Research article

Cytogenetic analysis of HER1/EGFR, HER2, HER3 and HER4 in 278 breast cancer patients

Andrea Sassen1, Justine Rochon2, Peter Wild3, Arndt Hartmann4, Ferdinand Hofstaedter1, Stephan Schwarz4* and Gero Brockhoff1*

1Institute of Pathology, University of Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany
2Center for Clinical Studies, University of Regensburg, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany
3Institute of Pathology, University Hospital Zurich, Schmelzbergstrasse 12, 8091 Zurich, Switzerland
4Institute of Pathology, University of Erlangen, Krankenhausstrasse 12, 91054 Erlangen, Germany

* Contributed equally

Corresponding author: Gero Brockhoff, gero.brockhoff@klinik.uni-regensburg.de

Received: 27 Jul 2007 Revisions requested: 9 Sep 2007 Revisions received: 25 Oct 2007 Accepted: 8 Jan 2008 Published: 8 Jan 2008

Breast Cancer Research 2008, 10:R2 (doi:10.1186/bcr1843)

Abstract

Introduction The HER (human EGFR related) family of receptor tyrosine kinases (HER1/EGFR (epidermal growth factor receptor)/c-erbB1, HER2/c-erbB2, HER3/c-erbB3 and HER4/c-erbB4) shares a high degree of structural and functional homology. It constitutes a complex network, coupling various extracellular ligands to intracellular signal transduction pathways resulting in receptor interaction and cross-activation. The most famous family member is HER2, which is a target in Herceptin™ therapy in metastatic status and also in adjuvant therapy of breast cancer in the event of dysregulation as a result of gene amplification and resulting protein overexpression. The HER2-related HER receptors have been shown to interact directly with HER2 receptors and thereby mutually affect their activity and subsequent malignant growth potential. However, the clinical outcome with regard to total HER receptor state remains largely unknown.

Methods We investigated HER1–HER4, at both the DNA and the protein level, using fluorescence in situ hybridisation (FISH) probes targeted to all four receptor loci and also immunohistochemistry in tissue microarrays derived from 278 breast cancer patients.

Results We retrospectively found HER3 gene amplification with a univariate negative impact on disease-free survival (hazard ratio 2.35, 95% confidence interval 1.08 to 5.11, \(p = 0.031\)), whereas HER4 amplification showed a positive trend in overall and disease-free survival. Protein expression revealed no additional information.

Conclusion Overall, the simultaneous quantification of HER3 and HER4 receptor genes by means of FISH might enable the rendering of a more precise stratification of breast cancer patients by providing additional prognostic information. The continuation of explorative and prospective studies on all HER receptors will be required for an evaluation of their potential use for specific therapeutic targeting with respect to individualised therapy.

Introduction Gene amplification of HER2 (HER2/neu, c-erbB2) receptor tyrosine kinase (RTK) is found in 10 to 25% of invasive breast carcinomas [1,2] and is associated with an unfavourable impact on the course of disease and reduced responsiveness to tamoxifen therapy, for example [3,4]. The HER2 receptor has frequently been described as dominantly triggering mitogenic signalling within the type 1 growth factor receptor family. As a ligandless orphan receptor, HER2 preferentially heterodimerises with its relatives [5,6] and thereby has an important role in signal triggering and amplification. Its malignant potential and its key role in enhanced cell proliferation, carcinogenesis, tumour progression and metastasis have

B = regression coefficient; CC = correlation coefficient; CI = confidence interval; DAPI = 4',6-diamidino-2-phenylindole; EGFR = epidermal growth factor receptor; FISH = fluorescence in situ hybridisation; HER = human EGFR related; HR = hazard ratio; IHC = immunohistochemistry; RTK = receptor tyrosine kinase; SSC = standard saline citrate; TMA = tissue microarray.
frequently been proved in numerous preclinical and clinical studies [7].

The overexpressed receptor protein is exploited as the therapeutic target for Herceptin™, known as the humanised monoclonal antibody trastuzumab, in metastatic breast cancer and has recently proved useful in designing adjuvant treatment for breast carcinoma [8]. Moreover, strong HER2 expression represents the decisive molecular basis for tumour therapy targeted at the same receptor. However, a therapeutic benefit in terms of tumour regression, prolongation of recurrence-free survival and even overall survival [9] is found for about 50% of patients [9-11] depending on previous therapies, antibody resistance and combination with other chemotherapeutics such as paclitaxel or docetaxel [12]. This observation reflects the substantial insufficiency of using HER2 gene amplification or HER2 protein overexpression to predict patient responsiveness to Herceptin.

Hence, the identification of clinicopathological and molecular characteristics of breast cancer to enable more accurate prognosis of the course of disease and prediction of therapy response to antibodies or small enzyme-inhibiting molecules [13-15], for example, is a continuing challenge in the field of diagnostic pathology. To this end, the three additional members of the HER (human EGFR related)–RTK family HER1 (epidermal growth factor receptor (EGFR), c-erbB1), HER3 (c-erbB3) and HER4 (c-erbB4) are of particular interest because of their ability to interact directly with HER2 [16]. On the basis of their common evolutionary origin these receptors share a high degree of structural and functional homology, which is the molecular basis for receptor interaction and cross-activation [17]. Thus, HER-receptor activity and functionality depend on one another and thus the impact on tumour cell proliferation and growth is likely to be dependent on HER-receptor coexpression and communication.

Several immunohistochemical studies have been undertaken to elucidate the coexpression profile of HER receptors in breast cancer, providing preliminary data on other HER receptors besides HER2, which may have an impact on the course of disease and therapy responsiveness in breast cancer patients [18-20].

In this study we performed a four-target fluorescence in situ hybridisation (FISH) analysis of the HER1, HER2, HER3 and HER4 gene loci together with centromere quantification using 278 primary breast cancer samples compiled into a tissue microarray (TMA). Additionally, we immunohistochemically stained the receptor proteins and categorised staining intensity in accordance with EGFR pharmDX™ and HercepTest™ scoring guidelines. Furthermore, the results were compared with the Ki-67 proliferation index, a prognostic marker in early breast cancer [21]. Our objective was to determine the potential association between HER1-HER4 gene amplification or altered protein expression and outcome and course of disease, as well as with known clinicopathological breast cancer prognosticators [22]. We addressed the question of whether alteration in the HER1, HER3 or HER4 genes or their protein products conveys any prognostic value that is complementary to or independent of HER2 that would allow a more precise rendering of breast cancer patients into subgroups with different clinical outcomes based on HER-receptor analysis.

Our data indicate additional HER3 and HER4 prognostic markers in breast cancer that should be prospectively explored in further detail. The integration of HER3 and HER4 analysis into routine cancer diagnosis would provide valuable additional information. Further descriptive and particularly functional studies are required to understand their impact on the course of disease at the molecular and cellular levels [16,23] and will provide the basis for designing specific targeted therapeutics in terms of individualised disease management.

Materials and methods
This study was approved by the Institutional Review Board of the University of Regensburg, Germany.

Breast tumour samples and patient characteristics
Formalin-fixed paraffin-embedded tissue blocks from 278 female patients with invasive lobular or ductal unilateral primary breast cancer (median age 55 years; range 25 to 82 years) were obtained from the archives of the Institute of Pathology, Regensburg, Germany, and were derived from a consecutive series of sporadic breast cancers. The patients were not involved in any clinical trial. Clinical data were acquired by the Tumour Centre Inc., Regensburg. All patients underwent surgery between 1992 and 2002. The histopathological characteristics are listed in Table 1. The median follow-up period was 125.6 months (95% confidence interval (CI) 120.3 to 131.0). A total of 106 (38.1%) patients died, and 136 (48.9%) had a recurrence of breast cancer.

Tissue microarray (TMA) construction
TMAs were prepared as described previously [24,25]. For each tumour a representative tumour section was selected from a haematoxylin/eosin-stained section of the donor block. Core cylinders with a diameter of 1.5 mm each were punched from this area with a thin-walled stainless steel tube and deposited into a recipient paraffin block. TMA sections were mounted on charged slides (SuperFrost™Plus; Menzel GmbH, Braunschweig, Germany). Haematoxylin/eosin-stained TMA sections were used for reference histology.

Fluorescence in situ hybridisation
FISH was performed on 5 μm sections of the TMAs with the use of directly labelled DNA probes for HER1, HER2, HER3 and HER4 (ZytoVision Ltd., Bremerhaven, Germany). The probes identified locus-specific sequences for both the genes.
and the corresponding centromeres 7, 17, 12 and 2 to differentiate between gene amplification and polysomy of the respective chromosome.

TMA sections were dewaxed for 40 minutes in an incubator at 72°C and twice for 10 minutes in xylene. After being rehydrated in a graded ethanol series and rinsed in distilled water, slides were placed in 0.01 M sodium citrate and steamed for 40 minutes in a water bath. Cell structures were digested in 0.1% pepsin (Sigma, Munich, Germany) and 0.01 M HCl for 10 minutes at 37°C. After washing in 2 × SSC (1 × SSC is 150 mM sodium chloride and 15 mM sodium citrate, pH 7) and water, slides were dehydrated in graded alcohols and air-dried. Respective DNA probe sets (10 μl each) were applied to the TMA area of each section. Sections were coverslipped and the edges were sealed with rubber cement. For co-denaturation of the probe and target DNA, slides were placed for 5 minutes on a hotplate preheated to 73°C and then transferred overnight to a warmed hybridisation chamber at 37°C. After hybridisation, the rubber cement was removed and the slides were immersed successively in 4 × SSC plus 0.3% Igepal (Serva, Heidelberg, Germany), 2 × SSC and 1 × SSC for 10 minutes at 50°C. The slides were rinsed briefly in Millipore water and air-dried. Nuclei were counterstained with anti-fading DAPI (4′,6-diamidino-2-phenylindole) Vectashield (Vector Laboratories, Burlingame, CA, USA) and were analysed by epifluorescence microscopy.

Microscopy, fluorescence in situ hybridisation scoring and digital imaging

Slides were imaged with an Axio Imager Z.1 (Zeiss, Göttingen, Germany) equipped with specific filter sets for DAPI fluorescence (excitation 365 ± 20 nm, emission 450 ± 25 nm; Zeiss), green fluorescence (excitation 500 ± 10 nm, emission 535 ± 15 nm) and red fluorescence (excitation 545 ± 15 nm, emission 610 ± 35 nm; AHF, Tübingen, Germany). Fluorescence images were obtained with a Plan-Apochromat lens (63×, numerical aperture 1.4) and recorded with a CCD (charge-coupled device) camera AxioCam MRm (Zeiss). The plug-in module ApoTome™ enabled the taking of pseudoconfocal, scattered out-of-focus light-free images using transmission grids and corresponding algorithms. To exclude the loss of FISH signals, three-dimensional z-stacks were generated. Each colour was recorded and digitally processed (filtering and contrast enhancement) using AxioVision 4.5 software (Zeiss). Corresponding images were superimposed.

FISH scoring was performed by counting fluorescence signals in 25 malignant, non-overlapping cell nuclei for each case by two independent interpreters (AS, MB). The FISH ratio was assessed as the number of genes proportional to the number of centromeres.

Immunohistochemistry

Immunostaining with anti-HER-receptor antibodies and MIB-1 (anti-Ki-67) was performed on 5 μm sections of the TMAs and applied in accordance with the manufacturer’s instructions. Table 2 shows the antibody-specific staining and scoring characteristics. MIB-1 was regarded as positive when 30% or more of the nuclei in the punched tissue were stained. Interpretation was performed independently by two experienced pathologists (SS, AH). Stably transfected mouse fibroblasts proved specific immunostaining (Figure 1).

Statistical analyses

The primary outcome measure, overall survival, was calculated as the time from the date of diagnosis to death from any cause or the date on which the patient was last known to be alive. Patients lost to follow-up were treated as censored cases on the basis of the date they were last known to be alive. The secondary outcome measure was the disease-free survival, the time from diagnosis to the date of tumour-related death. Two outcome-orientated approaches were used to determine the cut-off points for HER1–HER4 FISH with regard to overall survival. First, we examined plots of the martingale residuals against the single HER1–HER4 FISH variables using the PROC LOESS option in SAS and chose DIRECT SMOOTH with a smoothing parameter of 2/3. Second, we applied the Contal and O’Quigley method [26], which is based on the log

| Characteristic              | Node-positive, pN1–3 | Node-negative, pN0 | pNx |
|-----------------------------|----------------------|-------------------|-----|
| n = 131                     | n = 137              | n = 10            |
| pT                           |                      |                   |
| pT1                         | 32                   | 64                | 3   |
| pT2                         | 56                   | 66                | 4   |
| pT3                         | 14                   | 3                 | 1   |
| pT4                         | 29                   | 1                 | 2   |
| pTx                         | -                    | 3                 | -   |
| Histological grade          |                      |                   |
| G1                          | 14                   | 22                | 3   |
| G2                          | 52                   | 75                | 5   |
| G3                          | 69                   | 36                | 2   |
| Gx                          | -                    | 4                 | -   |
| ER status                   |                      |                   |
| 0                           | 26                   | 26                | 5   |
| 1–12                        | 79                   | 72                | 5   |
| ERx                         | 26                   | 39                | -   |

The median age of patients was 55 years (range 25 to 82 years).
rank statistic and provides p values corrected for examining multiple potential cut-off points. The cut-off points obtained were then used to divide patients into two groups: amplified and non-amplified. Survival curves were generated by using the Kaplan–Meier method, and log-rank tests compared the distributions between groups. In addition, hazard ratios (HR values) with 95% CIs were estimated for a single covariate (treated as continuous, and where appropriate as a dichotomous variable) using the Cox proportional-hazards model. Finally, multivariate Cox models were fitted to assess the prognostic significance of HER1, HER3 and HER4 irrespective of HER2. For cut-off point determination an adjusted 10% level of significance was used. In all other analyses, p ≤ 0.05 (two-

Table 2

| Characteristic                | HER1                        | HER2                        | HER3                        | HER4                        | Ki-67                        |
|-------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-------------------------------|
| Antibody                      | Mouse mAb                   | Rabbit pAb                  | Mouse mAb                   | Rabbit mAb                  | Mouse mAb                    |
| Origin                        | Dako                        | Dako                        | NanoTools                   | Cell Signaling              | Dako                          |
| Clone                         | 2–18C9                      | -                           | 5A12                        | 83B10                       | MIB-1                         |
| Concentration of primary antibody | EGFR pharmDX               | HercepTest (2.5 μg/ml)      | 5 μg/ml culture supernatant | 0.7 μg/ml                   | 1.6 μg/ml                     |
| Staining pattern              | Membrane                    | Membrane                    | Cytoplasm and membrane      | Cytoplasm and membrane      | Nucleus                       |
| Epitope retrieval             | Proteinas K                 | Heat induced, 10 mM citric acid buffer, pH 6.0 | Heat induced, 10 mM citric acid buffer, pH 7.3 | Heat induced, 10 mM sodium-citric acid pH 6.0 | Heat induced, 10 mM citric acid buffer, pH 7.2 |
| Blocking                      | Endogenous peroxidase blocking | Endogenous peroxidase blocking | Endogenous peroxidase blocking | Endogenous peroxidase blocking | Endogenous peroxidase blocking |
| Primary antibody              | Overnight, 4°C              | Overnight, 4°C              | Overnight, 4°C              | Overnight, 4°C              | 30 min, room temperature      |
| Detection system              | EnVision™ Dual Link System (Dako); DAB + chromogenic substrate | EnVision™ Dual Link System (Dako); DAB + chromogenic substrate | EnVision™ Dual Link System (Dako); DAB + chromogenic substrate | EnVision™ Dual Link System (Dako); DAB + chromogenic substrate | /VIEW™ DAB Detection Kit (Ventana) |
| Scoring in accordance with    | EGFR pharmDx guidelines     | HercepTest guidelines       | EGFR pharmDx guidelines     | EGFR pharmDx guidelines     | Manufacturer’s guidelines     |

mAb, monoclonal antibody; pAb, polyclonal antibody; DAB, diaminobenzidine; EGFR, epidermal growth factor receptor.
Results
Microarrays of paraffin-embedded breast cancer tissue from 278 patients were used to analyse gene amplification and protein expression of each member of the HER family. Furthermore, we analysed tumour and nodal status and tumour grading (Table 3) to indicate the representativeness of our patient collective (p < 0.001).

Table 3

Cox proportional hazards analysis for overall and disease-free survival

| Technique | Parameter | Overall survival | Disease-free survival |
|-----------|-----------|------------------|----------------------|
|           |           | HR   | 95% CI           | p        | HR   | 95% CI           | p        |
| FISH      | HER1      |      |                  |         |      |                  |         |
|           | Continuous 0.1 | 1.02 | 0.97–1.07        | 0.380   | 1.03 | 0.98–1.07        | 0.301   |
|           | Continuous 1.0 | 1.24 | 0.77–2.00        |         | 1.28 | 0.80–2.03        |         |
|           | HER2      |      |                  |         |      |                  |         |
|           | Continuous 0.1 | 1.02 | 1.01–1.02        | 0.001   | 1.02 | 1.01–1.03        | <0.001  |
|           | Continuous 1.0 | 1.16 | 1.06–1.26        |         | 1.18 | 1.08–1.29        |         |
|           | Dichotomous (≤1.5 vs. ≥1.6) | 2.07 | 1.33–3.21        | 0.001   | 2.33 | 1.48–3.68        | <0.001  |
|           | HER3      |      |                  |         |      |                  |         |
|           | Continuous 0.1 | 1.07 | 0.99–1.15        |         | 1.09 | 1.01–1.18        | 0.031   |
|           | Continuous 1.0 | 1.88 | 0.88–4.01        | 0.102   | 2.35 | 1.08–5.11        |         |
|           | HER4      |      |                  |         |      |                  |         |
|           | Continuous 0.1 | 0.90 | 0.75–1.09        | 0.289   | 0.92 | 0.76–1.12        | 0.395   |
|           | Continuous 1.0 | 0.36 | 0.06–2.36        |         | 0.43 | 0.06–3.03        |         |
| IHC       | HER1      |      |                  |         |      |                  |         |
|           | HER2      |      |                  |         |      |                  |         |
|           | HER3      |      |                  |         |      |                  |         |
|           | HER4      |      |                  |         |      |                  |         |
|           | HER2 divided |      |                  |         |      |                  |         |
|           | Ki-67     |      |                  |         |      |                  |         |
|           | ER        |      |                  |         |      |                  |         |
|           | PR        |      |                  |         |      |                  |         |
|           | Histology |      |                  |         |      |                  |         |
|           | pT        |      |                  |         |      |                  |         |
|           | pN        |      |                  |         |      |                  |         |
|           | Grading   |      |                  |         |      |                  |         |

Hazard ratios (HR), confidence intervals and p values of investigated parameters dependent on overall and disease-free survival (CI, confidence interval with lower and upper limits). All immunohistochemical and histological parameters were used as categorical variables. FISH, fluorescence in situ hybridisation; IHC, immunohistochemistry.
of 90.3% of values at a ratio of 1.0 and 1.1, an extremely high percentage compared with its receptor relatives (HER2, 59.3%; HER3, 28.1%; HER4, 77.7%).

With overall survival as the primary outcome measure, the plots of the martingale residuals versus HER1–HER4 FISH (Figure 4) implied the interpretation of HER1, HER3 and HER4 as continuous variables rather than dichotomising the

---

**Figure 2**

Anti-HER1–HER4 FISH in breast cancer tissue of one patient (dual probes). HER1: red cen 7, green loc 7p11 (diploid); HER2: red cen 17, green loc 17q12 (amplified); HER3: green cen 12, red loc 12q13 (moderately amplified); HER4: green cen 2, red loc 2q33 (diploid); 4',6-diamidino-2-phenylindole core staining blue. Cen, centromere; loc, gene locus.

---

**Figure 3**

Distribution of HER1–4 FISH ratios. (a) Boxplots of HER1–HER4 FISH ratios (gene/centromere). (b) Magnified extract of (a) to demonstrate the different distribution pattern of ratios for each HER-family member.
data on the basis of a cut-off point. In contrast, the smoothed curve for HER2 FISH was roughly zero up to about 1.5 and then increased rapidly. HER2 FISH was therefore converted into a categorical variable. The results from the Contal and O’Quigley method were consistent with the results from the graphical approach. For HER2 FISH there were 46 distinct values, any of which could be defined as a potential cut-off point. The most informative value of the log rank statistic occurred at the HER2 FISH ratio of 1.5 (adjusted $p = 0.086$). This suggests that the cut-off point obtained is related to overall survival. The patients were therefore divided into two groups: patients with HER2 FISH ratios of 1.5 or less, and patients with HER2 FISH ratios of at least 1.6. Using the Contal and O’Quigley method for HER1, HER3 and HER4 FISH, again no cut-off point related to overall survival could be defined (adjusted $p > 0.33$). Consequently, in further analyses HER1, HER3 and HER4 FISH were included as continuous variables, whereas HER2 FISH was assessed as both continuous and dichotomous variables.

The HER2 gene was amplified in most cases without evidence of polysomy, whereas both HER3 and HER4 gene alterations were usually found in combination with polysomic gene status. This finding partly explains lower FISH ratios for HER1 and, in particular, for HER3 and HER4 compared with HER2.

FISH-dichotomised HER2 results are presented in Table 4. HER2 showed 19.9% (46 of 231) positive cases and 80.1% (185 of 231) negative cases. HR values for continuous distribution of HER1–HER4 ratios for single (1.0) and one-tenth (0.1) units are given in Table 3. The HR displays either the increase (HR > 1.0) or the decrease (HR < 1.0) of the risk of mortality by an enlargement of HER1–HER4 FISH ratio. A HR value (single unit, 1.0) of 1.16 means that a patient with a HER2 FISH ratio of 3.0, for example, has a 1.16-fold higher risk (based on overall survival) than a patient with a ratio of 2.0. Of 46 amplified cases, 40 (87.0%) also overexpressed HER2 protein. A correlation analysis of HER2 FISH and immunohistochemistry (IHC; both dichotomous) was positive ($p < 0.001$;
correlation coefficient (CC) = 0.809). Reviewing the connection between HER3 FISH (continuous) and IHC (dichotomous), we found a significant weak correlation ($p = 0.038$, CC = 0.162) as well as for HER1 FISH and IHC ($p = 0.010$, CC = 0.200). HER4 FISH and IHC data showed no correlation ($p = 0.327$, CC = -0.075).

Patients with HER2 amplified breast cancer presented a significantly worse outcome for overall (HER2 dichotomised: HR = 2.07 (95% CI 1.33 to 3.21), $p = 0.001$; HER2 continuous: HR = 1.16 (95% CI 1.06 to 1.26), $p = 0.001$) and disease-free survival than patients with the non-amplified gene (Table 3 and Figure 5a2).

In addition, HER3-positive cases had a shorter disease-free survival (HR = 2.35 (95% CI 1.08 to 5.11), $p = 0.031$). Amplification of HER1 caused a negative trend for survival (HR = 1.24 (95% CI 0.77 to 2.00), $p = 0.380$), whereas HER4 resulted in a decrease in the HR (HR = 0.36 (95% CI 0.06 to 2.36), $p = 0.289$). For a comparison of overall and disease-free survival values see Table 3.

After performing univariate analysis based on overall survival, we calculated an individual HR for each patient ($HR_{ind} = \exp [B_{HER} \times HERx \text{ continuous}]$) and plotted against the respective receptor gene HER1 to HER4 (Figure 6). In this analysis, HER1, HER2 and HER3 displayed an increasing HR with raised FISH ratios (continuous), whereas the HER4 HR declined. HER3 had the steepest curve, followed by HER1 and HER2 as next steepest.

Exploring the effects of HER1, HER3 and HER4 in coexpression with HER2, HER2 results were separated into amplified and non-amplified cases (Figure 7). In a multivariate approach, dichotomised HER2 and continuous HER1, HER3 or HER4 were analysed. No significant interaction between HER2 and each of its three relatives was found. Thus, for each patient the individual HR without interaction was calculated ($HR_{ind} = \exp [B_{HER} \times HER2 \text{ dichotomised} + B_{HER} \times HERx \text{ continuous}]$). Amplified HER2 curves extend beyond non-amplified curves in every case, showing the greater impact of HER2 in multivariate Cox regression, visualised by adjusted HER2 HR values (Figure 7). HER1, HER3 and HER4 have additional relevance on the basis of increasing FISH ratio, given that the curves do not run parallel to the x-axis. Whereas the upper graph displays the impact of HER2 amplification dependent on increasing supplemental aberration of a second receptor, the lower curve demonstrates the exclusive impact of this receptor gene irrespective of HER2.

Figure 7 suggests that the main effects are additive in nature. Figure 7a,b shows the same monotonically increasing trend: higher HER1 and HER3 values were associated with higher risk of mortality. This was valid for patients both with and without HER2 gene amplification. In contrast, Figure 7c (HER4) showed a monotonically decreasing trend: higher HER4 values were associated with a lower risk of mortality applied to both the HER2 amplified and the HER2 non-amplified patient group.

Immunohistochemistry

Immunostaining of all four HER receptors was performed (Figure 1). The specificity of applied antibodies was proved by staining stably transfected mouse fibroblasts (NIH 3T3, kindly provided by Roche Diagnostics, Penzberg, Germany).

For IHC, 14.5% (26 of 178) were identified as HER1 positive (score 1+, 2+ and 3+), and 85.5% (152 of 178) as negative (Table 4). In 22.4% (48 of 214) HER2 was overexpressed (score 2+ and 3+), and 77.6% (166 of 214) were normal. IHC of HER3 resulted in 75.1% (130 of 173) positive (score 1+, 2+ and 3+) and 24.9% (43 of 173) negative patients. For HER4, 37.2% (71 of 191) of cases presented overexpression (score 1+, 2+ and 3+), whereas 62.8% (120 of 191) did not.

Table 4

| Evaluation characteristics | FISH  | Immunohistochemistry |
|---------------------------|-------|----------------------|
| HER2                      |       | HER1                 |
| $n = 231$                 |       | $n = 178$            |
| 0                         | n.r.  | 152 (85.4)          |
| 1+                        | n.r.  | 7 (3.9)             |
| 2+                        | n.r.  | 11 (6.2)            |
| 3+                        | n.r.  | 8 (4.5)             |
| Positive                  | 46 (19.9) | 26 (14.5)    |
| Negative                  | 185 (80.1) | 152 (85.5)   |

Numbers in parentheses are percentages. The total sample size was $n = 278$. Results in bold define data considered positive for immunohistochemistry. FISH, fluorescence in situ hybridisation; n.r. = non-relevant for FISH; cut-off point for HER2 FISH analysis ($\leq 1.5$ vs. $\geq 1.6$) distinguishes amplified (positive) or normal (negative) gene status.
Overexpressed HER2 receptors (Figure 5a1) were associated with decreased overall survival \( (p = 0.129; HR = 1.42 \text{ (95\% CI 0.90 to 2.25)}, p = 0.131) \), a well established observation that became unambiguously evident in cases that were scored IHC 2+ and HER2 FISH positive \( (p = 0.022; HR = 1.72 \text{ (95\% CI 1.07 to 2.75)}, p = 0.024) \). A comparison of positive and negative HER3 cases \( (p = 0.483; HR = 0.84 \text{ (95\% CI 0.51 to 1.37)}, p = 0.484) \) did not yield any supplemental information. Immunostaining of HER1 (Figure 5b) indicated a negative effect for patients with overexpressed levels \( (p = 0.067; HR = 1.66 \text{ (95\% CI 0.96 to 2.86)}, p = 0.070) \). In addition, HER4 protein overexpression (Figure 5d) tended to have a negative impact on disease \( (p = 0.068; HR = 1.48 \text{ (95\% CI 0.97 to 2.26)}, p = 0.070) \).

To examine the proliferation status in our patient cohort we assessed Ki-67 IHC. In 24% of cases \( (36 \text{ of } 150) \) we documented a positive staining (more than 30\% of nuclei) with a negative effect on overall survival, whereas 76\% of cases \( (114 \text{ of } 150) \) showed less or no staining resulting in a more favourable outcome \( (p = 0.142; HR = 1.49 \text{ (95\% CI 0.87 to 2.55)}, p = 0.145) \).

**Discussion**

Because screening of HER2 aberration as a prerequisite for Herceptin therapy \( [29-31] \) enables the prediction of neither the course of disease nor the individual response, the identification of additional prognostic and predictive parameters is of the utmost interest, primarily being those with immediate impact on HER2. Further investigation of the additional three, highly homologous HER2 cognate members
of the human EGFR (HER-) tyrosine kinases [32] is well founded. Here we present first-hand data of a four-target FISH and IHC analysis comprising all HER receptors in breast carcinomas by using TMA. The aim of this study was to identify HER2-related molecules with additional prognostic significance within the HER family.

We verified the known negative impact of HER2 amplification on the overall and disease-free survival of patients. The discrepancy of 13% of the patients showing amplification of the HER2 gene but not overexpression of the HER2 protein is consistent with the expected loss of IHC sensitivity associated with tissue fixation and embedding [33]. The positive correlation between HER2 FISH and HER2 IHC analysis is in accordance with the literature (up to 95% concordance between FISH and IHC) [2,6] as well as our own previous data (100% concordance between FISH and fluorescent IHC) [34].

Similar to our FISH and IHC analysis, Tsutsui and colleagues [19] found the combination of HER1 and HER2 expression in breast cancer to have a severe negative impact on disease outcome compared with normal protein levels, whereas the prognostic value of HER2 overexpression seemed more pronounced than HER1 overexpression. However, Diermeier and colleagues [16] provided evidence that the HER1 expression level in the breast cancer cell line SK-BR-3, coexpressed with overexpressed HER2, has a key role in mediating the anti-proliferative effect of Herceptin. Overall, HER1 gene amplification or HER1 protein overexpression in this study was found to be a rare event.

In contrast, the data presented in this study provide striking evidence for a significance of alterations in HER3 in breast cancer. This observation is supported by Holbro and colleagues [35], who identified the function of the HER2/HER3 dimer as an oncogenic unit in which HER3 couples active HER2 to the downstream signalling phosphoinositide 3-kinase/protein kinase B pathway. Blocking HER2 resulted in antiproliferative effects accompanied by a decrease in HER3 signalling activity [36]. Although HER3 has no intrinsic kinase activity to initiate the signalling process, ligand-bound or even ligand-independent HER3 may form heterodimers with HER2 that are potent signalling complexes [37,38]. According to Liu and colleagues [39], HER3 also contributes to HER2-associated tamoxifen resistance, and a decrease in HER3 levels restores sensitivity to tamoxifen. Jones and colleagues [40] provided a quantitative protein interaction network by applying protein microarrays comprising virtually every Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domain encoded in the human genome. They found a difference in the extent to which the HER receptors form protein–protein interactions when overexpressed, and consequently found the HER2–HER3 complex to have the most pronounced promiscuity with regard to activate intracellular signalling. Our results indicate the significance of the HER2/HER3 aberration in the increasing HR and therefore risk of mortality after multivariate analysis of HER2 and HER3 (Figure 7).

We detected a positive impact of HER4 on disease outcome with FISH but not with IHC analysis, as reflected by the lack of correlation between FISH and IHC data. HER4 protein overexpression has previously been described as a positive prognostic factor, a suggestion based on investigative approaches.
These positive effects can most probably be attributed to growth controlling and differentiation signalling. Barnes and colleagues [43] showed that HER4 decreases HER2 signalling activity by leading to decreased proliferation activity and increased apoptosis. In accordance with these data, we were also able to show via FISH analysis a HER2-compensating effect of HER4 represented by a decreased HER2 HR in the presence of alteration of HER4 (Figure 7). Furthermore, in support of a potential impact of HER4 amplification on improved outcome, we found that HER4 amplification detected by FISH was correlated with a positive oestrogen receptor status (p = 0.001, CC = 0.266) [18,44,45]. Significant correlation of HER4 positivity with low bromodeoxyuridine-derived proliferation indices as described by Tovey and colleagues [18] is associated with a good prognosis in breast cancer tumours. Contrary conclusions were reported by Vogt and colleagues [46], who found that HER4 amplification and ER activity were negatively correlated. These differences may occur as a result of the variable responses by HER4 to its activating ligand Heregulin, resulting in either proliferation or differentiation, and perhaps influenced by homodimerisation or heterodimerisation with other HER-family members [47].

Vidal and colleagues considered that the HER4 cell-killing intracellular domain 4ICD might be responsible for association with overall improved patient prognosis [48] by accumulating in mitochondria, causing an efflux of cytochrome c and resulting in mitochondria-regulated apoptosis [49]. It is known that HER-receptor activity and signalling is variable and depends on a particular receptor coexpression profile, potentially explaining the unequivocally strong correlation between HER4 alteration/enexpression and tumour grade or proliferation index [50]. To discover a potential relationship between the proliferation index and HER4 IHC-associated positive or HER4 IHC-associated negative patient outcome, we performed Ki-67 immunostaining. Positive MIB-1 IHC resulted in a negative trend (overall survival) in a univariate analysis, which is consistent with the literature [21]. In fact, Her4 IHC and Ki-67 were positively correlated (r = 0.202, CC = 0.256), whereas no correlation was found between HER4 FISH and Ki-67 (r = 0.267, CC = -0.095). Detailed functional studies addressing the impact of HER4 in the context of well-described coexpression patterns will elucidate the importance of HER4 within the HER-receptor family.

Information on HER1, HER3 and HER4 protein overexpression is extremely variable in the literature [20,44,51]; for example 16 to 36% for HER1, 18 to 26% for HER3 and 12 to 82% for HER4, but is usually similar for HER2 (23 to 27%). Our data fell within these ranges, except for HER3 IHC results (75%). With regard to a HER2 coexpression profile, 18.4% of cases additionally overexpressed HER1, 85.0% HER3 and 71.4% HER4. Although the protein statuses of HER2 and HER3 (p = 0.013, CC = 0.197) and HER2 and HER4 (p < 0.001, CC = 0.446) were significantly correlated with one another, no such correlation was found between HER2 and HER1 IHC (p = 0.654, CC = 0.035), an observation supported by the results of Hudelist and colleagues [41].

Overall, the quality of immunohistochemical studies seems to be highly inconsistent because of several factors such as individual tissue preparation, the application of different detection antibodies with different binding specificity, and user-dependent interpretation of staining pattern and intensity [52]. Hence, as demonstrated for HER2, in contrast to FISH, IHC is most probably the less reliable tool for discriminating patients on the basis of alterations in HER receptors [33,53]. Particularly with regard to HER3 and HER4, numerous antibodies are commercially available from which we could prove only one to be specific for each receptor (Figure 1). Furthermore, HER-receptor overexpression can change during breast cancer development, and both a decrease and an increase in expression have been observed [42], additionally challenging the interpretation of staining results.

In addition, the prognostic value of HER-family mRNA expression has been a matter of controversy. Bleche and colleagues [55], using real-time quantitative RT-PCR in patients with known long-term survival, found HER1 to be underexpressed in 82.3% of cases, HER2 (16.9%) and HER3 (46.2%) to overexpressed and HER4 both underexpressed (29.2%) and overexpressed (24.6%). Among patients with high HER4 mRNA levels, a shorter recurrence-free survival was found, suggesting that HER4 mRNA status might reflect a marker of poor outcome. However, Zaczek and colleagues [56] recently linked HER4 amplification (differential-display PCR) to favourable characteristics, as well as higher levels of HER3. RNA–RNA in situ hybridisation might clarify any discrepancy between gene and protein states and might fill an information gap.

Further subdivision of the patient cohort into subgroups with regard to individual patient treatment or additional clinical parameters was not considered in the study presented here but should be investigated in a larger patient cohort to examine the predictive value of HER1 to HER4.

Conclusion

FISH with hybridisation probes targeted to all genes encoding HER receptors turned out to be a sensitive and reliable tool for detecting potential alterations in breast cancer. Although the dominant importance of HER2 over other HER receptors is globally accepted [19], we were able to show a substantial impact of HER3 amplification on outcome of breast cancer disease (disease-free survival) even at low amplification rates. Our data provide initial evidence for the integration of HER3 as well as HER4 analysis into the diagnosis of breast cancer. Investigations are currently under way to determine the clinical importance of individual but interrelated alterations in HER in breast cancer at both the gene and protein levels. An inte-
grated quantification of individual patterns of HER-receptor alterations may enable optimised patient stratification with respect to disease outcome. The quantification of the activated receptor relative to the unactivated protein is a promising approach, particularly with regard to therapeutic response. Further descriptive and functional studies of HER receptors will serve to characterise the disease in terms of a given molecular HER-receptor equivalent, thus providing an essential basis for individualised therapy.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AS wrote the manuscript, performed the image analysis and FISH evaluation and analysed the statistical data. JR analysed the statistical data and helped to write the manuscript. PJW performed the histological analysis. AH performed the statistical data and helped to write the manuscript. PJW, AS wrote the manuscript, performed the image analysis and interpreted the data. FH provided material interpreted the data. FH provided material and interpreted the data. GB is the research group leader and senior author and evaluated and interpreted the data.

Acknowledgements
The authors thank Marietta Bock for her excellent technical assistance. We also sincerely thank Dr Monika Klinkhammer-Schalke, Tumour Centre Inc., Regensburg, for providing the clinical data. This study was supported by the Bayerische Forschungsstiftung (grant number 585/03), Munich, Germany, and Zytovision Ltd., Bremerhaven, Germany.

References
1. Menard S, Casalini P, Campiglio M, Pupa S, Agresti R, Tagliabue E: HER2 overexpression in various tumor types, focussing on its relationship to the development of invasive breast cancer. Ann Oncol 2001, 12(Suppl 1):S15-S19.
2. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levis WJ, Stuart SG, Udove J, Ullrich A: Studies of the HER-2/new proto-oncopogene in human breast and ovarian cancer. Science 1989, 244:707-712.
3. Tovey S, Dunne B, Witton CJ, Forsyth A, Cooke TG, Bartlett JM: Can molecular markers predict when to implement treatment with aromatase inhibitors in invasive breast cancer? Clin Cancer Res 2005, 11:4835-4842.
4. Kirkegaard T, McGlynn LM, Campbell FM, Muller S, Tovey SM, Dunne B, Nielsen KV, Cooke TG, Bartlett JM. Amplified in breast cancer 1 in human epidermal growth factor receptor-positive tumors of tamoxifen-treated breast cancer patients. Clin Cancer Res 2007, 13:1405-1411.
5. Graus-Porta D, Beerli RR, Daly JM, Hynes NE: ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J 1997, 16:1647-1655.
6. Holbro T, Civenni G, Hynes NE: The ErbB receptors and their role in cancer progression. Exp Cell Res 2003, 284:99-110.
7. Yarden Y: The EGFR family and its ligands in human cancer: signalling mechanisms and therapeutic opportunities. Eur J Cancer 2001, 37(Suppl 4):S3-S8.
8. Pritchard KI, Shepherd LE, O'Malley FP, Andrusi IL, Tu D, Bramwell VH, Levine MN: HER2 and responsiveness of breast cancer to adjuvant chemotherapy. N Engl J Med 2006, 354:2103-2111.
9. Slamon DJ: Herceptin: increasing survival in metastatic breast cancer. Eur J Oncol Nurs 2000, 4:24-29.

10. Plosker GL, Keam SJ: Trastuzumab: a review of its use in the management of HER2-positive metastatic and early-stage breast cancer. Drug Des Devel Ther 2007, 1:449-476.
11. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, et al.: Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 2001, 344:783-792.
12. Nahta R, Esteva FJ: HER2 therapy: molecular mechanisms of trastuzumab resistance. Breast Cancer Res 2006, 8:215.
13. Normanno N, Di Maio M, De Maio E, De Luca A, de Matteis A, Giordano A, Perrone F: Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. Endocr Relat Cancer 2005, 12:721-747.
14. Esteva FJ: Monoclonal antibodies, small molecules, and vaccines in the treatment of breast cancer. Oncologist 2004, 9(Suppl 3):4-9.
15. Nahta R, Hortobagyi GN, Esteva FJ: Growth factor receptors in breast cancer: potential for therapeutic intervention. Oncologist 2003, 8:5-17.
16. Diermeier S, Horvath G, Knechel-Clarke R, Hofstaetter F, Szollosi G, Brockhoff G: Epidermal growth factor receptor coexpression modulates susceptibility to Herceptin in HER2/new overexpressing breast cancer cells via specific erbB-receptor interaction and activation. Exp Cell Res 2005, 304:604-619.
17. Schlessinger J: Cell signaling by receptor tyrosine kinases. Cell 2000, 103:211-225.
18. Tovey SM, Witton CJ, Bartlett JM, Stanton PD, Reeves JR, Cooke TG: Outcome and human epidermal growth factor receptor (HER) 1–4 status in invasive breast carcinomas with proliferation indices evaluated by bromo-oxuridine labelling. Breast Cancer Res 2004, 6:R246-R251.
19. Tsutsui S, Ohno S, Murakami S, Katoaka A, Kinoshita J, Hachitanda Y: Prognostic value of the combination of epidermal growth factor receptor and c-erbB-2 in breast cancer. Surgery 2003, 133:219-221.
20. Witton CJ, Reeves JR, Going J, Cooke TG, Bartlett JM: Expression of the HER-1–4 family of receptor tyrosine kinases in breast cancer. J Pathol 2003, 200:290-297.
21. de Azambuja E, Cardoso F, de Castro G Jr, Coloza M, Mano MS, Duarte V, Sotiriou C, Larnsont D, Piccart-Gebhart MJ, Paesmans M: Ki-67 as prognostic marker in early breast cancer: a meta-analysis of published studies involving 12,155 patients. Br J Cancer 2007, 96:1504-1513.
22. Trama A, Agostara B, Marasa L, Calabro M, Zarcone M, Carruba G: HER2/neu expression in relation to clinicopathologic features of breast cancer patients. Ann NY Acad Sci 2006, 1089:159-167.
23. Brockhoff G, Heckel B, Schmidt-Bruecken E, Plander M, Hofstaetter F, Vollmann A, Diermeier S: Differential impact of Cetuximab, Pertuzumab and Trastuzumab on BT474 and SK-BR-3 breast cancer cell proliferation. Cell Profil 2007, 4:488-507.
24. Klopocki E, Kristiansen G, Wild PJ, Klaman I, Castanos-Velez E, Singer G, Stohr R, Simon R, Sauter G, Leibiger H, et al.: Loss of SFRP1 is associated with breast cancer progression and poor prognosis in early stage tumors. Int J Oncol 2004, 25:641-649.
25. Bubendorf L, Nocito A, Moch H, Sauter G: Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies. J Pathol 2001, 195:72-79.
26. Contal C, O’Quigley J: An application of changepoint methods in studying the effect of an adjuvant survival in breast cancer. Comput Statist Data Anal 1999, 30:253-270.
27. Mandrekar JN, Mandrekar SJ, Cha SS: Cutpoint determination methods in survival analysis using SAS®. SAS Users Group 28, Seattle, Washington; March 30-April 2, 2003:261-280.
28. Williams BA, Mandrekar JN, Mandrekar SJ, Cha SS, Furth AF: Finding optimal cutpoints for continuous covariates with binary and time-to-event outcomes. Mayo Foundation Technical Report Series, no. 79. Rochester, MN: Department of Health Sciences Research, Mayo Clinic 2006 [http://ndc.mayo.edu/mayo/research/biostat/upload/79.pdf].
29. Liberato NL, Marchetti M, Barosi G: Cost-effectiveness of adjuvant trastuzumab in human epidermal growth factor receptor 2-positive breast cancer. J Clin Oncol 2007, 25:625-633.
30. Kurian AW, Thompson RN, Gao AF, Arias S, Ortiz R, Garber AM: A cost-effectiveness analysis of adjuvant trastuzumab regimens.
in early HER2/neu-positive breast cancer. J Clin Oncol 2007, 25:364-373.
31. Norum J, Olsen JA, Wist EA, Lonning PE: Trastuzumab in adjuvant breast cancer therapy. A model based cost-effectiveness analysis. Acta Oncol 2007, 46:153-164.
32. Yarden Y, Slwikowski MX: Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001, 2:127-137.
33. Pauelli G, Dandeker S, Rong H, Ramos I, Peng H, Seshadri R, Slamon DJ: Assessment of methods for tissue-based detection of the HER-2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. J Clin Oncol 2000, 18:3651-3664.
34. Lottner C, Schwartz S, Darmeyer S, Hartmann A, Knuechel R, Hofstaedter F, Brockhoff G: Simultaneous detection of HER2/neu gene amplification and protein overexpression in paraffin-embedded breast cancer. J Pathol 2005, 205:577-584.
35. Popov T, Beerli RR, Maurer F, Koziczak M, Barbas CF, Hynes NE: The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. Proc Natl Acad Sci USA 2003, 100:8933-8938.
36. Neve RM, Sutterlty H, Pullen N, Lane HA, Daly JM, Krek W, Hynes NE: Effects of an ErbB4 oncogenic ErbB2 on G1 cell cycle regulators in breast tumour cells. Oncogene 2000, 19:1647-1656.
37. Citri A, Skaria KB, Yarden Y: The dead and the dumb: the biology of ErbB-2 and ErbB-3. Exp Cell Res 2003, 284:54-65.
38. Wallasch C, Weiss FU, Niederfellner G, Jallal B, Issing W, Ullrich A: Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3. EMBO J 1995, 14:4267-4275.
39. Liu B, Ordonez-Ercan D, Fan Z, Edgerton SM, Yang X, Thor AD: Downregulation of erbB3 abrogates erbB2-mediated tamoxifen resistance in breast cancer cells. Int J Cancer 2007, 120:1874-1882.
40. Jones RB, Gordus A, Krall JA, MacBeath G: A quantitative protein interaction network for the ErbB receptors using protein microarrays. Nature 2006, 439:168-174.
41. Hudelist G, Singer CF, Manavi M, Koziczak M, Barbas CF, Hynes NE: Effects of an oncogenic ErbB2 on G1 cell cycle regulators in breast tumour cells. Oncogene 2000, 19:1647-1656.
42. Fuchs IB, Siemer I, Buhler H, Schmider A, Henrich W, Lichtenerger W, Schaller G, Kuemmel S: Epidermal growth factor receptor changes during breast cancer metastasis. Anticancer Res 2006, 26:4397-4401.
43. Barnes NL, Khawari S, Boland GP, Cramer A, Knox WF, Bundred NJ: Absence of HER4 expression predicts recurrence of ductal carcinoma in situ of the breast. Clin Cancer Res 2005, 11:2163-2168.
44. Suo Z, Riasberg B, Kalason MG, Willman K, Tierens A, Skovlund E, Nesland JM: 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.
45. Bacus SS, Chin D, Yarden Y, Zelick CR, Stern DF: Type 1 receptor tyrosine kinases are differentially phosphorylated in mammary carcinoma and differentially associated with steroid receptors. Am J Pathol 1996, 148:549-558.
46. Vogt U, Bielawski K, Schlottmer CM, Bosse U, Falkiewicz B, Podhajska AJ: Amplification of erbB-4 oncogene occurs less frequently than that of erbB-2 in primary human breast cancer. Gene 1996, 223:375-380.
47. Sartor CI, Zhou H, Kozloskwa E, Guttridge D, Kawata E, Caskey L, Harrelson J, Hynes NE, Ethier S, Calvo B, et al.: Her4 mediates ligand-dependent antiproliferative and differentiation responses in human breast cancer cells. Mol Cell Biol 2001, 21:4265-4275.
48. Vidal GA, Clark DE, Marrero L, Jones FE: A constitutively active ERBB4/HER4 allele with enhanced transcriptional coactivation and cell-killing activities. Oncogene 2007, 26:462-466.
49. Naresh A, Long W, Vidal GA, Wimley WC, Marrero L, Sartor CI, Tovey S, Cooke TG, Bartlett JM, Jones FE: The ERBB4/HER4 intracellular domain 4CD is a BH3-only protein promoting apoptosis of breast cancer cells. Cancer Res 2006, 66:6412-6420.
50. Riese DJ, van Raaij TM, Plowman GD, Andrews GC, Stern DF: The cellular response to neuregulins is governed by complex inter-