Differential spatial expression and activation pattern of EGFR and HER2 in human breast cancer

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Abstract. Overexpression of HER family members is a well established prognostic factor and identifies potential targets for antibody-based receptor blocking strategies. While several studies have analyzed the expression of HER2 and other HER-family members in malignant tumors, considerably less is known about their expression and activation in non-involved breast tissue from breast cancer patients. We have therefore investigated the differential expression of EGFR, HER2, and their tyrosine-kinase activated forms (ptyr-1248 Her-2 and ptyr-845 EGFR) in 63 tumor specimen containing: a) malignant epithelium, b) in non-malignant tissue located at the peritumoral margin, and c) in uninvolved breast tissue obtained from tissue distant from the tumor. Using immunohisto-chemistry (IHC), we found significantly higher HER2 protein expression levels in malignant epithelium than in marginal and peripheral non-malignant epithelium (p=1.3x10^{-10} Fisher's exact test). Epithelial EGFR expression did not differ between the three tissue types, but stromal EGFR protein was significantly more common in marginal and peripheral tissues when compared to tumor tissues (p=0.008, Fisher's exact test). When analyzing activated receptor forms, we found epithelial ptyr-1248 HER2 expression in one tumoral, one marginal and one peripheral sample. We did not observe ptyr-845 EGFR in any of the samples analyzed. We found a significant overall correlation between epithelial and stromal EGFR expression (r=0.442; p<0.0001; Spearman's Rho), and between stromal EGFR expression and normal tissue type (r=0.170; p<0.02; Spearman's Rho). Epithelial HER2 expression and normal tissue type (r=0.492; p<0.0001; Spearman's Rho) were inversely correlated. Taken together, we have observed a differential expression pattern of EGFR, HER2, and activated HER2 that is dependent on the spatial relation to a malignant tumor. Our findings of decreased intratumoral EGFR expression and the absence of activated EGFR suggests that, in contrast to HER2, EGFR inhibition might not be an ideal target for antibody therapy.

Introduction

The HER-family is comprised of the membrane-bound receptors EGFR, HER2, HER3 and HER4, which all share a high degree of structural homology. The proteins are characterized by an extracellular ligand-binding domain which is responsible for ligand recognition and high affinity binding, a single membrane-spanning region, and a cytoplasmic protein tyrosine kinase domain with phosphorylation-triggered signaling properties. While physiological HER receptor activation is associated with cellular proliferation and differentiation, the amplification and consecutive overexpression of HER2 has been found to play a key role in malignant transformation and promotion of breast cancer.

With the exception of HER2, receptor activation is a result of ligand binding to the extracellular domain of HER receptors, which leads to the formation of both receptor homo- and heterodimers (1). Dimerization, in turn, stimulates the intrinsic tyrosine kinase activity and triggers phosphorylation of specific, C-terminal tyrosine residues within the cytoplasmic domain. The ensuing signal transduction occurs through two distinct pathways: the phosphatidylinositol-3-kinase (PI3K) pathway which leads to activation of protein kinase C and increased intracellular calcium concentration, and the ras protein cascade which leads to activation of the MAP kinase. Both pathways ultimately trigger mitogenic responses (2-4).

A number of studies have investigated the expression of EGFR and HER2 in malignant tumors and have found that the co-expression of the two receptors is associated with resistance to endocrine therapies and poor prognosis (5-9). However, both proteins have also proved to be hopeful targets for receptor blocking antibodies such as trastuzumab (Herceptin®) and gefitinib (Iressa®) in selected malignancies.

While most studies have addressed the expression of HER family members in malignant breast tumors (10-12), HER2 expression has also been described in a variety of epithelial cells. HER-2 protein expression levels in these normal tissues were similar to the levels found in non-amplified breast cancers

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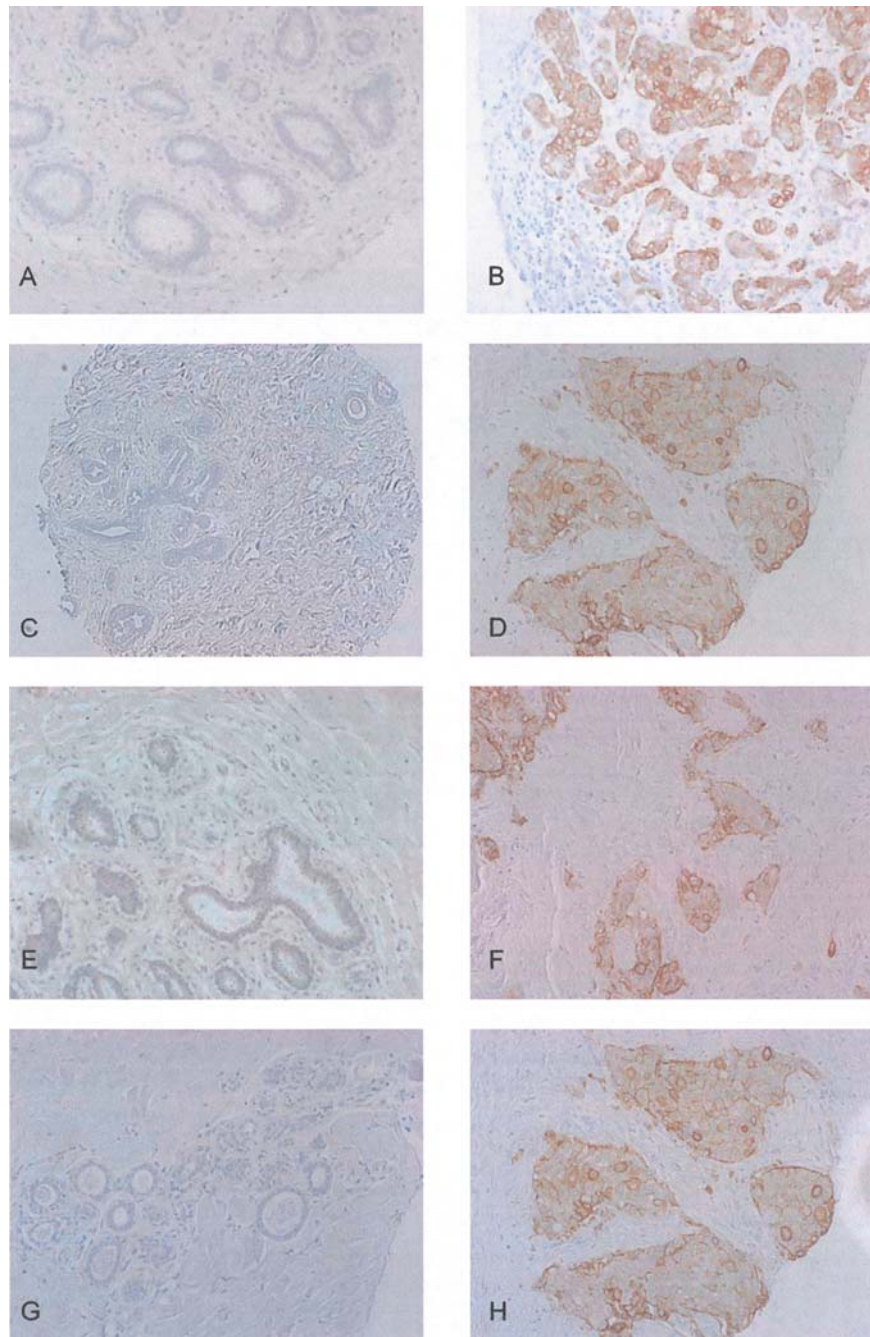E-mail: christian.singer@meduniwien.ac.at

Figure 1. Immunhistochemical analysis of malignant (right) and non-malignant (left) breast tissue (obtained from marginal or peripheral breast tissue) using the EGFR [negative in (A), positive in (B)], HER2 [no staining in (C), strong staining in (D)], ptyr-845 EGFR [negative in (E), positive control in (F)], and the ptyr-1248 HER2 [no staining in (G), strong staining in (H)], antibodies as described in Materials and methods. (Positive pEGFR control slide, not part of the tissue array).

and breast cancer cell lines (13). By contrast, several studies also demonstrated a higher level of EGFR expression in normal and benign diseased breast tissue than malignant breast tissue (14-18). This is also in line with one publication which describes higher levels of EGFR in marginal tissue (17).

Nevertheless, relatively little is still known about their presence in marginal and peripheral tissues. More specifically, it is not known, whether there is a differential expression and activity pattern of EGFR/HER-family members dependent on the spatial relation within the involved breast.

We have therefore examined the presence and activation status of EGFR and HER2 in three sets of breast biopsies from breast cancer patients. Tissues were obtained directly from a malignant tumor, from non-malignant marginal tissue, and from non-malignant peripheral breast tissue located at a distant from a malignant tumor. Protein expression of both receptors and their active (ptyr-1248 and ptyr-845) forms were analyzed by immunohistochemistry, and expression profiles in the three sets were correlated.

Materials and methods

Tissue specimen and patient characteristics. Tissue arrays containing 63 paraffin-embedded sets of tissues obtained

Table I. Immunohistochemical analysis of EGFR, HER2, pEGFR, pHER2 both in normal, tumoral and marginal tissue and in epithelial (epi) and stromal (str) cells of human breast tissue. 0, no expression; +, weak expression; ++, moderate expression; +++, high expression as assessed by the IRS (0, 0-2; +, 3-5; ++, 6-9; +++, 10-12).

Type	Score	HER2EPI (%)	HER2STR (%)	PHER2EPI (%)	PHER2STR (%)	EGFREPI (%)	EGFRSTR (%)	PEGFREPI (%)	PEGFRSTR (%)
Normal	0	52 (100)	65 (100)	34 (97.1)	65 (100)	46 (88.5)	55 (84.6)	53 (100)	65 (100)
	+	0	0	1 (2.9)	0	5 (9.6)	10 (15.4)	0	0
	++	0	0	0	0	1 (1.9)	0	0	0
	+++	0	0	0	0	0	0	0	0
Tumor	0[a]	40 (62.5)	65 (100)	47 (97.9)	64 (100)	59 (92.2)	63 (98.5)	64 (100)	65 (100)
	+	9 (14)	0	0	0	1 (1.6)	1 (1.5)	0	0
	++	8 (12.5)	0	0	0	2 (3.1)	0	0	0
	+++	7 (11)	0	1 (2.1)	0	2 (3.1)	0	0	0
Margin	0	47 (98)	59 (100)	34 (97.1)	59 (100)	45 (93.8)	54 (91.5)	48 (100)	59 (100)
	+	1 (2)	0	1 (2.9)	0	3 (6.2)	5 (8.5)	0	0
	++	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0

directly from the malignant tumor ('tumor'), from microscopically uninvolved tissue <15 mm distant from the invasive front of a malignant tumor ('margin'), and from peripheral breast tissue ('peripheral') were purchased from Biomax (Biomax Inc., Rockville, MD). The presence or absence of malignant tumor cells and of epithelial cells was confirmed by an experienced pathologist of our department. The patients had a mean age of 51.5 years (range 31-72 years) and 59 of the 63 were post-menopausal (94%). Of the 63 tumor-derived specimens 57 contained tumor cells and were comprised of 41 invasive-ductal, 1 invasive-lobular, 2 medullary, 1 mucinous carcinoma, 10 NOS, 1 ductal carcinoma *in situ* and 1 cystosarcoma phylloides of the breast. Forty-five of 63 cases (70%) were estrogen receptor positive.

Immunohistochemistry. Immunohistochemical HER2 protein expression was assessed by utilizing the Herceptest® according to the manufacturer's recommendations (Dako, Glostrup, Denmark). Expression of EGFR protein was assessed by using the EGFR PharmDX® kit according to the manufacturer's recommendations (Dako). The phosphorylated forms of HER2 and EGFR (ptyr-1248 HER2 and ptyr-845 EGFR) were immunodetected with the monoclonal antibodies PN2A (anti-ptyr-1248 HER2, Dako) and EGFR-12A3 (anti-ptyr-845 EGFR, Nanotools, Munich, Germany) as previously described (7,19). The sensitivity and specifity of EGFR-12A3 has been described previously (20). In brief, paraffin-embedded sections were deparaffinized, tissues were rehydrated, and antigen retrieval was performed by microwaving sections in 10 mmol/l citrate buffer (pH 6.0) for 15 min. Slides were then washed with PBS, blocked with Ultra V Block (Lab Vision, Fremont, CA), and incubated with the primary antibodies at a dilution of 1:30 at 4˚C overnight. Sections were sequentially incubated with biotinylated goat anti-polyvalent (Lab Vision) and streptavidin-HRP (Lab Vision) at RT for 30 min, and the immunoreaction was visualized with 3-amino-9-ethylcarbazole (AEC, Sigma). EGF-treated and thus ptyr-1248 HER2, ptyr-845 EGFR and ptyr-173 EGFR positive cells were used as controls. The specificity of both phosphoantibodies has been confirmed previously (19).

HER2 overexpressing SKBR3 human mammary carcinoma cells were stimulated with 100 ng/ml to achieve receptor phosphorylation at the tyr-1248 HER2 site and were used as positive controls for ptyr-1248 HER2 as previously described (21). One hundred ng/ml EGF-stimulated human epithelial A-431 carcinoma cells were used as positive controls for ptyr-845 EGFR (data not shown) (7,19). Expression of both receptors and their phosphorylated forms was assessed using the HercepScore® according to the manufacturer's recommendations (Dako) and only membrane-specific staining was considered positive. Staining was independently assessed by two experienced pathologists of our department. Representative photomicrographs of immunohistochemical analysis are shown in Fig. 1.

Statistical analysis. Fisher's exact test was used to identify significant differences in receptor protein expressions in tumoral, marginal, and peripheral biopsies of total and activated HER2 and EGFR. Associations between protein expression of the investigated receptors were evaluated by Spearman's Rho test. SAS statistical software system (SAS Inc., Cary, NC, version 8.1) was used for all calculations and a two-sided p-value of <0.05 was considered statistically significant.

Results

Fig. 1 and Table I show the expression of EGFR, HER2, ptyr-845 EGFR and ptyr-1248 HER2 in tumoral, marginal and peripheral breast tissue as measured by immunohisto-

Table II. Correlation coefficient (r) and p-value between EGFREPI (epithel), EGFRSTR (stroma), HER2EPI (epithel), HER2STR (stroma) and HISTO (malignant vs non-malignant breast tissue).

	EGFREPI	EGFRSTR	HER2EPI	HER2STR	HISTO
EGFREPI	1	r=0.442 p<0.0001	r=-0.056 p=0.49	- -	r=0.055 p=0.49
EGFRSTR	-	1 -	r=-0.124 p=0.11	- -	r=0.170 p<0.02
HER2EPI	-	-	1 -	- -	r=-0.491 p<0.0001
HER2STR	-	- -	- -	1 -	- -

chemical analysis. When present, receptor staining was exclusively found in the cell membranes, regardless of the antibody used (Fig. 1). A strong epithelial HER2 expression was found in 7 tumor samples (11%), while intermediate expression was observed in 8 (12.5%), and weak expression was seen in 9 (14%) cases of the 64 tumor tissues investigated. By contrast, none of the 52 normal epithelial tissues expressed HER2, and only one of the 48 tissues obtained from the tumoral margin exhibited weak HER2 expression (2%). Furthermore, none of the stromal cells stained for HER2, regardless of their relation to a malignant tumor (Table I). When the activated form of HER2 was analyzed in the same tissue samples, we found one case of high epithelial pHER2 expression in tumoral tissue (2.1%), as well one case of weak expression in normal and marginal epithelium each (2.9 and 2.9%, respectively). Epithelial EGFR was found in 5 of 64 tumor samples (7.8%), while 6 of the normal breast biopsies (11.5%) and 3 of the marginal samples (6.2%) expressed the protein. The stromal EGFR expression was observed in only one of the 64 tumor samples (1.5%) while in normal tissues we found EGFR expression in 10 of 65 cases (15.4%) and in marginal tissue in 5 of 59 cases (8.5%). We did not observe any pEGFR staining, regardless of cell type and sample. We did not observe HER2 or EGFR staining in the cystosarcoma phyllodes case (Table I).

When the presence of EGFR, HER2 and their activated forms was compared in tumor, marginal and peripheral tissue, we found that HER2 expression in malignant epithelium was significantly more common than in normal ductal epithelium (0/52; p=6.2x10^{-6}, Fisher's exact test), and as well as in epithelium obtained from the tumor margin (1/47; p<0.00001, Fisher's exact test).

While there was no difference in the epithelial pHER2 expression, and in the epithelial EGFR expression in benign and malignant tissues (p=n.s. and p=n.s., respectively), stromal EGFR was significantly more common in normal tissue when compared to tumor samples (p=0.008, Fisher's exact test).

Correlations. We then looked at possible correlations between the different parameters (Table II). We found a significant overall correlation between stromal EGFR expression and normal tissue (r=0.170; p<0.02; Spearman's Rho), and between epithelial and stromal EGFR expression (r=0.442; p<0.0001; Spearman's Rho). Epithelial HER2 expression was inversely correlated with normal tissue (r=0.491; p<0.0001; Spearman's Rho).

Discussion

Co-expression of HER2 and EGFR has been observed in 7-8% of malignant breast tumors and has been shown to be associated with shorter DFS and OS in patients with early and advanced breast cancer (6,7,16). While most studies have concentrated on intratumoral receptor expression little is known about peritumoral expression in surrounding, histologically uninvolved tissue. We present herein the first systematic study which investigates the spatial expression of EGFR, HER2 and their acitvated forms in tumoral, marginal, an peripheral breast tissue from breast cancer patients. Our finding of intratumoral HER2 and pHER2 expression supports a wealth of previous studies, in which the receptor was observed in 10-40% of human early breast cancers (22) and its activated form in 3-5% (23,24). We have, however, also detected overexpression of HER2 in histologically normal breast epithelium from the vicinity of a malignant tumor. A small constitutive amount of HER2 expression in normal breast tissue is consistent with reports described (13), but the HER2 expression in marginal breast tissue has not been reported previously. In contrast to normal breast tissue in benign tumors, especially in fibroadenomas, the HER2 expression is discussed controversially (25,26).

Similar to HER2, EGFR expression in human breast carcinomas has also been associated with an unfavourable prognosis (27-32) but the incidence of EGFR expression in breast cancer reported varies between 14-65% (33). However, it has been widely reported that EGFR is expressed more frequently in non-malignant than malignant breast tissues (15,16,33,34). EGFR expression can be observed in a variety of normal cells including many epithelial cell types (35,36-41). Non-epithelial cell types that express EGFR include smooth muscle cells, fibroblasts, and perineurium (42).

In our study, epithelial EGFR expression did not differ between normal, tumor and marginal tissue, whereas stromal EGFR was significantly more common in marginal and peri-

pheral tissue than in the actual tumor samples (p=0.008, Fisher's exact test). Our results are somewhat contradicted by findings from Möller *et al*, who did not find EGFR positive stromal cells in normal breast tissue but in fibroadenomas stroma (25%). This group did, however show EGFR positive epithelial cells in the close vicinity of malignant tumor samples of the breast while stroma remained negative (43). Similar findings were published by others (16,44,45).

There is no previous information on the activity of EGFR in the general population of breast cancer patients. While we were unable to observe activated EGFR in any of the tissues investigated, Gschwantler *et al* found in a highly selected patient population, HER2 overexpressing malignant breast tumors, an EGFR expression in 35% and activated EGFR expression in 13% of cases (19). Hudelist *et al* showed in this patient population that the presence of pHER2 and pEGFR was a strong predictor of both response to trastuzumab-based treatment and clinical benefit (20). EGFR expression and activated EGFR expression was investigated also in other tissue types: in node-positive colorectal cancer patients Cunningham *et al* found that EGFR was expressed in 76% of the cases and pEGFR was positive in 8% (46). In untreated non-small cell lung cancer (NSCLC) the expression rate of EGFR, pEGFR and HER2 was 97.2, 44.4 and 86.1%, respectively and the overexpression rate was 80.6, 0.0 and 27.8%. They demonstrated that neither overexpression of EGFR nor HER2 correlated with the time to progression or overall survival, while EGFR phosphorylation showed an inverse correlation with regard to time to progression and overall survival in the patients with NSCLC. These results suggest that the phosphorylation of EGFR might be an important predictor for clinical outcome of NSCLCs, possibly due to variant III mutations within the TK domain of the EGFR, which might be responsible for the higher response rate following treatment with EGFR inhibitors (47).

The success of TK inhibitors in NSCLCs is in striking contrast to results from several studies which have analyzed the activity of TK inhibitors in metastatic breast cancers: while some of these studies demonstrate modest single-agent activity, they are somewhat disappointing, presumably because until now, activating EGFR mutations have not been described in breast cancer, and because EGFR signaling does not seem to be critically involved in local invasion and tumor progression (48; Albain K, *et al*, Breast Cancer Res Treat 76: 33, abs. 20, 2002; Robertson JFR, *et al*, Proc Annu Meet Am Soc Clin Oncol 22: 7, abs. 23, 2003; Baselga J, *et al*, Proc Annu Meet Am Soc Clin Oncol 22: 7, abs. 24, 2003).

In conclusion, we report herein for the first time the differential expression pattern of EGFR, HER2 and their activated forms depending on the spatial relation to a malignant tumor. The fact that intratumoral EGFR expression is decreased in comparison to peritumoral expression, and the lack of activated EGFR in any of the samples suggests that, in contrast to HER2, EGFR inhibition might not be an ideal target for antibody therapy. The topic of EGFR mutations is clearly an interesting and important one, and further research should be undertaken in the general population of breast cancer patients.

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