Incomplete surgical resection of ductal carcinomas *in situ* results in activation of ERBB2 in residual breast cancer cells

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Abstract

ERBB2 amplification and consecutive overexpression is a predictor for poor prognosis in breast cancer patients. In addition, incomplete resection of ERBB2-overexpressing tumors leads to increased proliferation of residual breast cancer cells. While the local release of cytokines is thought to be responsible for the malignant behavior of remaining tumor tissue, the exact mechanism is still unknown. We have analyzed epidermal growth factor receptor (EGFR), activated (p)EGFR, and activated (p)ERBB2 protein expression in ERBB2-overexpressing and in non-ERBB2-overexpressing tumors from patients who underwent breast surgery and consecutive re-excision for involved margins, and compared expression levels by immunohistochemistry. While overall ERBB2 protein expression in the initial and the re-excised sample were comparable, we observed an increase in pERBB2 in ductal carcinomas *in situ* in both, ERBB2-overexpressing (16/21 vs 24/24; $P=0.018$, χ^2 test) and non-ERBB2-overexpressing tumors (3/28 vs 5/12; $P=0.025$, χ^2 test). pERBB2 was not increased in invasive tumors, regardless on whether the samples had been taken from a ERBB2-overexpressing (9/25 vs 6/17; $P=0.261$, χ^2 test) or a non-ERBB2-overexpressing tumor (1/27 vs 0/8; $P=0.581$, χ^2 test). EGFR expression was only detected in 1/47 ERBB2-overexpressing primary tumors and 2/48 non-ERBB2-overexpressing tumors, and was undetectable in re-excised specimen. Taken together, we have demonstrated an increase in ERBB2 receptor activation in incompletely resected preinvasive breast cancer. We hypothesize that receptor phosphorylation is caused by growth factor stimulation in response to intraoperative tissue damage, and perioperative inhibition of specific cytokines could become a promising therapeutic strategy.

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Introduction

ERBB2 (ERBB2, c-ERBB2) is a member of the family of epidermal growth factor receptors (EGFR), and intratumoral *ERBB2* gene amplification and consecutive ERBB2 protein overexpression are strong predictors of poor outcome for breast cancer patients (Slamon *et al.* 1987). ERBB2 overexpression does, however, also identify a subset of patients who are likely to benefit from therapeutic inhibition of ERBB2-mediated signal transduction and who are eligible for trastuzumab-based treatment strategies (Gonzalez-Angulo *et al.* 2006). The receptor is overexpressed in ~25–30% of invasive breast tumors, although its prevalence in ductal carcinomas *in situ* (DCIS) is even higher, reaching up to 60% (Latta *et al.* 2002). As of yet, ERBB2 is an orphan receptor for which a ligand has not yet been identified. In lieu of ligand-dependent activation, ERBB2 can be activated through heterodimerization with EGFR, ERBB3, or ERBB4, when any of these proteins are co-expressed and activated by their respective ligands (Yarden & Sliwkowski 2001). An alternative mechanism of receptor activation is ligand-independent cleavage of the ERBB2 extracellular domain through matrix metalloproteinases (Molina *et al.* 2001). Both

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mechanisms result in autophosphorylation of tyrosine residues that are located on the intracellular domain of the receptor, which in turn leads to the activation of specific signal transduction pathways (Zwick *et al*. 2001).

Given these considerations, the number of autophosphorylated receptors, rather than the total number of receptors, should be biologically more important. Indeed, several observations support this hypothesis: Thor *et al*. (1999) have demonstrated that receptor activation, as measured by phosphorylated (p)ERBB2 expression in nodal-positive breast cancer provides the most significant prognostic value by multivariate analysis, thereby exceeding the prognostic value of mere ERBB2 protein overexpression. Activated ERBB2 also identifies a subgroup of tumors that are resistant to single-agent taxanes in the metastatic setting with an almost doubling the likelihood of direct progression of disease during treatment in pERBB2-positive tumors. We have recently been shown that the presence of pERBB2, but also of pEGFR, allows to identify patients with metastatic breast cancer who derive a specifically profound benefit from trastuzumab-based therapy: ERBB2-overexpressing tumors that also stained positive for pERBB2 had a median 11.7-month progression-free survival (PFS) as opposed to patients a 4.5-month PFS for patients whose tumors were pERBB2 negative. The presence of pEGFR further added to the prognostic value of pERBB and allowed to predict the efficacy of trastuzumab even better than pERBB2 expression alone (Hudelist *et al*. 2006).

While the exact mechanism by which ERBB2 activation results in poor outcome is still unknown, it is well known that several ERBB2-activated signaling pathways including the mitogen-activated protein kinase, protein kinase C, the signal transducer and activator of transcription, the Jun N-terminal kinase, and the phosphatidylinositol 3-kinase pathway respectively, result in enhanced cellular motility, proliferation, and evasion of apoptosis (Marmor *et al*. 2004). In this context, Tagliabue *et al*. have demonstrated that residual breast carcinomas that had been surgically removed within 48 days after first surgery showed a significant increase in proliferation if they were ERBB2 positive. In addition, both wound drainage fluid and postsurgical serum samples from patients stimulated growth of ERBB2-overexpressing breast carcinoma cells *in vitro*, presumably through EGF. Treatment of ERBB2-positive tumor cells with trastuzumab before adding the growth stimulus abolished drainage-fluid-induced proliferation, which further corroborates that ERBB2 activation in breast carcinoma cells may have a role in the postsurgery stimulation of growth of breast carcinoma cells (Tagliabue *et al*. 2003). We have therefore analyzed activated (p)ERBB2, activated (p)EGFR, and EGFR protein expression in ERBB2-overexpressing and non-ERBB2-overexpressing tumor samples that were resected during the initial surgery and a secondary re-excision for involved margins in order to find out whether incomplete resection of ERBB2-overexpressing breast cancer can result in ERBB2 activation of remaining tumor tissue.

Materials and methods

Patients and samples

Patients who had undergone breast conserving therapy for invasive and/or preinvasive DCIS breast cancer and in whom a second surgical procedure had been performed between 2000 and 2005 at the Medical University of Vienna Hospital because of involved excision margins were retrospectively identified by chart review. The presence of tumor tissue (DCIS and/or invasive) was then confirmed by an experienced pathologist, and only cases in which the interval between initial operation and second surgery was less than 4 weeks were further analyzed. In order to compare corresponding tumor areas in the primary and the secondary specimen, ERBB2 and pERBB2 expression analysis in the primary resectate was confined to the tumor area that was adjacent to the incompletely resected margin. The study was approved by the local internal review board.

ERBB2- and non-ERBB2-overexpressing tumors were matched by first identifying incompletely resected, ERBB2-overexpressing cases and consecutive matching with non-ERBB2-overexpressing tumors according to the parameters age, ER and PR status, and grading.

Determination of ERBB2 overexpression

Assessment of ERBB2 overexpression and determination of ERBB2 phosphorylation were performed independently by two experienced pathologists blinded to the clinical course of patients and the results of other tests performed. ERBB2 protein expression was evaluated on paraffin embedded tissue using the HercepTest kit (DAKO A/S, Glostrup, Denmark) for immunoenzymatic staining in accordance with the protocol described in the manufacturer's guide. Positive controls consisting of freshly cut breast cancer cases known to express ERBB2 and a control slide consisting of three pelleted, formalin-fixed, paraffin-embedded human breast cell lines with staining intensity scores of 0, 1+, and 3+ (supplied by the kit manufacturer) were included in each staining run. Negative controls were performed by substitution of the

ERBB2 primary antibody by normal rabbit serum (DAKO Negative Control Reagent). Only membrane staining intensity and pattern were evaluated and expression levels were graded from 0 to 3+ according to the HercepTest kit scoring guidelines.

Determination of EGFR, pEGFR, and pERBB2 in breast cancer tissues

ERBB2 activity was evaluated using the monoclonal anti-phospho ERBB2 antibody PN2A (NeoMarkers, Westinghouse Drive, Fremont, CA, USA). The PN2A antibody specifically recognizes phosphorylation of the major autophosphorylation site Tyr-1248 (P-Tyr1248) without cross reactivity with c-ERBB1 (EGFR), c-ERBB3 or c-ERBB4 or unphosphorylated ERBB2. EGFR was detected as described previously (Sainsbury *et al*. 1985; Fig. 1). The phosphorylated form of EGFR was detected with the monoclonal antibody EGFR-12A3 as described previously (Hudelist *et al*. 2006). The antibody specifically recognizes phosphorylation of the src-kinase-dependent tyrosine residue tyr-845 (anti-ptyr-845) without cross reactivity with c-ERBB1 (EGFR), c-ERBB3 or c-ERBB4 or unphosphorylated tyr-1173 and tyr-845 EGFR. Four micrometer formalin-fixed, paraffin-embedded tissue sections were deparaffinized, rehydrated, and endogenous peroxidase blocked with 2% hydrogen peroxide. Antigen retrieval was performed by placing sections in 10 mmol/l citrate buffer (pH 6.0) and microwave treatment for 15 min. Slides were allowed to cool to room temperature (RT), washed with PBS and distilled water, and blocked with Ultra V Block (Lab Vision, Westinghouse Drive, Fremont, CA, USA). PN2A (6 μg/ml) was applied and sections were incubated at 4 °C overnight. After two additional PBS washes, sections were sequentially incubated at RT for 30 min with biotinylated goat anti-polyvalent (Lab Vision) and streptavidin-HRP (Lab Vision). Subsequently, slides were incubated with 3-amino-9-ethylcarbazole (AEC, a widely used chromogen), counterstained with hematoxylin, and cover slipped.

The expression of EGFR, pEGFR, and pERBB2 was visually assessed using the same scoring system applied for determination of ERBB2 overexpression (see above). In contrast to the evaluation of receptor overexpression of ERBB2 (considering grade 2+ and grade 3+ tumors positive), tumors exhibiting a clearly discernible positive signal for receptor phosphorylation (≥ grade 1+ using Herceptest guidelines) on cellular membranes were considered positive, because even weak staining for phosphorylation of the EGFR or ERBB2 molecule might represent tyrosine kinase activity, i.e., active receptor signaling. By contrast, faint cytoplasmatic in the absence of membranous staining was not considered positive. For each assay, pelleted, formalin-fixed, paraffin-embedded human T47D, and rhEGF-stimulated SKBR3 breast cancer cell lines (American Type Culture Collection, Rockville, MD, USA) were used as positive controls. ER, PR, and Ki67 staining were performed according to the local laboratory standard procedures. Results were manually evaluated by an experienced breast pathologist according to the Allred score (ER and PR), and according to the percentage of cells with nuclear staining (Ki67). Tumors with ER and PR protein expression of Allred score 3 and more were considered positive.

ERBB2 and pERBB2 protein expression in SKBR3 breast cancer cells in response to heregulin

ERBB2 activation in SKBR3 monocultures in response to heregulin was analyzed by pERBB2 immunohistochemistry. Cells were cultured in DMEM high-glucose medium with L-glutamine (PAA GmbH, Linz, Austria) to which 10% heat-inactivated FCS (Gibco, BRL) had been added. Cells were then serum starved for 24 h before 25 ng/ml heregulin (Weitzmann Institute, Rehovot, Israel) was added for 7 min. Cells were then washed with PBS and formalin fixed before permeabilization with 0.1% Triton-X 100/PBS (Sigma) was performed. Immunostaining was carried out using either the DAKO ERBB2 kit antibody or the PN2A antibody for ERBB2 and for pERBB2 detection respectively. The primary antibody was detected by the Ultravision LP System (LabVision) and the immunoreaction was visualized with AEC chromogen.

Statistical analysis

The χ^2 test was used to identify differences in ERBB2 and pERBB2 protein expression levels in tissues obtained from the original surgical specimen and the consecutive surgical resections. Correlations between ERBB2 and pERBB2 were analyzed by Spearman's test (two-sided). For all analyses, a $P<0.05$ was considered statistically significant. Correction for multiple testing was performed. Win-SAS V 8 (SAS Institute GmbH, Heidelberg, Germany) statistical software system was used for all calculations.

Results

Patient characteristics

Patient characteristics are shown in Table 1. Patients with ERBB2-negative tumors had a median age of 58 years, while the median age of women with ERBB2-positive

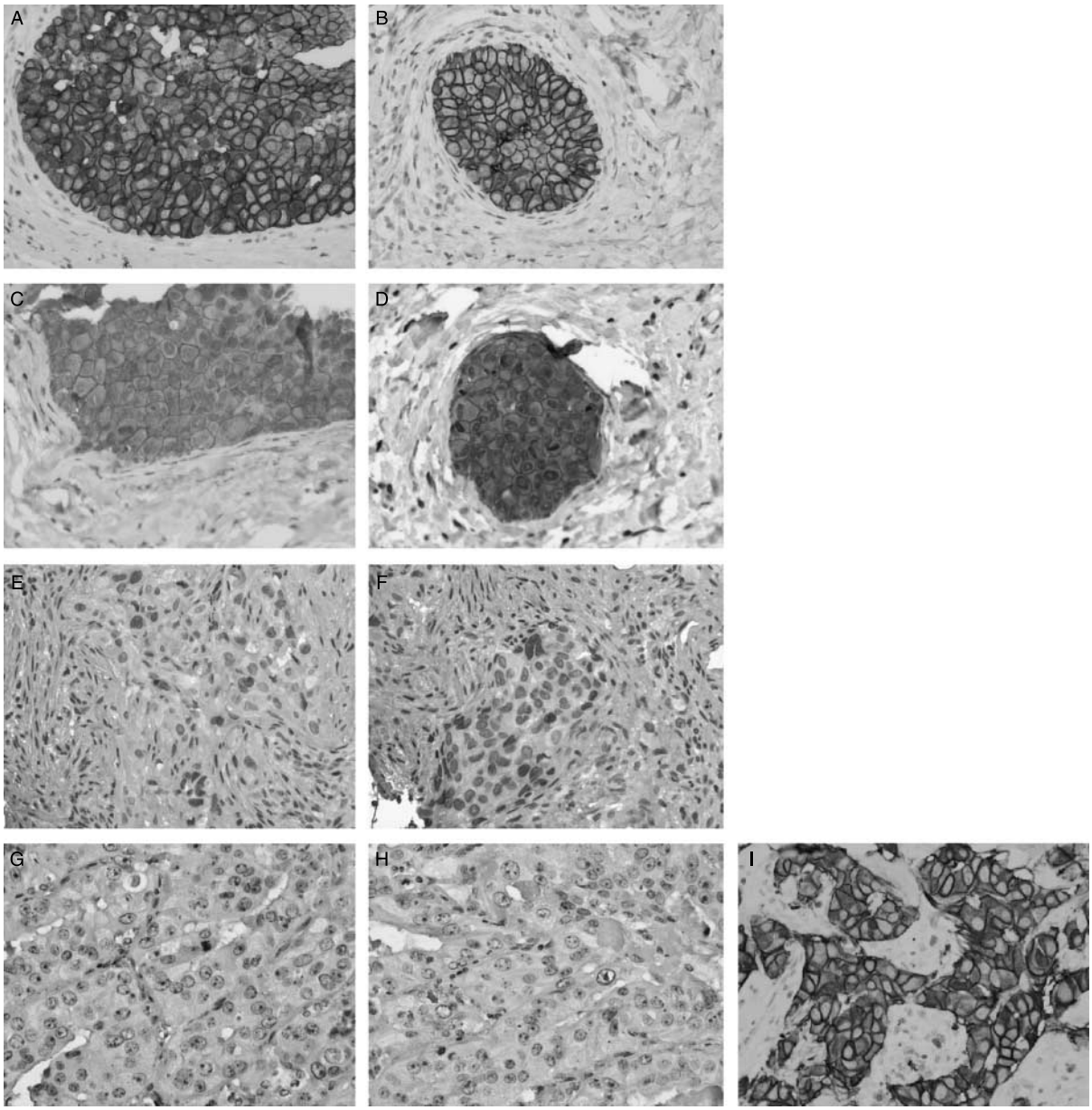

Figure 1 Immunohistochemical ERBB2, pERBB2, and EGFR protein expression in representative tumor samples obtained during the initial operation (A, C, E, and G, I; 250×) and during a second surgical procedure that became necessary for involved margins (B, D, F, and H, 250×). Strong membranous ERBB2 staining in DCIS is shown in a ERBB2-positive tumor in the primary resectate in A and in the re-excised sample in B. ERBB2 staining is absent in an invasive tumor in both, the initial (E) and the re-excised sample (F). Intermediate pERBB2 staining is shown in a representative DCIS in C and strong pERBB2 staining is shown in a sample obtained from the same tumor 2 weeks later in D. Unspecific pERBB2 staining is shown in the initial resectate of an invasive tumor in G and in the re-excised sample in H. Strong membranous EGFR staining in DCIS is also shown in one of the few EGFR-positive tumors in the primary resectate in I.

tumors was 61 years. As depicted in Table 1, ERBB2-negative cases consisted 46/54 DCIS and 8/54 IBC cases, whereas ERBB2-positive cases consisted of 39/51 DCIS and 12/51 invasive breast cancer (IBC) cases. In both groups, ductal carcinomas were most common with 85% (ERBB2-negative) and 76% (ERBB2-positive) cases. Most tumors were T2 and T3 in both groups. Thirty-three percent of axillary lymph nodes were involved in ERBB2-negative and 27% were involved in ERBB2-positive tumors. ER was positive in 83% of ERBB2-negative tumors while 43% stained positive in ERBB2-overexpressing breast cancers.

Table 1 Patient characteristics

	ERBB2 neg ($n=54$)	ERBB2 pos ($n=51$)
Age (years)		
Median	58	61
Range	32–81	38–80
Grading		
1	16/54 (30%)	10/51 (20%)
2	26/54 (48%)	14/51 (27%)
3	12/54 (22%)	27/51 (53%)
Histology		
DCIS	46/54/85%)	39/51 (76%)
IDC	7/54 (13%)	12/51 (24%)
Other invasive	1/54 (2%)	0/51 (0%)
Clinical stage		
T1	17/54 (31%)	12/51 (24%)
T2	23/54 (43%)	16/51 (31%)
T3	11/54 (20%)	22/51 (43%)
T4	3/54 (6%)	1/51 (2%)
N0	27/54 (50%)	11/51 (22%)
N1	18/54 (33%)	14/51 (27%)
N unknown	9/54 (27%)	26/51 (51%)
Hormone receptors		
ER+	44/54 (83%)	22/51 (43%)
PR+	36/54 (67%)	14/51 (27%)
ER unknown	2/54 (4%)	4/51 (8%)
PR unknown	2/54 (4%)	4/51 (8%)

EGFR, pEGFR, and pERBB2 expression in malignant breast tissue and in residual tumor tissue in ERBB2-negative breast cancer

Table 2 depicts the results of intratumoral ERBB2 and pERBB2 staining in the original tumor samples and in the specimen that had been removed from the tumor site within 3 weeks after initial surgery for incomplete primary resection in both, ERBB2-overexpressing and ERBB2-negative tumors.

We first confirmed ERBB2 immunostaining according to the DAKO score. As expected, none of the tumors originally classified as 'ERBB2 negative' by Herceptest was found to be ERBB2 positive with the anti ERBB2 antibody. This held true for both, the invasive and the DCIS component. Activated ERBB2 (pERBB2) was detected in 1 out of the 27 (4%) of invasive breast cancers, and in 3 out of the 28 (11%) of DCIS cases obtained during the initial operation. Staining was weak in all DCIS cases and the one pERBB2-positive invasive breast tumor showed intermediate expression.

Likewise, ERBB2 protein expression in both DCIS and invasive tumors obtained from the re-excised sample was comparable with the initial sample. None of the 32 cases in which residual DCIS tissue was histologically confirmed were ERBB2 positive. In analogy, ERBB2 expression in residual tumors obtained from invasive breast cancer was also undetectable in all 20 cases. pERBB2 expression in invasive tumors that had been obtained during the second operation was also comparable with that seen in the initial invasive carcinoma (8 out of the 8 negative versus 26 out of the 27 negative, $P=0.581$, χ^2 test). In DCIS, however, pERBB2 activity was significantly up-regulated in the re-excision samples: 5 out of the 12 DCIS stained positive for pERBB2 as opposed to 3 out of the 28 DCIS in primary resection specimens, $P=0.025$, χ^2 test). No difference in ER or PR expression was detected when invasive tumor components from the original tumor sample were compared with re-excision samples ($P=0.57$, χ^2 test), or when the respective DCIS components were compared ($P=0.24$, χ^2 test). Grading was also not affected by excision and initial and re-excised tumor samples exhibited comparable grading in both DCIS and invasive tumor components ($P=0.754$ and $P=0.259$ respectively, χ^2 test). The same was also true for Ki67 which did not differ significantly when 40 primary resectates were compared with 25 re-excised samples ($P=0.13$ and $P=0.47$ respectively, χ^2 test). EGFR was only present in the DCIS and the invasive component of 2 out of the 48 samples, while pEGFR expression was not detectable at all (data not shown).

pERBB2, EGFR, and pEGFR expression in malignant breast tissue and in residual tumor tissue in ERBB2-overexpressing breast cancer

Using the PN2A antibody, we detected ERBB2 phosphorylation in 36 out of the 41 (88%) ERBB2-overexpressing DCIS cases. In one case (2%), ERBB2 expression was found to be weak and in two cases (5%) protein expression was intermediate. Two out of the 41 cases that had been considered ERBB2-overexpressing tumors did not show protein expression when analyzed according to the Herceptest. In invasive tumor components, 5 out of the 15 (30%) samples were ERBB2 negative and in one case (7%), ERBB2 expression was intermediate. Nine out of the fifteen (60%) of invasive tumor components overexpressed ERBB2 protein in the initial surgical specimen. Activated (p)ERBB2 was found in 16 out of the 28 (57%) DCIS components analyzed, and was weak in 11 out of the 28 (39%) samples, and intermediate in 5 out of the 28 (18%) samples. None of the DCIS analyzed expressed high levels of pERBB2. In initial breast tumor specimen that contained areas of invasion, 9 out of the 25 (36%) samples expressed pERBB2, and expression was weak in six (24%) cases, intermediate in two (8%) cases, and strong in one (4%) case.

Table 2 ERBB2 and pERBB2 protein expression in breast cancer biopsies at initial excision and re-excision

	DCIS component				Invasive component			
	ERBB2[a]	pERBB2	ER	PR	ERBB2[a]	pERBB2	ER	PR
ERBB2 overexpression								
Excision								
0	2/41 (5%)	12/28 (43%)	6/21 (28%)	8/21 (38%)	5/15 (33%)	16/25 (64%)	29/51 (57%)	37/51 (73%)
+	1/41 (2%)	11/28 (39%)	7/21 (33%)	2/21 (10%)	0/15 (0%)	6/25 (24%)	8/51 (16%)	6/51 12%)
++	2/41 (5%)	5/28 (18%)	4/21 (19%)	7/21 (33%)	1/15 (7%)	2/25 (8%)	7/51 (14%)	6/51 (12%)
+++	36/41 (88%)	0/28 (0%)	4/21 (19%)	4/21 (19%)	9/15 (60%)	1/25 (4%)	7/51 (14%)	2/12 (4%)
Re-excision								
0	2/39 (5%)	2/26 (8%)	5/17 (29%)	9/16 (%)	2/9 (22%)	11/17 (65%)	3/7 (43%)	3/6 (50%)
+	2/39 (5%)	14/26 (54%)	5/17 (29%)	2/16 (%)	2/9 (22%)	4/17 (24%)	2/7 (29%)	3/6 (50%
++	1/39 (3%)	8/26 (31%)	6/17 (35%)	5/16 (31%)	2/9 (22%)	2/17 (12%)	2/7 (29%)	0/6 (0%)
+++	34/39 (87%)	2/26 (8%)	1/17 (6%)	0/16 (0%)	3/9 (33%)	0/17 (0%)	0/7 (0%)	0/6 (0%)
No ERBB2 overexpression								
Excision								
0	44/44 (100%)	25/28 (89%)	9/23 (39%)	5/23 (22%)	25/25 (100%)	26/27 (96%)	9/53 (17%)	18/54 (33%)
+	0/44 (0%)	3/28 (11%)	7/23 (30%)	5/23 (22%)	0/25 (0%)	0/27 (0%)	3/53 (6%)	6/54 (11%)
++	0/44 (0%)	0/28 (0%)	2/23 (9%)	11/23 (47%)	0/25 (0%)	1/27 (4%)	22/53 (42%)	22/54 (41%)
+++	0/44 (0%)	0/28 (0%)	5/23 (22%)	2/23 (9%)	0/25 (0%)	0/27 (0%)	19/36 (36%)	8/54 (15%)
Re-excision								
0	32/32 (100%)	7/12 (58%)	2/3 (67%)	3/3 (100%)	20/20 (100%)	8/8 (100%)	1/2 (50%)	0/2 (0%)
+	0/32 (0%)	5/12 (42%)	0/3 (0%)	0/3 (0%)	0/20 (0%)	0/8 (0%)	0/2 (0%)	0/2 (0%)
++	0/32 (0%)	0/12 (0%)	1/3 (33%)	0/3 (0%)	0/20 (0%)	0/8 (0%)	1/2 (50%)	1/2 (50%)
+++	0/32 (0%)	0/12 (0%)	0/3 (0%)	0/3 (0%)	0/20 (0%)	0/8 (0%)	0/2 (0%)	1/2 (50%)

[a]Analyzed according to the DAKO score.

Samples from corresponding secondary resections exhibited a fairly similar expression pattern of ERBB2 in both DCIS and invasive components when compared with the initial sample: strong ERBB2 expression was detected in 34 out of the 39 (87%) of DCIS samples, while intermediate expression was found in one (3%) and weak expression in two cases (5%). In invasive tumor components, weak expression was found in two out of the nine (22%) cases, intermediate expression in two (22%), and strong expression in three (33%) cases. No significant difference was seen when ERBB2 expression in invasive and DCIS components were compared with the initial tumor sample ($P=0.244$, and $P=0.288$ respectively, χ^2 test). A significantly different pattern was, however, observed for pERBB2, when compared with the initial resectate. In DCIS 14 (54%) of the samples obtained during secondary resection exhibited weak staining, eight (31%) intermediate, and two (8%) strong staining ($P=0.018$, χ^2 test). By contrast, in invasive tumors, 6 out of the 17 invasive tumor samples were positive for the activated receptor that did not translate into a statistically significant difference when compared with the initial invasive component ($P=0.261$, χ^2 test). EGFR was detected in 1 out of the 47 samples (in both the DCIS and the invasive component of the tumor), while pEGFR expression was not found (data not shown). Corresponding images are depicted in Fig. 1.

Furthermore, no difference in ER or PR expression was detected when invasive tumor components from the original tumor sample were compared with re-excision samples ($P=0.644$ and $P=0.483$, χ^2 test). While in DCIS components, ER expression was similar in primary tumors when compared with re-excised samples, we observed a trend toward a decreased PR protein expression in re-excised samples ($P=0.054$, χ^2 test). Grading was also not affected by re-excision and initial and re-excised tumor samples exhibited comparable grading in both DCIS and invasive tumor components ($P=0.933$ and $P=0.309$ respectively, χ^2 test). The same was also true for the Ki67 labeling index in the initial ($n=38$) and re-excised ($n=27$) samples in both tumor components ($P=0.22$ and $P=0.51$, χ^2 test).

Correlation of ERBB2 and phosphor ERBB2 with clinicopathological parameters

Results are in Table 3: within the group of ERBB2-negative tumors (i.e., DCIS and invasive tumors), we found the overall ERBB2 expression in DCIS to be very well correlated with ERBB2 expression in invasive tumor components ($r=1.0$; $P<0.0001$, Spearman correlation). While we found no correlation between pERBB2 expression in DCIS versus pERBB2 expression in invasive parts of the tumor, we did, however, find an inverse correlation between pERBB2 in DCIS and

Table 3 Correlation between ERBB2 and hormone receptors in (a) ERBB2 non-overexpressing tumors and (b) ERBB2-overexpressing tumors (initial excision and consecutive resection combined)

	ERBB2 DCIS	ERBB2 invasive	pERBB2 DCIS	pERBB2 invasive	ER	PR
ERBB2 non-overexpressing tumors						
ERBB2 DCIS	–	–	–	–	–	–
ERBB2 invasive		–	–	–	–	–
pERBB2 DCIS				–	$r=0.329$ $P=0.081$	$r=0.361$ $P=0.055$
pERBB2 invasive					$r=-0.110$ $P=0.586$	$r=-0.255$ $P=0.200$
ER						$r=0.383$ $P=0.004$
ERBB2-overexpressing tumors						
ERBB2 DCIS	$r=-0.465$ $P=0.353$	$r=0.312$ $P=0.042$	$r=0.039$ $P=0.881$	$r=0.185$ $P=0.248$	$r=0.197$ $P=0.218$	
ERBB2 invasive		$r=-0.485$ $P=0.093$	$r=0.250$ $P=0.685$	$r=0.111$ $P=0.694$	$r=0.445$ $P=0.097$	
pERBB2 DCIS			$r=0.396$ $P=0.084$	$r=-0.331$ $P=0.085$	$r=0.375$ $P=0.049$	
pERBB2 invasive				$r=-0.123$ $P=0.638$	$r=-0.459$ $P=0.064$	
ER					$r=0.536$ $P<0.0001$	

Correlations between ERBB2 and pERBB2 were analyzed by Spearman's test (two-sided). For all analyses, a $P<0.05$ was considered statistically significant.

grading of the invasive component of the same tumor ($r=-0.519$; $P=0.013$, Spearman correlation). We also observed a direct correlation between pERBB2 in DCIS and ER and PR expression in adjacent invasive tumor components ($r=0.330$; $P=0.081$ and $r=0.361$; $r=0.055$, Spearman correlation respectively). Furthermore, grading in the invasive and in the DCIS components were well, albeit inversely, correlated ($r=0.848$; $P<0.0001$). Not surprisingly, within the invasive components, ER and PR were also correlated ($r=0.383$; $P=0.004$).

In the group of ERBB2-overexpressing tumors, ERBB2 and pERBB2 protein expression in DCIS components was correlated ($r=0.312$; $P=0.042$, Spearman correlation), but no such association was found for invasive components, which was probably due to the low number of ERBB2-positive cases in the secondary resectates. Finally, PR expression in invasive components was associated with both, grading in DCIS components ($r=0.476$; $P=0.004$), and ER expression ($r=0.536$; $P<0.0001$). No other correlation was observed when applying Spearman correlation analysis.

ERBB2 receptor activation in response to heregulin

We then went on to investigate whether ERBB2 can be activated in by locally expressed cytokines that are known to be expressed in response to tissue damage (Fig. 2). SKBR3 breast cancer cells are known to express ERBB2 protein, and were cultivated in monocultures until they reached subconcluency. The cells were then serum starved and maintained in either without (A, C) or with (B, D) recombinant human heregulin for 7 min. While exposure to heregulin did not appear to have an effect on intracellular ERBB2 protein expression (B), it was able to significantly up-regulate pERBB2, thereby resulting in a strong membranous signal in tumor cells (D) in comparison with untreated cells (A).

Discussion

We have analyzed ERBB2, EGFR, pEGFR, and pERBB2 protein expression pattern in biopsies obtained during breast conserving surgery for breast cancer and compared them with samples removed during a second operation which had become necessary because of involved margins.

We found ERBB2 to be activated in 57% of the original tumor samples in which DCIS components overexpressed ERBB2 protein. This observation is almost identical with findings by DiGiovanna *et al.* (2002) who, using the same antibody, described pERBB2 protein expression in 58% of ERBB2-overexpressing DCIS. We were, however, unable to find the inverse correlation between the activated receptor in DCIS and hormone receptor expression that the authors had described previously. By contrast, only 11% of tumors

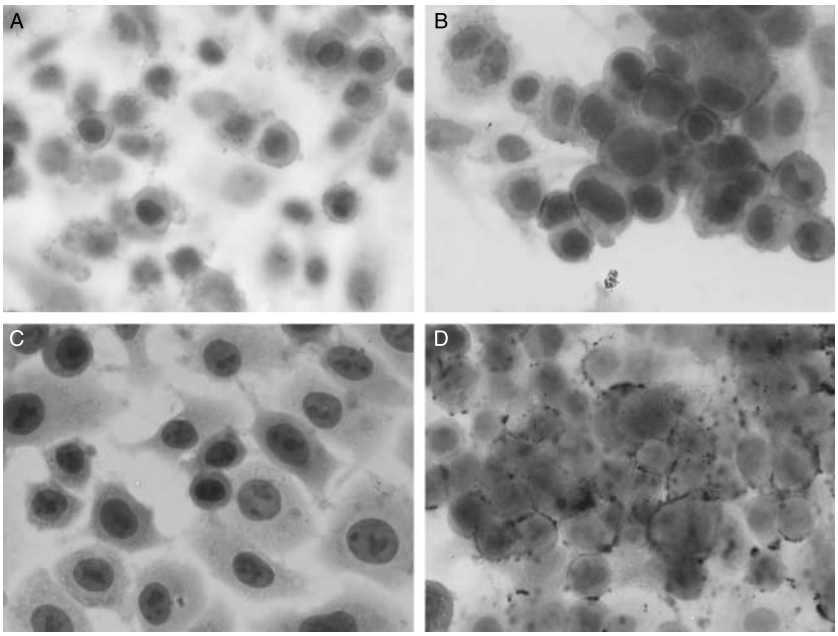

Figure 2 Immunocytochemical ERBB2 and pERBB2 protein expression in SKBR3 breast cancer cells in response to heregulin treatment. SKBR3 cells were cultured in serum-free medium and in the absence (A and C, 500×) and in the presence (B and D) of 25 ng/ml heregulin. Cells were stained with either an anti ERBB2 antibody (A and B, 500×) or an anti-pERBB2 antibody (C and D, 500×). While the addition of heregulin does not affect ERBB2 protein expression, it results in a strong membranous staining pattern of pERBB2.

in the ERBB2 non-overexpressing DCIS group, and only one out of the 27 (4%) ERBB2 non-overexpressing invasive tumors exhibited detectable, albeit weak, pERBB2 staining, thus suggesting that receptor activation and consecutive signal transduction is not strictly dependent on ERBB2 overexpression.

While we did not observe significant differences in ERBB2 protein expression between the original and the re-excised tissue samples in both ERBB2-overexpressing and ERBB2-negative tumors, we found a significant up-regulation of activated (p)ERBB2 in DCIS components. This held true for both ERBB2-positive and ERBB2-negative tumors. Interestingly, we did not find such an up-regulation in invasive tumor components. Although it is possible that ERBB2 receptors in preinvasive lesions exhibit a different biological behavior than in invasive lesions, it cannot be excluded that the lack of significance is simply caused by the relatively small number of tumors in which both the initial and the re-excised specimen contained invasive tumor components. The low incidence of invasive tumor components in re-excised tumor samples is mainly caused by two tumor biological features: a) ERBB2 overexpression in DCIS is considerably more common than in invasive breast cancer and b) in contrast to invasive breast cancer, which is more often confined to a relatively well-circumscribed area, areas affected by DCIS are more difficult to delineate and usually spread out to a larger area of the breast (Allred *et al*. 1992, Dillon *et al*. 2007). The combination of the two attributes has lead to a somewhat unbalanced distribution, and explains why the vast majority of the residual tumors from ERBB2-overexpressing breast cancer contain DCIS tissue. It is somewhat interesting that grading was inversely correlated in DCIS and invasive components in ERBB2 non-overexpressing tumors and not in overexpressing tumors. To our knowledge, this has never been demonstrated before and we have no explanation for this observation.

Our findings are somewhat limited by the fact that the evaluation of residual tumor cells proved extremely difficult. This has to do with the fact that, although attempted, we were not always able to receive consecutive slides for ERBB2 and pERBB2 staining. Thus, in some slides, we might only have had evaluable invasive components on a slide that was used for ERBB2 staining, whereas in a slide from the same tumor that was used for pERBB2 staining we might have had both invasive and non-invasive components available for analysis, and vice versa. Also, owing to the nature of DCIS, we found considerably more DCIS tumor residues than invasive residues.

In contrast to our own findings in ERBB2-overexpressing metastatic breast cancer, we detected EGFR 1 out of the 47 (2%) cases analyzed, despite using the same antibody and protocol (Gschwantler-Kaulich *et al*. 2005). Others have similarly found a low

prevalence of EGFR-overexpressing tumors (6%, regardless of ERBB2 status), while yet another group observed EGFR protein expression in 33% of ERBB2 amplified and in 16% of ERBB2 non-amplified tumors (Bhargava *et al.* 2005, Park *et al.* 2007). The lack of pEGFR protein expression in any of the tumor samples is also somewhat contrasting our own findings in metastatic breast cancer, where we were able to detect pEGFR in 13% of ERBB2-overexpressing tumors (Hudelist *et al.* 2006).

A number of growth factors and cytokines are released during wound healing. Among them, EGF, IGF1, PDGF, and TGFB1 appear to be essential factors for tissue remodeling in response to laceration. TGFB1, for example, has been shown to induce the motility of ERBB2-transfected MCF-10A cells, a mechanism that most likely involves type 1-TGFB1 receptor-associated activation of ERBB2 signaling (Ueda *et al.* 2004). EGF has similarly been demonstrated to accelerate epithelial cell migration and wound healing *in vitro* and *in vivo* (Shirakata *et al.* 2005, Katz *et al.* 2007). In addition, EGF also induces the degradation of type 1 procollagen protein through the up-regulation of MMP-1 expression, which is a key step in wound healing-associated tissue matrix remodeling (Singer *et al.* 1999, Shirk & Kuver 2005).

Neu differentiation factor (NDF, heregulin) is another cytokine that has been shown to result in tyrosine phosphorylation of the ERBB2 receptor via binding to either the ERBB3 or ERBB4 receptor. NDF contains a receptor-binding EGF-like domain and is a member of the EGF family. It is expressed in the stromal compartment and acts on ERBB expressing epithelium via a paracrine mechanism. In human skin, it results in epidermal migration and integrin expression in excisional wounds (Danilenko *et al.* 1995). It is thus quite feasible that intraoperative tissue damage results in the release of ERBB2-activating cytokines as part of a wound healing process that is being initiated. Unfortunately, the very same cytokines also result in a proliferative stimulus for ERBB2 expressing remaining tumor cells and can facilitate local tissue lysis and matrix remodeling (Ram *et al.* 1995, Normanno & Ciardiello 1997, Schroeder & Lee 1997, Wells *et al.* 2002, Buck & Knabbe 2006, Mimura *et al.* 2006). The co-expression of TGFB1 and EGF has recently even been demonstrated to exert synergistic effects on the oncogenic properties of human epithelial cells, although their potential influence on ERBB2 activation has not been investigated (Uttamsingh *et al.* 2008).

These observations have lead to the hypothesis that postoperative reactive tissue repair might also result in proliferation of remaining tumor cells. Hofer *et al.* have suggested that the time interval during which the sequelae of events in wound healing occur in relation to the time interval of tumor recurrence may be important and may provide a basis for defining new therapeutic strategies that can interfere with tumor outgrowth without affecting wound healing processes. Tagliabue *et al.* (2003) have demonstrated increased proliferation in re-excised blocks of ERBB2-overexpressing breast cancer which further supports this hypothesis. While we were unable to detect a similar up-regulation in our ERBB2-overexpressing tumors in our samples, this could be due to the relatively small number of invasive tumors in which Ki67 staining was available for both the initial and the re-excised samples. However, even in DCIS cases, where Ki67 was available in a considerably larger set of initial and consecutive re-excised samples, no such correlation was observed. Nevertheless, the findings by Hofer and Tagliabue could potentially lead to new therapeutic approaches that are aimed at targeting potentially hazardous repair mechanisms following surgery or other invasive procedures in cancer patients (Hofer *et al.* 1999).

Taken together, our results suggest that the increased in ERBB2 activation in re-excised DCIS specimen suggests an activation of ERBB2 signaling through processes, such as heregulin release, which are physiologically associated with postoperative wound healing. ERBB2 activation could well serve as one possible mechanism by which the phenotype of residual tumor cells is altered towards a more aggressive one, and emphasizes the need of complete resection of the primary tumor in preinvasive lesions.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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