). HER-4 was overexpressed in 57% of patients. There was a trend for expression of higher levels of HER-4 in tumors larger than 2 cm (62%) compared with smaller tumors (38%). This difference did not reach statistical significance ($p = 0.09$). There was no correlation between levels of HER-4 expression and age, number of involved axillary lymph nodes and hormone receptor status of the primary tumors.

Heregulin immunoreactivity was characterized by diffuse cytoplasmic pattern (*Figure 5*). Heregulin was overexpressed in 48% of patients. The expression of heregulin was independent of age, tumor size, number of axillary lymph nodes and hormone receptor status. Overexpression

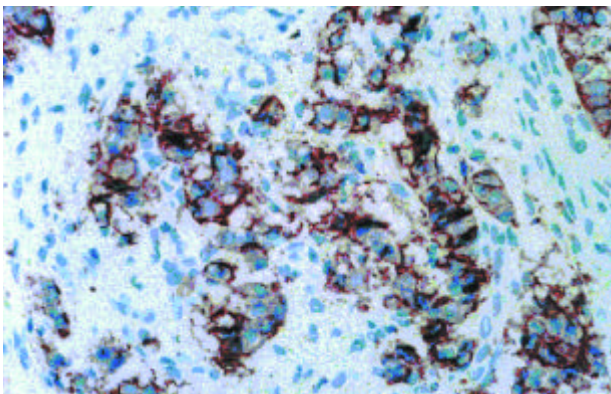


Figure 2. HER-2 staining pattern

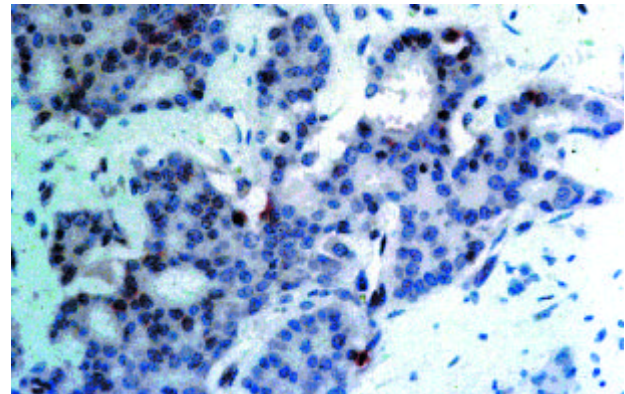


Figure 3. HER-3 staining pattern

of the estrogen receptor was noted in 64% of heregulin overexpressing tumors and 50% of heregulin negative tumors ($p = 0.49$). An inverse trend was noted for heregulin and progesterone receptor expression, although it was not statistically significant ($p=0.99$).

The staining pattern for p-p38 revealed nuclear positivity (Figure 6). High levels of phosphorylated P38 were noted in 55% of patients younger than 50 years of age and in 45%

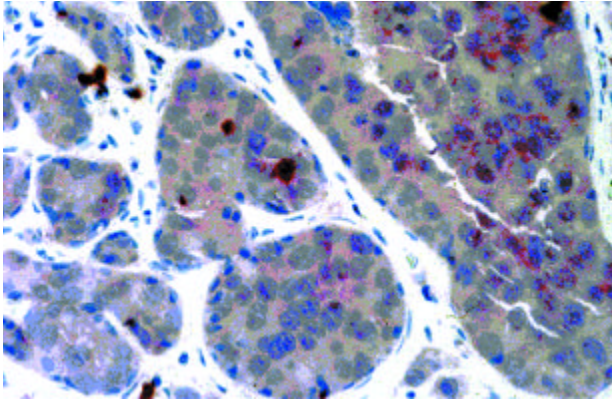


Figure 4. HER-4 staining pattern

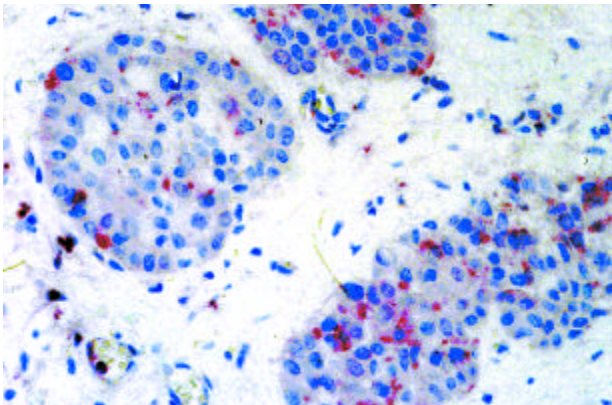


Figure 5. Heregulin staining pattern

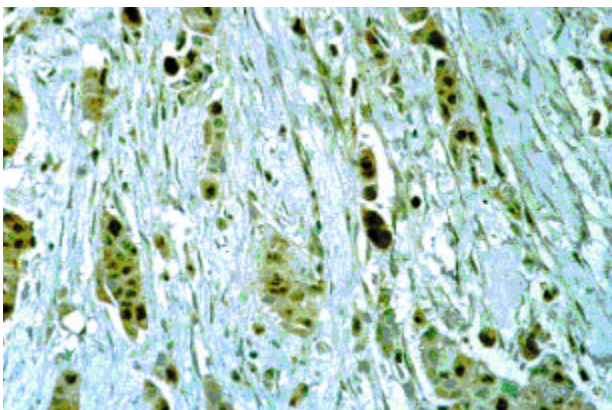


Figure 6. P-p38 staining pattern

of patients older than 50 ($p=0.45$). There was no correlation between P38 expression and tumor size, number of involved axillary lymph nodes and hormone receptor status.

Discussion

The identification of markers associated with the biological and clinical behavior of breast cancer may eventually be useful to predict a tumor's response to adjuvant chemotherapy. This would improve the management of patients with early-stage breast cancer. Patient selection for adjuvant chemotherapy is purely empirical and commonly based on crude risk factors such as tumor size or lymph node status. Preclinical data suggest that the evaluation of EGFR and HER-2 are associated with chemotherapy response. Little is known about the other two members of the family, namely HER-3 and HER-4 and their associated signal transduction pathways. Elucidation of the involvement of all the HER family of receptors and their ligands may lead to the identification of markers (or groups of markers) that would allow specific therapies to be tailored to individual patients. In this study, we analyzed the expression of EGFR (HER-1), HER-2, P-HER-2, HER-3, HER-4, heregulin, P-p38, and P-MAPK in 35 invasive breast carcinomas from patients who had axillary lymph node involvement. We evaluated the relationships between these markers and their associations with clinicopathologic features.

HER-3 is a cell-surface receptor with a dysfunctional intracytoplasmic tyrosine kinase. Binding of heregulin to the HER-3 protein may result in the formation of HER-3/HER-2 heterodimers that activate the HER-2 tyrosine kinase and downstream signal transduction pathways.¹⁴⁻¹⁶ There are limited data regarding the prognostic role of HER-3 in breast cancer.¹⁶ HER-4 is a gene that encodes a 180-kD transmembrane protein that is structurally similar to EGFR, HER-2, and HER-3.^{21,22} HER-4 can be activated by heregulin, and this interaction may play a role in breast cancer pathogenesis.²³ Knowlden et al²⁴ reported that HER-4 overexpression correlated with ER expression and improved DFS (disease-free survival).

Heregulin was initially identified as a 44-kD protein that was able to induce HER-2 phosphorylation.²⁵ *In vitro* data showed that heregulin does not bind HER-2 directly.²⁶ Instead, heregulin binds the HER-3 and HER-4 receptors, which leads to transphosphorylation of the HER-2 protein through heterodimer formation. Heregulin activates signaling pathways that are involved in breast cancer invasion and metastasis.²⁷ However, the role of heregulin as a prognostic marker in breast cancer patients is unknown. Experimental evidence suggests that heregulin signaling pathways are involved in the progression of breast cancer cells to a more aggressive phenotype.²⁸ Heregulin has been shown to activate the phosphatidylinositol 3-kinase (PI3K).²⁹⁻³¹ PI3K has been shown to be associated with

cellular survival as well as cellular proliferation and transition from G1 to S phase of the cell cycle, thus contributing to more proliferation activity in these cancers through activation of the oncogene Raf and its downstream signals MAPK, and the extracellular signal-regulated kinase 1 (ERK-1) and ERK-2.³² In addition, heregulin activates the AKT pathway and nuclear factor kappa B (NFB), which have been associated with invasiveness and drug resistance in breast cancer cells.³³⁻³⁷ Thus, patients whose tumors express heregulin and its receptors HER-3 and HER-4 may be more resistant to doxorubicin-based therapy, whereas patients whose tumors overexpress HER-2 have been shown to benefit from doxorubicin-based therapy.⁷ Studies in our laboratory indeed indicate that use of the PI3K inhibitor Wortmanin augmented the cytotoxicity activity of doxorubicin.³⁸ In addition, HER-4 and HER-3 have been shown to be receptors to other growth factors and to augment their activity.³⁹⁻⁴¹ This probably contributes to cellular growth and drug resistance in breast cancers that express high levels of HER-3 and HER-4.

Phosphorylation activity of p38 has been associated with apoptosis and drug-induced toxicity.^{8,18,29} Using the p38 inhibitor SB203580 totally stops paclitaxel and doxorubicin-induced cytotoxicity.³⁸ The ability of p38 to be phosphorylated and activated in some human cancers probably makes these cancers more susceptible to chemotherapeutic drugs such as doxorubicin.

Our study points to the fact that the different HER family of receptors and their ligand are regulated independently, and their levels of expression are important. A pattern is emerging in which human epidermal growth factor receptors and their ligands can activate downstream apoptotic and survival signals that are linked not only to proliferation and carcinogenicity, but also may contribute to drug sensitivity and resistance.

It is clear that the multiplicity of HER receptors and ligands constitute an additional area of investigation in the HER signaling network.⁴⁰ Although this preliminary study evaluates a small group of proteins, it sets the stage to begin to understand the role that they play in the clinical outcome of patients with breast cancer.

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