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Phosphorylation of tyrosine 1248-ERBB2 measured by chemiluminescence-linked immunoassay is an independent predictor of poor prognosis in primary breast cancer patients

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ABSTRACT

ERBB2 (HER2/Neu) gene amplification and overexpression is associated with increased risk of metastases and shorter survival in breast cancer. Tyrosine 1248 is a major phosphorylation site of ERBB2 and reflects the activation status of the receptor. The aim of this study was to investigate the relationships between quantitative levels of pY1248-ERBB2 (p-ERBB2) and the expression of epidermal growth factor receptor (EGFR)-family members, and whether p-ERBB2 could provide additional prognostic value compared with established prognostic markers. For this purpose we developed a highly sensitive chemiluminescence-linked immunoassay (CLISA) and detected p-ERBB2 levels in 70 primary breast cancer biopsies. Phosphorylated ERBB2 correlated with EGFR and ERBB2, and inversely with oestrogen receptor (ER), progesterone receptor (PgR) and ERBB4 expression levels. Additionally, p-ERBB2 was associated with poor clinical outcome in univariate and multivariate Cox regression analysis. Further studies are needed to evaluate the predictive value of p-ERBB2.

1. Introduction

ERBB2 (HER2/Neu) is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, which comprises EGFR (HER1, ERBB1), ERBB3 (HER3) and ERBB4 (HER4).[1–3] Upon ligand binding, homo- and heterodimeric complexes are formed, with ERBB2 as the preferred dimerisation partner.[4] This leads to autophosphorylation of specific tyrosine residues, activation of downstream signalling cascades and, finally, initiation of biological processes such as proliferation.[5] ERBB2 contains five major tyrosine autophosphorylation sites, including Y-1248.[6]

In primary breast cancer, ERBB2 is amplified and overexpressed in 15–30% of patients and has been associated with poor prognosis.[7–9] Trastuzumab (Herceptin™), a humanised monoclonal anti-ERBB2 antibody is the first clinically available oncogene-targeted therapeutic agent for treatment of solid tumours, and is approved for use in metastatic breast cancer patients.[10] First-line trastuzumab in combination with chemotherapy resulted in a 25% improvement in overall

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survival compared with chemotherapy alone. However, only up to 40% of patients respond to the therapy, suggesting that more accurate biomarkers are required to identify patients who are likely to respond to treatment such as trastuzumab. It was also reported that an inverse relationship exists between oestrogen receptor (ER) and ERBB2 expression, where ERBB2 overexpression is associated with decreased ER/progesterone receptor (PgR) levels and reduced sensitivity, possibly even resistance to endocrine therapy.[9,11,12]

ERBB2 gene amplification or overexpression per se may not reflect adequately the activated status of the ERBB2 receptor. It was hypothesised that the percentage of phosphorylated ERBB2, and thus activated receptor, could be different between tumours expressing similar amounts of ERBB2.[13]

The aim of the present study was to investigate the prognostic value of pY1248-ERBB2 detected with a newly developed chemiluminescence-linked immunoassay (CLISA), its association with protein and mRNA expression levels of the EGFR-family members including established prognostic markers in a set of 70 primary breast cancer patients.

2. Patients and methods

2.1. Patients and tumour characteristics

For all tumour samples the Stiftung Tumorbank Basel (STB) received a representative piece of fresh frozen tissue containing more than 65% tumour cells after surgery and pathological examination. Specimens were immediately processed or cryopreserved (−80 °C). For this study, 70 primary breast tumour samples were selected according to ERBB2 protein expression levels detected by enzyme immunoassay (EIA) at time of surgery. Tumours with ERBB2 protein levels >260 ng/mg total protein were considered positive, which corresponds to a previously published cut-off value of 500 U/mg total protein and correlates with the immunohistochemistry (IHC) DAKO 3+[8] as well as ERBB2 amplification detected by fluorescence in situ hybridisation (FISH) (Urban P, et al., submitted). ERBB2-negative tumours showed protein expression levels between 100 and 260 ng/mg. All patients underwent primary surgery before January 1996. Twenty-four patients (34%) relapsed within the median follow-up time of 55 months (range 30–89 months). Thirty-seven (53%) were nodal-positive, 50 (71%) were ER-positive and 40 (57%) patients were ERBB2-positive. None of the patients received neoadjuvant therapy.

STB is a non-profit organisation with an official Swiss permit that guarantees ethical issues and patient confidentiality. Patients and tumour characteristics are summarised in Table 1.

Table 1 – Tumour and patients characteristics

Feature	Number of patients (%)
Patients	70 (100)
Histology type	
Ductal	48 (69)
Lobular	11 (16)
Other	11 (16)
Tumour size	
T1	18 (26)
T2	42 (60)
T3–4	10 (14)
Lymph node status	
Node-negative	33 (47)
Node-positive	37 (53)
Histopathological grade	
I + II	27 (39)
III	34 (48)
Not analysed	9 (13)
Oestrogen receptor	
Positive (>20 fmol/mg)	50 (71)
Negative (⩽20 fmol/mg)	20 (29)
Median/mean (fmol/mg)	72/139
Progesterone receptor	
Positive (>20 fmol/mg)	38 (54)
Negative (⩽20 fmol/mg)	32 (46)
Median/mean (fmol/mg)	28/128
ERBB2	
Positive (>260 ng/mg)	40 (57)
Negative (<260 ng/mg)	30 (43)
Median/mean (ng/mg)	307/298

2.2. Cell lines and tissue culture

SKBr3 breast cancer cells were cultured in improved minimal essential medium with zinc option (IMEM-ZO) supplemented with 5% foetal bovine serum (FBS) and L-glutamine at 37 °C in a 5% CO_2 incubator. For the phospho-standard preparation sub-confluent SKBr3 cells were serum-starved for 48 h in serum-free medium, treated with NaF and Na_3VO_4 for 1 h, then with 10% FBS for 10 min. Cells were lysed in EB lysis buffer (0.5 M NaCl, 10 mM EDTA, pH 8, 1% Triton × 100, 20 mM Tris–Cl, pH 7.0, 20 mM NaF, 20 mM glycerophosphate, 2 mM Na_3VO_4, proteinase inhibitor cocktail, Roche) for 5 min on ice, centrifuged at 20,000*g* for 5 min and the supernatant stored at −80 °C.

2.3. Measurement of ER, PgR, ERBB2 and EGFR protein levels

Tissue homogenates were prepared in accordance with standard procedures for tumour marker EIA measurement, as described previously.[8] In brief, frozen tissues were powderised in liquid nitrogen (Micro-Dismembrator U, B. Braun AG, Melsungen, Germany) and homogenised (tissue homogeniser, Ultra-Turrax; Janke and Kunkel, IKA-Werke, Staufen, Germany) for 20 s in three volumes of ice-cold extraction buffer. The homogenate was centrifuged at 800*g* for 30 min at 2 °C, and the resulting supernatant recentrifuged in an ultracentrifuge (Beckman Instruments, Fullerton, CA, United States of America (USA)) at 100,000*g*. The resulting supernatants (cytosols) were used for measurement of the hormone receptors (ER, PgR by Abbott Laboratories, Abbott Park, IL, USA), while the membrane fractions were used for EIA measurement of ERBB2 (Oncogene Science Human HER-2/neu Quantitative ELISA Kit, Bayer, Leverkusen, Germany). Quantification of EGFR was done by radioligand binding assay (LBA) as described previously.[14] Quality control of ER and PgR measurements were carried out in collaboration with the Receptor

Biomarker Group of the European Organisation for Research and Treatment of Cancer (EORTC).

2.4. *pY1248-ERBB2 immunoassay*

p-ERBB2 levels were measured with a two-site CLISA. Black 96-well microtitre plates (Nunc Black MaxiSorp Surface; Nalgen Nunc International, Rochester, NY, USA) were coated with antihuman activated *Neu/c-ERBB2* antibody (#06-229, lot #15916; Upstate Biotechnology, Lake Placid, NY) at a concentration of 4 mg/ml of coating buffer (phosphate buffered saline (PBS) with 0.6 mM EDTA) in a volume of 100 μl/well and kept at 4 °C overnight. This antibody is virtually identical to the monoclonal antibody clone PN2A that recognises only pY1248-ERBB2.[13,15] Tumour extracts were prepared in the presence of Na_3VO_4. Before sample application, coated microtitre plates were washed five times with 200 μl/well of washing buffer (25 mM HEPES, pH 7.4, 300 mM NaCl, 0.05% Tween-20) and then blocked for 2 h at room temperature with 250 μl blocking buffer (25 mM HEPES, pH 7.4, 300 mM NaCl, 0.05% Tween-20, 3% TopBlock, Juro AG, Switzerland). The blocked wells were washed five times with 300 ml blocking buffer. Then 100 μl of the diluted membrane extracts or reference material was added to the wells and incubated overnight at 4 °C. SKBr3 cell extract was used as reference for each assay as described above. First, SKBr3 cell membrane extract was sequentially diluted with sample dilution buffer at ratios of 1×, 0.75×, 0.5×, 0.25×, 0.125× and 0.025×. Subsequently, 100 μl aliquots were incubated on each microtitre plate together with the tumour extracts or controls (dilution buffer only). After incubation, wells were washed five times with 300 μl washing buffer at room temperature to eliminate unbound particles. Biotinylated detection antibody (HER-2/Neu Microtiter ELISA kit, Oncogene Science) was added to the wells, incubated for 2 h at room temperature and complex detected with horseradish peroxidase (HRP)-conjugated streptavidin using SuperSignal WestPico substrate (Pierce) in a glow luminometer. A curve was fitted to the data of the reference dilution series and used for quantification of tumour extracts. The value of the undiluted SKBr3 extract was denominated as 100 U/ml. The standard curve and additional specification are shown in Fig. 1.

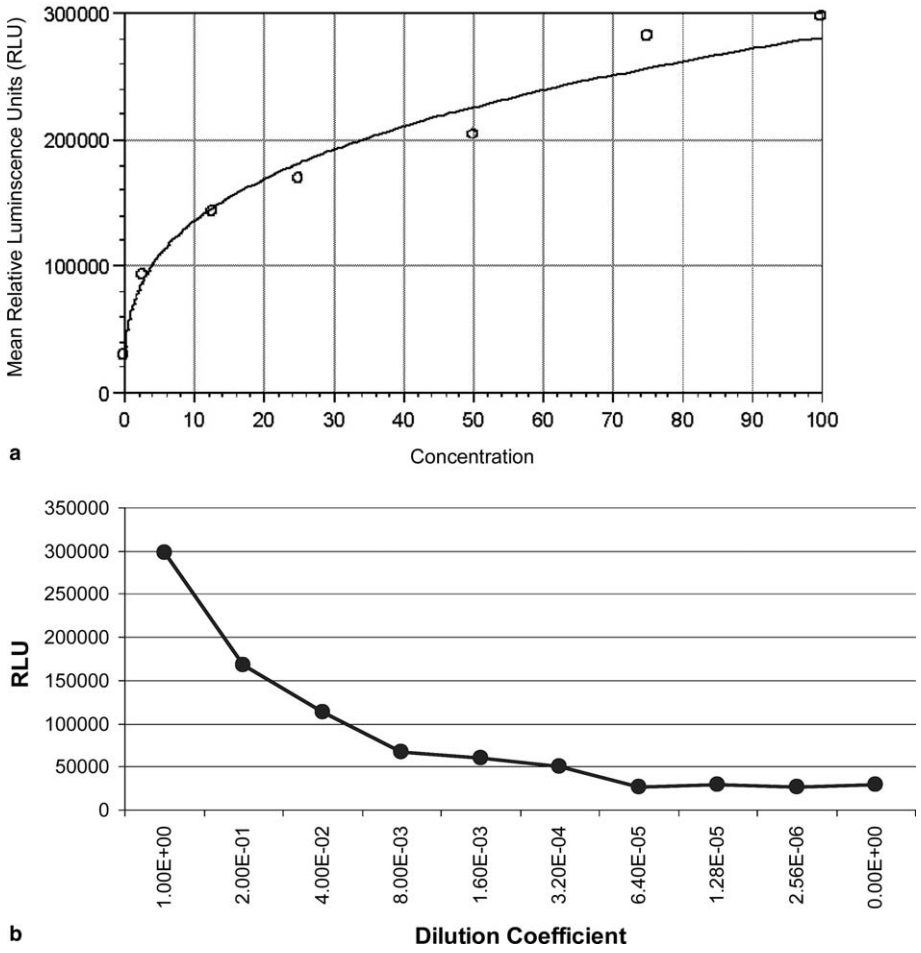

Fig. 1 – Supplementary assay specifications: (a) standard curve for pY1248-ERBB2 immunoassay as described in Section 2. A concentration of 100 (*x*-axis) corresponds to 100 μl undiluted standard (1×), a concentration of 50 to a dilution of 0.5×, etc. Standard curve was fitted using SoftMax software (Molecular Devices, CA, USA). Limit of detection (LOD) and limit of quantification (LOQ) was calculated from repeated measurements without analyte. LOD (mean + 2SDs) value was 31088 RLU or 0.07 U/ml, and LOQ (mean + 10SDs) was 39203 RLU or 0.41 U/ml, respectively. The undiluted value of SKBr3 standard was denominated 100 U/ml; (b) dilution curve for SKBr3 standard (see Section 2).

Table 2 – Primer sequences

Gene	RefSeq	Forward primer	Reverse primer
ESR1	NM_000125	CTTGCTCTTGGACAGGAACCA	CAAACTCCTCTCCCTGCAGATT
PgR	NM_000926	TGTCGAGCTCACAGCGTTTC	TACAGATGAAGTTGTTTGACAAGATCA
EGFR	NM_005228	GGACTATGTCCGGGAACACAA	CCAAGTAGTTCATGCCCTTTGC
ERBB2	NM_004448	CTGAACTGGTGTATGCAGATTGC	TTCCGAGCGGCCAAGTC
ERBB3	NM_001982	AATAAAAGGGCTATGAGGCGATACT	AGCTTCCTTAGCTCTGTCTCTTTGA
ERBB4	NM_005235	GTCCAGATAGCTAAGGGAATGATGTAC	CTAGCCCAAAATCTGTGATTTTCAC

PgR, progesterone receptor; EGFR, epidermal growth factor receptor.

2.5. *Quantitative real-time PCR (Qrt-PCR) for ER, PgR and EFGR-family*

Total RNA was extracted using RNeasy kit (Qiagen, Hilden, Germany). RNA quality and quantity was checked for all samples on Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA). All genes were measured using SYBR Green I method and a Taqman 7000 (Applied-Biosystems, Foster City, CA, USA). Relative quantification (delta ct-value) was obtained by normalisation to the ribosomal 18S gene, and by a standardisation step using Human Universal Standard RNA (Stratagene, La Jolla, CA, USA). Quantitative real-time polymerase chain reactino (Qrt-PCR) results were expressed in arbitrary units of reverse transcribed RNA (U/ug rt-RNA). Primer sequences are listed in Table 2.

2.6. *Statistical methods*

Correlations between continuous values were assessed by the Spearman rank correlation coefficient (r_s). Statistical significance between p-ERBB2 and dichotomous variables was calculated using Mann–Whitney *U* test. In the present study patients were dichotomised and defined as p-ERBB2-negative/positive according to median value of p-ERBB2. Alternatively, an optimised cut-off value for p-ERBB2 with respect to prognosis was searched for by classification and regression trees (CART) analysis.[16] Relationships between categorical data were assessed using Fisher's exact test. The prognostic significance between p-ERBB2 and other variables was tested in univariate and multivariate Cox regression analysis and likelihood ratio test. Hazard rates and confidence intervals (CIs) were summarised in tables. Survival curves were estimated by the Kaplan–Meier method and statistical significance compared by means of log-rank test. All tests were performed using S-PLUS statistical software (Insightful, Version 6).

3. Results

3.1. *Distribution of p-ERBB2 and association with ERBB2*

p-ERBB2 levels quantified by CLISA ranged from 0 to 127 U/mg total protein, with a median of 1.096 U/mg (mean 7.49 U/mg). After log-transformation, values became almost normally distributed (Fig. 2). Correlation between p-ERBB2 and expression levels of ERBB2 was 0.62 for mRNA and 0.53 for protein (Fig. 3 and Table 3). p-ERBB2 levels were significantly higher

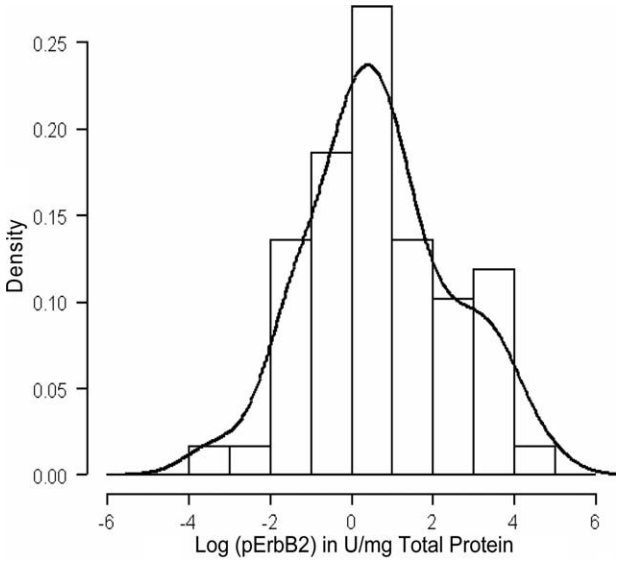

Fig. 2 – Distribution of log (p-ERBB2) levels in U/mg total protein; zero values were omitted.

in ERBB2-positive tumours than in ERBB2-negative tumours (Fig. 3). When taking the median value of p-ERBB2 as a cut-off for p-ERBB2 status, 8 out of 30 (27%) ERBB2-negative and 27 out of 40 (68%) ERBB2-positive tumours were p-ERBB2-positive ($P = 0.001$). Similar results were obtained when p-ERBB2 levels were compared with ERBB2 mRNA expression levels (Fig. 4). Of note, ERBB2-negative tumours included in the present study represent the upper third of all ERBB2-negative tumours (Fig. 5). No significant association was found in p-ERBB2 levels with respect to nodal status, either when analysing it as a continuous variable or after dichotomisation (Fig. 3).

3.2. *p-ERBB2 and expression of ER/PgR*

Quantitative p-ERBB2 expression levels correlated inversely with ER ($r_s = -0.54$) and PgR ($r_s = -0.46$) mRNA as well as protein expression level ($r_s = -0.67$ and $r_s = -0.45$, Table 3), and there was a significant difference in ER and PgR protein levels in p-ERBB2-negative as compared with p-ERBB2-positive tumours: median ER levels were almost 6-fold ($P < 0.001$) and PgR near 7-fold ($P = 0.005$) lower in p-ERBB2-positive tumours (Fig. 6). With respect to the hormone receptor status, 30 out of 35 (86%) p-ERBB2-negative tumours were ER-positive

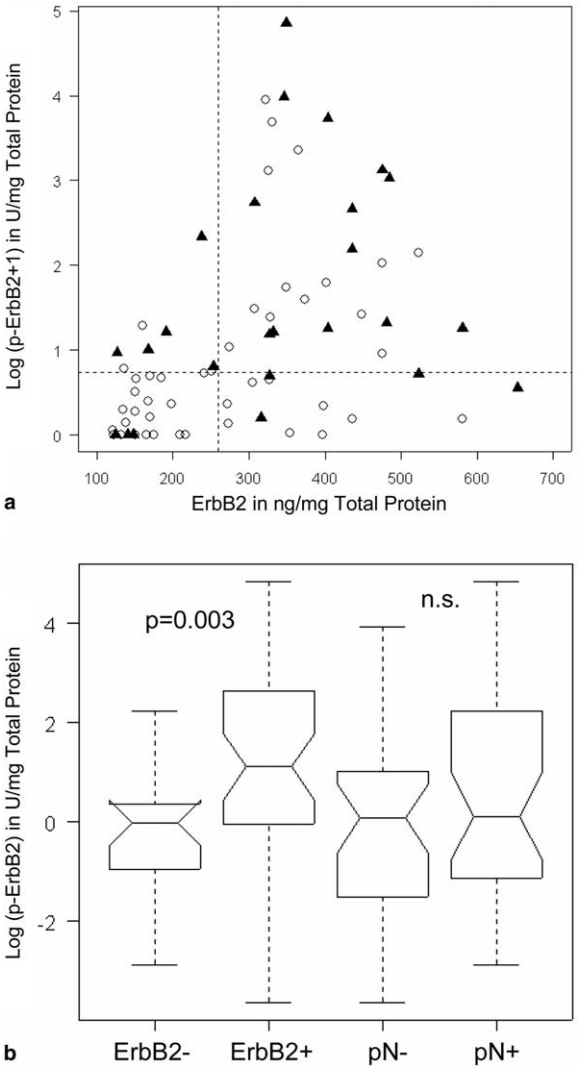

Fig. 3 – Scatter plot of p-ERBB2 (CLISA) versus ERBB2 (ELISA) protein expression levels. p-ERBB2 expression levels were log-transformed and one added as constant in order log-transformed values of zero correspond to zero values in the raw data. Tumours with ERBB2 >260 ng/mg and p-ERBB2 > median are considered positive (dashed lines): (a) circles and triangles indicate patients without and with relapse, respectively; (b) significant differences of p-ERBB2 levels in ERBB2-negative and ERBB2-positive tumours but no difference with respect to nodal status.

Table 3 – Correlation between p-ERBB2 levels and other quantitatively assessed markers (Spearman correlation coefficient r_s)

Correlation with	mRNA expression	Protein expression
ER	−0.54 ($P < 0.001$)	−0.67 ($P < 0.001$)
PgR	−0.46 ($P < 0.001$)	−0.45 ($P < 0.001$)
EGFR	0.26 ($P = 0.049$)	0.43 ($P = 0.005$)
ERBB2	0.62 ($P < 0.001$)	0.53 ($P < 0.001$)
ErBB3	−0.22 ($P = 0.080$)	n.d.
ErBB4	−0.47 ($P < 0.001$)	n.d.

n.d., not determined; ER, oestrogen receptor; PgR, progesterone receptor; EGFR, epidermal growth factor receptor.

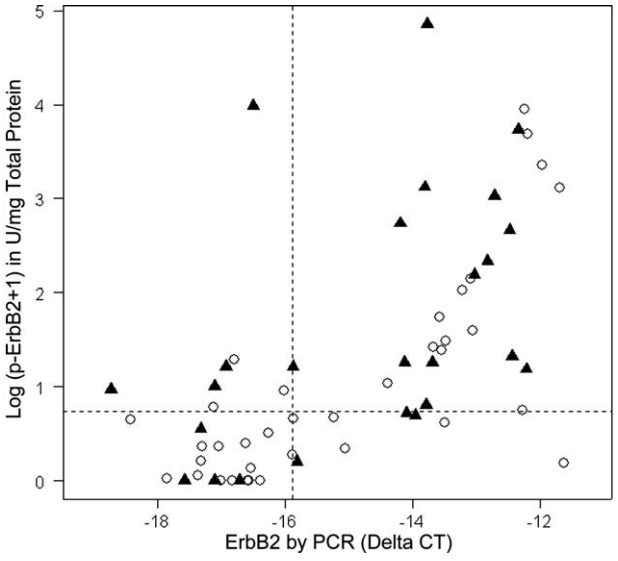

Fig. 4 – Scatter plot of p-ERBB2 (CLISA) versus ERBB2 expression levels measured by polymerase chain reaction (PCR) (Delta CT). p-ERBB2 expression levels were log-transformed and one added as constant so that log-transformed values of zero correspond to zero values in the raw data. Dashed lines indicate the cut-off value for ERBB2 status by PCR (M. Labuhn et al., submitted) and median expression value of p-ERBB2, respectively. Circles and triangles indicate patients without and with relapse, respectively.

(>20 fmol/mg) and 24 (69%) PgR-positive (>20 fmol/mg). In contrast, only 20 out of 35 (57%) p-ERBB2-positive tumours were ER-positive and 22 (43%) PgR-positive ($P = 0.016$), respectively.

3.3. *Correlation of p-ERBB2 with mRNA expression of EGFR-family members, and EGFR and ERBB2 protein expression*

p-ERBB2 levels were correlated with quantitative mRNA expression levels of all four EGFR-family members (Table 3). p-ERBB2 was positively correlated with EGFR ($r_s = 0.26$) and ERBB2 ($r_s = 0.62$) and inversely correlated with ERBB-4 ($r_s = -0.47$). A negative correlation between p-ERBB2 and ERBB3 mRNA was not statistically significant. Similar results were obtained for protein expression level of EGFR ($r_s = 0.43$) and ERBB2 ($r_s = 0.53$, Table 3). Distributions and statistical differences of the median mRNA expression levels for all EGRF-family members in p-ERBB2-negative versus p-ERBB2-positive tumours are summarised in Fig. 6. ERBB2 expression was significantly higher whereas ERBB4 expression was significantly lower in p-ERBB2-positive patients. Despite their correlation with p-ERBB2, EGFR and ERBB3 were not significantly differentially expressed between p-ERBB2-positive and -negative samples.

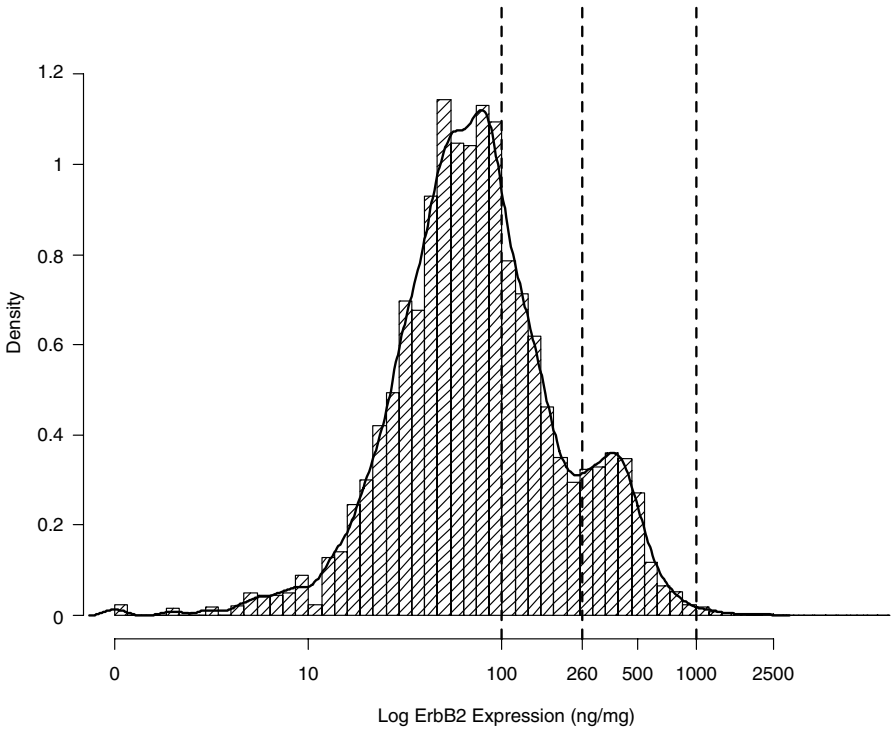

Fig. 5 – Distribution of ERBB2 protein expression levels (enzyme-linked immunosorbent assay (ELISA)) in over 3200 primary breast cancer patients.

3.4. *Prognostic significance of p-ERBB2*

p-ERBB2 was first tested in univariate Cox regression analysis, revealing significant correlation with patient disease-free survival (DFS) and overall survival (OS, Table 4). Notably, p-ERBB2 retained significant prognostic value in univariate Cox regression analysis in both ERBB2-negative and ERBB2-positive groups of patients. Subsequently, Kaplan–Meier survival analysis was performed for p-ERBB2-negative versus p-ERBB2-positive tumours (Fig. 7). Eighteen out of 35 (51%) patients with p-ERBB2-positive tumours developed disease recurrence, whereas this was the case for only 7 out of 35 (20%) in the p-ERBB2-negative group ($P = 0.004$). Five-year DFS was 45% (CI 31–67%) in the p-ERBB2-positive group and 82% (CI 70–96%) in the p-ERBB2-negative group. In OS analysis 9 out of 35 (26%) patients with p-ERBB2-positive tumours died compared with 4 out of 35 (11%) in p-ERBB2-negative tumours ($P = 0.079$, Fig. 7). Differences in survival remained significant when stratified according to nodal status. However, the results were more significant in nodal-positive than nodal-negative patients (Fig. 7). Moreover, p-ERBB2 status was significantly associated with DFS in ERBB2-negative tumours (Fig. 7). Five out of 8 p-ERBB2-positive/ERBB2-negative tumours relapsed. Finally, p-ERBB2 was an independent and significant prognostic factor in multivariate Cox regression analysis, which included ER, EGFR, ERBB2, tumour size (pT), lymph node status (pN) and age. The results are summarised in Table 4.

4. Discussion

This is the first study to measure quantitative levels of activated pY1248-ERBB2 applying a newly developed immunoassay, and investigating the relationship with mRNA and protein expression levels of the EGFR-family, established prognostic markers and survival. Compared with IHC, p-ERBB2 expression levels assessed by CLISA yield quantitative, highly sensitive and reproducible results. In addition, CLISA results obtained from fresh frozen tissue extracts avoid potential antigen damage due to formalin fixation, paraffin embedding and sample storage. However, phosphorylation reflects a dynamic process and potential alterations in phosphorylation levels require careful sample handling and the use of phosphatase inhibitors. Further, a two-site (sandwich) CLISA assay as used in this study ensures increased specificity compared with single-antibody assays such as IHC and Western blotting.

Several studies have investigated the role of phosphorylated ERBB2 in breast tumour samples using IHC.[13,15,17] We could reconfirm the association with ERBB2 status, poor prognosis and the inverse correlation with hormone receptor expression. Although there is good correlation between ERBB2 and its phosphorylation levels, we identified cases with high p-ERBB2 levels in ERBB2-negative tumours. This is in contrast to previously published data by Thor and colleagues,[13] which could not detect p-ERBB2 in ERBB2-negative tumours using IHC. A possible reason for these findings might be differences in assay sensitivity, this being higher in CLISA. We observed 8 (27%) of ERBB2-negative tumours to express p-ERBB2 values above the median, a cut-off that also revealed significant prognostic value in survival analysis. These were unlikely p-ERBB2-positive samples classified falsely as ERBB2-negative using protein-based ERBB2 status since the same number of p-ERBB2-positive cases was found using mRNA-based ERBB2 status determination (Fig. 4). We further report that p-ERBB2

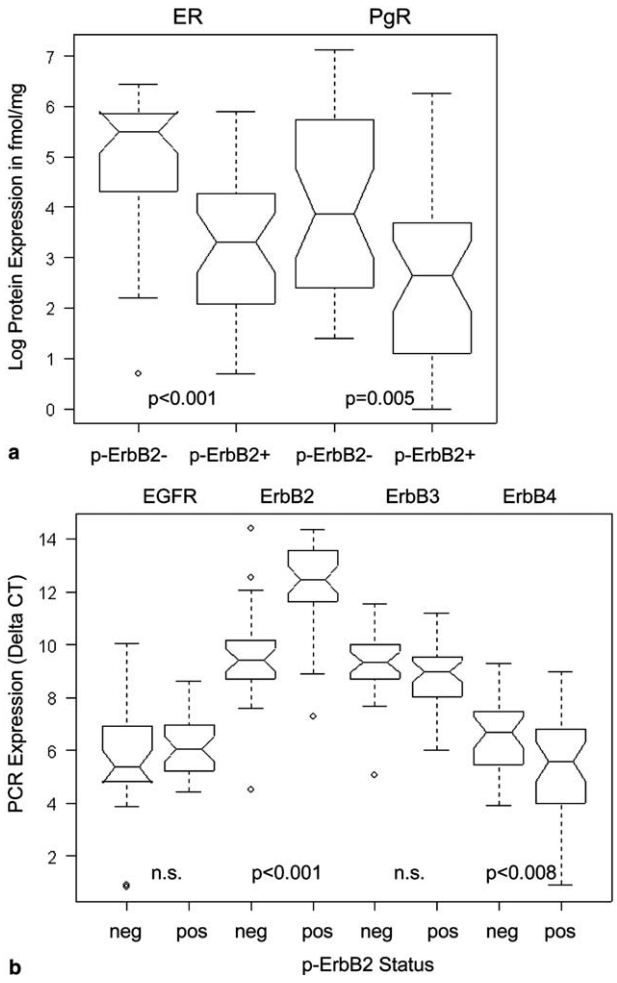

Fig. 6 – Box plot showing the differences in: (a) distribution of ER/PgR protein levels, and (b) mRNA expression levels of the epidermal growth factor receptor (EGFR)-family members in p-ERBB2-negative/-positive tumours.

was significantly associated with survival in both ERBB2-negative and ERBB2-positive patients. Finally, multivariate analysis including ERBB2, EGFR, ER, nodal status, age and tumour size demonstrated independent prognostic value for p-ERBB2, suggesting that p-ERBB2 is providing additional information despite being associated with ERBB2 – a finding which was also recognised by Thor and colleagues.[13] Besides p-ERBB2, nodal status (pN) and tumour size (pT) were independent prognostic markers in multivariate analysis. Grade was not included because of too many missing values.

We used the median value of p-ERBB2 to define p-ERBB2-negative/-positive tumours. Alternatively, we tested an optimised cut-off with respect to prognosis searched for by CART analysis, which classified 33 tumours in the high-risk group instead of 35 when using the median. This optimised cut-off, however, did not change the overall results. It is noteworthy that we selected the samples a priori according to ERBB2 protein expression levels and explicitly enriched the population with ERBB2-positive samples. The high correlation between ERBB2 and p-ERBB2 and the fact that there are approximately as many ERBB2-positive as ERBB2-negative patients in the population studied might explain why using the median as cut-off is reasonable in this setting. However, the number of p-ERBB2-positive tumours is expected to be significantly lower in a randomised situation. Moreover, our ERBB2-negative population had ERBB2 protein expression levels ranging from 100 to 260 ng/mg total protein. This correspond to the upper third of ERBB2 expression levels among ERBB2-negative patients when compared with the distribution of ERBB2 protein expression levels observed in a large study population (Fig. 5[8]). Thus, the percentage of p-ERBB2-positive tumours among ERBB2-negative patients is probably overestimated.

Because EGFR-family receptors have to homo- and/or hetero-dimerise to become activated, we sought to identify potential candidates for the preferred partner for ERBB2 in this process. We report correlation between p-ERBB2 and EGFR expression at both mRNA and protein level, suggesting a potential role for EGFR in ERBB2 phosphorylation and signalling towards a more aggressive phenotype. Indeed, it was proposed that ERBB2 in a heterodimer with EGFR is involved in signalling pathways required for a human breast cancer cell to become metastatic.[18,19] Despite this correlation with EGFR there was no significant difference in EGFR expression levels between p-ERBB2-negative and p-ERBB2-positive tumours. However, we observed that p-ERBB2 expression was significantly higher in tumours having high EGFR protein levels compared with tumours with low EGFR protein levels (Figs. 6 and 8). Conversely, ERBB3, ERBB4 and ER correlated negatively with p-ERBB2 and were expressed at significantly lower

Table 4 – Uni- and multi-variate Cox regression analysis against patient disease-free survival (DFS)

Factor	Univariate		Multivariate	
	P-value	HR (95% CI)	P-value	HR (95% CI)
p-ERBB2	0.004	3.4 (1.4–8.1)	0.010	4.1 (1.4–11)
ERBB2	0.041	1.9 (1.0–3.4)	n.s.	–
EGFR	n.s.	–	n.s.	–
ER	0.012	0.7 (0.6–0.9)	n.s.	–
pT	<0.001	1.9 (1.3–2.7)	0.008	2.1 (1.2–3.7)
pN	0.011	2.0 (1.2–3.3)	0.049	1.9 (1.0–3.7)
Age	n.s.	–	n.s.	–

n.s., not significant; ER, oestrogen receptor; EGFR, epidermal growth factor receptor; HR, hazard ratio; CI, confidence interval; pT, tumour size; pN, lymph node status.

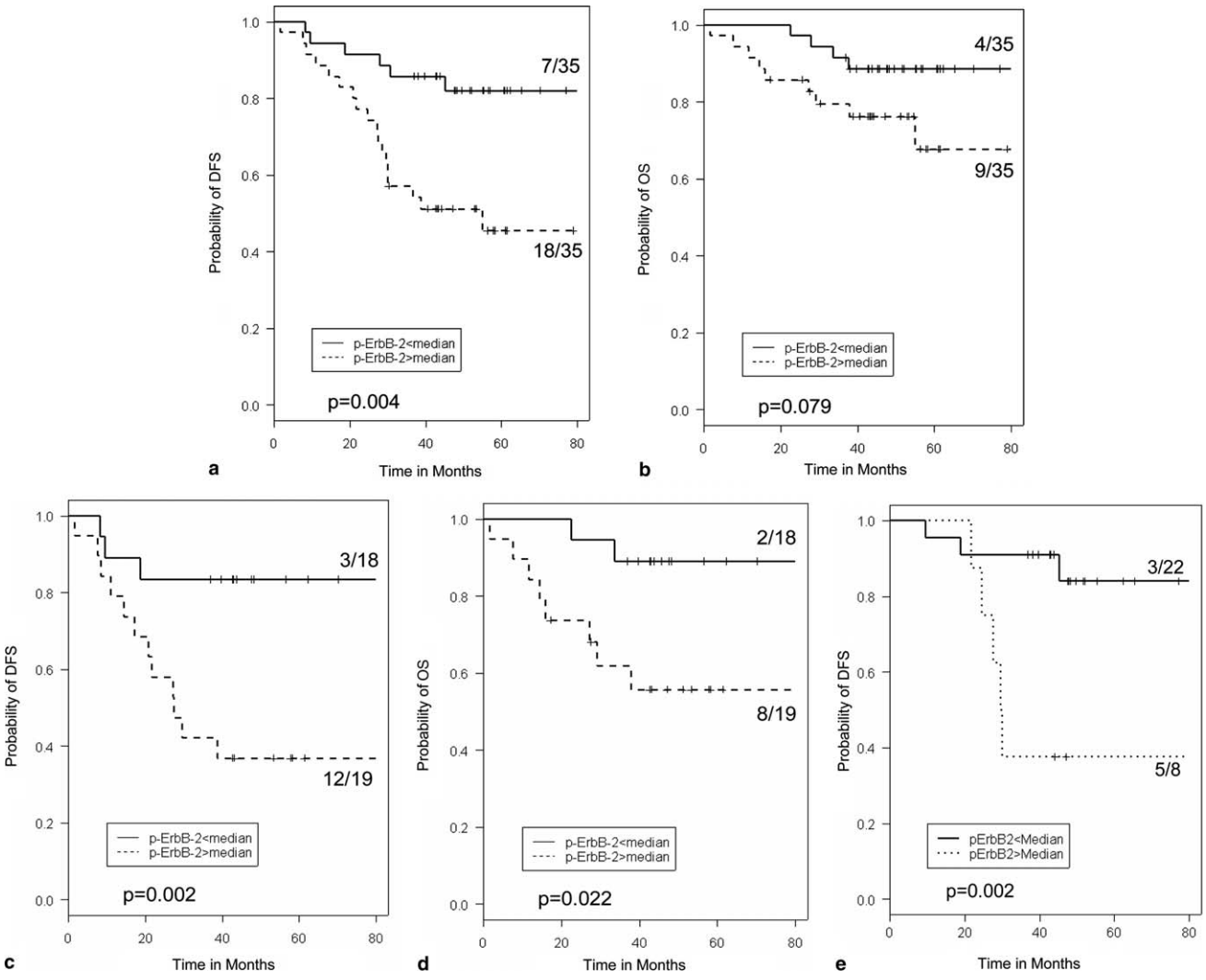

Fig. 7 – Kaplan–Meier survival curves for p-ERBB2. Patients were dichotomised according to the median expression value of p-ERBB2. Disease-free survival (DFS) and overall survival (OS) in (a, b) all patients, in (c, d) nodal-positive patients and (e) DFS in ERBB2-negative patients.

levels in tumours having high levels of p-ERBB2 (Fig. 6). Analogous findings were made in the subset of ERBB2-negative patients (Fig. 9). With respect to ERBB4 mRNA expression, it was shown to be associated with good prognosis in a number of studies,[20–22] whereas there is conflicting data about the role of ERBB3. ERBB3 together with ERBB2 promotes tumour cell proliferation in vitro,[23] but ERBB3 mRNA expression was also shown to correlate with good prognosis.[20,21] The latter findings are in accordance with our results, where ERBB3 and ERBB4 are good prognostic factors (data not shown).

In conclusion, we show that p-ERBB2, measured quantitatively by CLISA, is a marker of poor prognosis independent of ERBB2, ER/PgR, tumour size, nodal status and age. In addition, p-ERBB2 correlated with expression of EGFR and ERBB2, and inversely with ER, PgR, ERBB3 and ERBB4. Hormone receptors and ERBB4 were significantly lower in tumours expressing high p-ERBB2. These findings might be of interest with respect to the selection of appropriate treatment strategies. For trastuzumab, it was demonstrated that tumours expressing p-ERBB2 (IHC) have significantly longer survival when compared with tumours lacking p-ERBB2.[24] The positive correlation between p-ERBB2 and EGFR might suggest that p-ERBB2 may not only be a predictor of trastuzumab response but also predictive of novel EGFR-family targeted treatments such as ERBB2-dimerisation inhibitors and dual-specific tyrosine-kinase inhibitors.[25,26] The significantly lower levels of hormone receptors in p-ERBB2-positive patients might further suggest relative resistance to anti-ER targeted treatment.[9] However, since this study addressed mainly the prognostic value of p-ERBB2 and its relationship with other EGFR-family members, future research should further investigate the predictive value of p-ERBB2.

Conflict of interest statement

The authors declare no financial interest. Quantitative real-time PCR was performed at OncoScore AG.

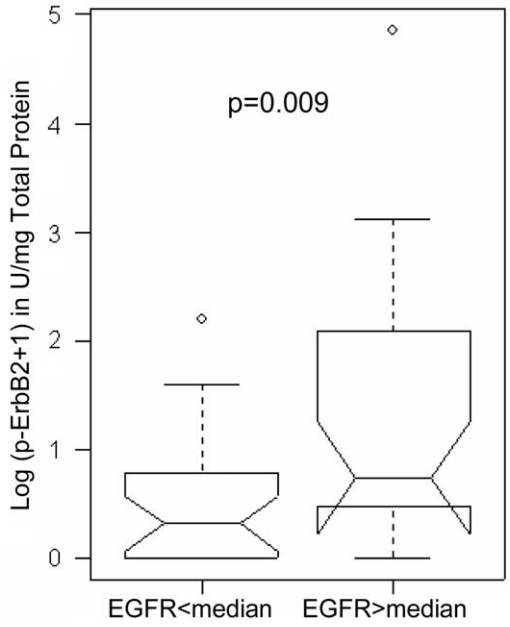

Fig. 8 – Distribution of p-ERBB2 expression levels in tumour expression epidermal growth factor receptor (EGFR) protein levels below and above the median value, respectively. p-ERBB2 expression levels were log-transformed and one added as constant so that log-transformed values of zero correspond to zero values in the raw data.

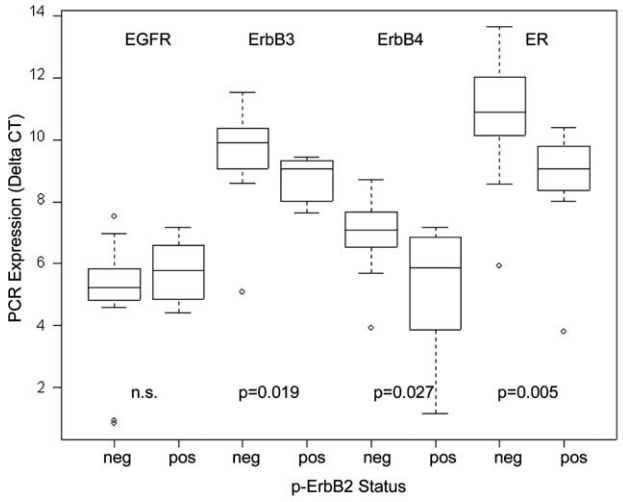

Fig. 9 – Distribution of epidermal growth factor receptor (EGFR)-family members and oestrogen receptor (ER) expression in ERBB2-negative patients with respect to their p-ERBB2 status.

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REFERENCES

1. Schechter AL, Hung MC, Vaidyanathan L, et al. The neu gene: an erbB-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. *Science* 1985;**229**:976–8.
2. Bargmann CI, Hung MC, Weinberg RA. Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. *Cell* 1986;**45**: 649–57.
3. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;**2**:127–37.
4. Tzahar E, Waterman H, Chen X, et al. A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor. *Mol Cell Biol* 1996;**16**:5276–87.
5. Schlessinger J. Cell signaling by receptor tyrosine kinases. *Cell* 2000;**103**:211–25.
6. Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. *Cell* 1990;**61**:203–12.
7. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987;**235**:177–82.
8. Eppenberger-Castori S, Kueng W, Benz C, et al. Prognostic and predictive significance of ErbB-2 breast tumor levels measured by enzyme immunoassay. *J Clin Oncol* 2001;**19**:645–56.
9. Konecny G, Pauletti G, Pegram M, et al. Quantitative association between HER-2/neu and steroid hormone receptors in hormone receptor-positive primary breast cancer. *J Natl Cancer Inst* 2003;**95**:142–53.
10. Leyland-Jones B. Trastuzumab: hopes and realities. *Lancet Oncol* 2002;**3**:137–44.
11. Osborne CK, Bardou V, Hopp TA, et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst* 2003;**95**:353–61.
12. De Laurentiis M, Arpino G, Massarelli E, et al. A meta-analysis on the interaction between HER-2 expression and response to endocrine treatment in advanced breast cancer. *Clin Cancer Res* 2005;**11**:4741–8.
13. Thor AD, Liu S, Edgerton S, et al. Activation (tyrosine phosphorylation) of ErbB-2 (HER-2/neu): a study of incidence and correlation with outcome in breast cancer. *J Clin Oncol* 2000;**18**:3230–9.
14. Costa S, Stamm H, Almendral A, et al. Predictive value of EGF receptor in breast cancer. *Lancet* 1988;**2**:1258.
15. DiGiovanna MP, Stern DF. Activation state-specific monoclonal antibody detects tyrosine phosphorylated p185neu/erbB-2 in a subset of human breast tumors overexpressing this receptor. *Cancer Res* 1995;**55**:1946–55.
16. Breimann L, Friedman JH, Olsen RA. *Classification and regression trees*. Monterey, CA: Wadsworth and Brooks Publishing; 1984.
17. DiGiovanna MP, Chu P, Davison TL, et al. Active signaling by HER-2/neu in a subpopulation of HER-2/neu-overexpressing ductal carcinoma in situ: clinicopathological correlates. *Cancer Res* 2002;**62**:6667–73.
18. Brandt BH, Roetger A, Dittmar T, et al. c-erbB-2/EGFR as dominant heterodimerization partners determine a motogenic phenotype in human breast cancer cells. *FASEB J* 1999;**13**:1939–49.

19. Dittmar T, Husemann A, Schewe Y, et al. Induction of cancer cell migration by epidermal growth factor is initiated by specific phosphorylation of tyrosine 1248 of c-erbB-2 receptor via EGFR. *FASEB J* 2002;**16**:1823–5.
20. Knowlden JM, Gee JM, Seery LT, et al. c-erbB3 and c-erbB4 expression is a feature of the endocrine responsive phenotype in clinical breast cancer. *Oncogene* 1998;**17**:1949–57.
21. Pawlowski V, Revillion F, Hebbar M, et al. Prognostic value of the type I growth factor receptors in a large series of human primary breast cancers quantified with a real-time reverse transcription-polymerase chain reaction assay. *Clin Cancer Res* 2000;**6**:4217–25.
22. Suo Z, Risberg B, Kalsson MG, et al. EGFR family expression in breast carcinomas. c-erbB-2 and c-erbB-4 receptors have different effects on survival. *J Pathol* 2002;**196**:17–25.
23. Holbro T, Beerli RR, Maurer F, et al. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci USA* 2003;**100**:8933–8.
24. Hudelist G, Kostler WJ, Attems J, et al. Her-2/neu-triggered intracellular tyrosine kinase activation: in vivo relevance of ligand-independent activation mechanisms and impact upon the efficacy of trastuzumab-based treatment. *Br J Cancer* 2003;**89**:983–91.
25. Wakeling AE. Inhibitors of growth factor signalling. *Endocr Relat Cancer* 2005;**12**(Suppl. 1):S183–7.
26. Agus DB, Gordon MS, Taylor C, et al. Phase I clinical study of pertuzumab, a novel HER dimerization inhibitor, in patients with advanced cancer. *J Clin Oncol* 2005;**23**: 2534–43.