HER1-4 protein concentrations in normal breast tissue from breast cancer patients are expressed by the same profile as in the malignant tissue

Dorte Aa. Olsen[1,*], Birthe Østergaard[2], Susanne Bokmand[3], Peter A. Wamberg[3], Erik H. Jakobsen[4], Anders Jakobsen[4] and Ivan Brandslund[1,5]

[1] Department of Clinical Biochemistry, Vejle County Hospital, Vejle, Denmark
[2] Department of Pathology, Vejle County Hospital, Vejle, Denmark
[3] Breast Cancer Centre, Vejle County Hospital, Vejle, Denmark
[4] Department of Oncology, Vejle County Hospital, Vejle, Denmark
[5] The Faculty of Health Sciences, University of Southern Denmark, Odense, Denmark

Abstract

Background: The epidermal growth factor receptor HER2 is overexpressed or amplified in 25%–30% of patients with breast cancer. The mechanism behind HER2 amplification is unknown, but may be a pathophysiological phenomenon caused by continuous stimulation and activation of the HER1-4 system. We have mapped the protein concentrations of HER1-4 in breast cancer tissue, autologous reference tissue, normal breast tissue and serum samples, to see whether non-cancer cells from these patients express a protein profile indicating general activation.

Methods: Tissue samples from malignant and adjacent normal breast tissue (autologous reference tissue) were collected from 118 women consecutively admitted for surgical treatment of breast cancer. In addition, 26 samples of normal breast tissue were collected from healthy women having breast reduction surgery. The tissue samples were homogenized and the proteins extracted. The tissue and serum concentrations of HER1-4 were determined quantitatively using a commercially available enzyme linked immunosorbent assay (ELISA) method.

Results: HER1 was down regulated in cancer tissue when compared to autologous reference tissue ($p = 8 \times 10^{-6}$), while HER2 ($p < 10^{-7}$) and HER3 ($p = 3 \times 10^{-5}$) were up regulated. Comparing autologous reference tissue with normal tissue showed down regulation of HER1 ($p = 0.122$) and up regulation of HER2 ($p = 10^{-6}$), HER3 ($p < 10^{-7}$) and HER4 ($p < 10^{-7}$).

*Corresponding author: Dorte Aa. Olsen, Department of Clinical Biochemistry, Vejle County Hospital, Kabbeltoft 25, 7100 Vejle, Denmark
Phone: +45-79406633, Fax: +45-79406871,
E-mail: Dorte.Aalund.Olsen@slb.regionsyddanmark.dk

Furthermore, we observed that correlations between the receptor combinations HER1-2, HER1-3 and HER1-4 were maintained from normal breast tissue to autologous reference breast tissue, but were lost in cancer tissue.

Conclusions: We suggest that these findings indicate that breast cancer is a systemic disease where the HER1-4 system in autologous reference tissue is continuously activated, thus favoring the subsequent development of cancer.

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Introduction

The epidermal growth factor family of tyrosine kinases includes four receptor members: HER1 [epideral growth factor receptor (EGFR)/ErbB1], HER2 (ErbB2/Neu), HER3 (ErbB3) and HER4 (ErbB4). All receptors are structurally similar and consist of an extracellular region binding ligands, a transmembrane domain and an intracellular tyrosine kinase domain. A number of ligands have been identified which induce homo- or heterodimerization of the receptors following binding. This results in phosphorylation and activates the next step in the pathway leading to cell growth and proliferation (1).

HER2 has been thoroughly investigated and it is well established that this receptor is expressed at higher than normal concentrations in 25%–30% of patients with breast cancer (2–4). It has been documented that HER2 overexpression is associated with shorter disease free survival and overall survival (3–5). Treatment using the anti-HER2-specific humanized monoclonal antibody trastuzumab (Herceptin®) has been shown to increase survival time in these patients (6, 7). The possible role of HER1, HER3 and HER4 in breast cancer needs further elucidation. They are interesting because of their ability to heterodimerize with HER2 and theoretically, HER2 being without any known ligand, overexpression of HER2 could be caused by HER1 stimulation.

The aim of this study was to investigate the protein concentrations of HER1-4 in breast cancer tissue and blood, autologous reference tissue and normal breast tissue. We have previously shown that the concentration of HER2 in autologous reference tissue is higher

than normal (8). This has prompted us to investigate if this phenomenon also was true for the other receptors, indicating systemic activation of the cells. The HER1-4 findings were compared with estrogen receptor (ER) status, tumor grade, axillary node status, tumor size, disease free survival, and their inter-relationship was evaluated.

Materials and methods

Patients

We obtained approval by the Regional Science Ethics Committee of Fyns and Vejle Counties (no S-VF-20040101). Fresh breast tissue samples of malignant and adjacent normal autologous reference tissue were collected from 118 women admitted consecutively for surgical treatment of primary breast cancer between 2004 and 2005. The patients underwent surgery according to standard Danish Breast Cancer Guidelines (DBCG) (www.DBCG.dk). The autologous reference tissue was collected at least 1 cm away from the tumor and determined macroscopically to be tumor free by a pathologist (Birthe Østergaard), while micro ductal carcinoma in situ (DCIS) cells were not searched for. The pathological characteristics of the patients are summarized in Table 1. The tissue samples were immediately frozen and stored at $-80^{\circ}C$. In addition, normal breast tissue was collected from 26 healthy women who underwent cosmetic breast surgery. Tissue samples were homogenized as described in Olsen et al. (8) and the protein concentration of each sample was determined using the bicinchoninic acid protein assay (Pierce, Rockford, USA). Blood samples were available for 114 cancer patients. These were collected prior to surgery and left to coagulate for 30–60 min, followed by centrifugation at $2000 \times g$ for 10 min. Serum samples were immediately processed and stored at $-80^{\circ}C$.

HER1 ELISA

A commercially available enzyme linked immunosorbent assay (ELISA) (Oncogene Science, Cambridge, MA, USA)

Table 1 Pathological data in 118 breast cancer patients.

	Number of patients (%)
Tumor type	
Ductular	107 (91)
Lobular	6 (5)
Others	5 (4)
Age, years	
Range	34–91
Median	59
Tumor size, mm^3	
Range	0.7–233
Median	7.36
Tumor grade	
1	20 (17)
2	51 (43)
3	41 (35)
Undetermined	6 (5)
Node status	
–	49 (42)
+	69 (58)
Estrogen receptor status	
–	21 (18)
+	97 (82)

was used to quantify HER1 in breast tissue and in serum. Serum was diluted $50\times$ according to the manufacturer's instructions and tissue extracts were diluted in sample diluent provided with the kit to achieve a final protein concentration of 70 µg/mL. Diluted tissue samples along with standards and controls were added to a 96 well microtiter plate coated with mouse monoclonal anti- (HER1) antibody and incubated for 1.5 h at 37°C. After this incubation step, plates were washed and incubated with an alkaline phosphatase-labeled mouse monoclonal anti- (HER1) antibody for 0.5 h at room temperature (RT). Enzymatic reactions were performed at RT by adding BluePhos substrate and the reaction was terminated after 1.0 h by the addition of stop solution. Color development was measured at 650 nm using a plate reader V_{max} (Molecular devices, Sunnyvale, CA, USA) and the HER1 concentrations in the unknown samples were estimated from the standard curve. All samples were analyzed in duplicate and the average recorded. The total coefficient of variation was below 7%.

HER2 ELISA

A commercially available ELISA kit (Oncogene Science, USA) was used to quantify HER2 in tissue as described previously (8). In brief, samples were diluted to a final protein concentration of 70 µg/mL and dispensed, along with standards and controls, in duplicate into a 96 well plate coated with a monoclonal anti-HER2 antibody. The total coefficient of variation was <8%. Serum samples were analyzed with the HER2/neu assay using the ADVIA Centaur system (Bayer, Leverkusen, Germany) according to the manufacturer's instructions.

HER3 ELISA

A commercially available DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN, USA) was used to quantify HER3 in breast tissue and in serum. The plate was coated with a mouse anti-human HER3 antibody using 4.0 µg/mL and left to stand overnight (ON) at RT. The plate was washed in phosphate buffered saline (PBS) containing 0.05% Tween®20, blocked for 1 h using PBS with 1% bovine serum albumin (BSA) and washed again. Tissue extracts were diluted in PBS with 1% BSA to a final protein concentration of 70 µg/mL and serum samples were diluted $10\times$. Diluted samples, along with standards and controls, were dispensed in duplicate into a 96 well plate and incubated for 2 h at RT. The plate was washed and incubated for 2 h at RT in the presence of a biotinylated mouse anti-human HER3 antibody using 0.5 µg/mL. After washing horseradish peroxidase (HRP), conjugated Streptavidin was added and the plate was left to stand for 30 min at RT. The plate was washed and developed using a 1:1 mixture of tetramethylbenzidine and H_2O_2. The reaction was stopped after a 30-min incubation at RT using 2 N H_2SO_4. Bound HER3 was detected by measuring the absorbance at 450 nm using a plate reader V_{max} (Molecular devices, USA). The HER3 concentration in the samples was determined from the standard curve. The total coefficient of variation was <14%.

HER4 ELISA

A commercially available DuoSet ELISA Development kit (R&D Systems, USA) was used to quantitate HER4 in breast tissue and serum. The procedure was identical to the HER3 assay described previously, with a few adjustments. The coating antibody was a mouse anti-human HER4 antibody at 4.0 µg/mL, and the detection antibody was a biotinylated

mouse anti-human HER4 antibody at 0.25 μg/mL. Breast tissue extracts were diluted to a protein concentration of 80 μg/mL, and serum was applied undiluted. The total coefficient of variation was <13%.

ER status by immunohistochemistry (IHC)

IHC was performed on formalin-fixed paraffin-embedded breast tissue sections. The slides were deparafinized and rehydrated in graded alcohol solutions. Slides were incubated with a 1:150 dilution of a mouse anti-human estrogen receptor mab (clone 1D5, Dakocytomation, Glostrup, Denmark) for 30 min. Visualization of the reaction was performed using Super Sensitive Polymer-HRP IHC kit (Biogenix, San Ramon, CA, USA). Tumors with staining of 10% or more of the nuclei were considered to be ER positive according to standard Danish Breast Cancer Guidelines (www.DBCG.dk).

Statistical methods

Data were evaluated using NCSS software (version 2004, Kaysville, UT, USA) to perform the following statistical analyses: Wilcoxon Signed Ranks test, Mann-Whitney U-test, Spearman's ρ, Kruskal-Wallis test, Kaplan-Meier and log-rank test.

Results

HER1-4 measurements in breast tissues

The level of HER1 was decreased by 20% in autologous reference tissue when compared to normal tissue. HER2, HER3 and HER4 were significantly increased (Table 2, Figure 1). In cancer tissue, HER1 was significantly reduced when compared to normal and autologous reference tissue, while HER2 and HER3 were significantly increased. The level of HER4 was increased to the same degree in the autologous reference and cancer tissue (Table 2, Figure 1).

Dividing patients into negative and positive groups according to HER2 status as determined by IHC and fluorescence in situ hybridization (FISH) showed no significant differences for HER1, 3 and 4 in the autologous reference tissue. In addition, the differences

with respect to the normal tissue were maintained. In HER2 negative and positive cancer tissue, the concentration of HER1 and 3 showed no differences, while HER4 was significantly lower in the HER2 positive group (p=0.004). In the HER2 negative paired groups of autologous reference tissue and cancer tissue, significant differences were found for HER1 (p=4×10^{-5}) and HER3 (p=7×10^{-5}) but not for HER4. In the HER2 positive paired groups of autologous reference tissue and cancer tissue, no differences were observed.

HER1-4 correlations

The HER1-4 receptors in normal tissues were correlated significantly between HER1-2, HER1-3, HER1-4 and HER2-3 (Table 3). Furthermore, associations between receptors in autologous reference tissue were studied and significant correlation was found between all receptors (Table 3). In cancer tissue, significant correlations were found between HER2-3 and HER3-4 (Table 3).

HER1-4 measurements in serum

HER1-4 concentrations were quantitated in 114 serum samples (Table 2). The values were compared with corresponding HER1-4 measurements in cancer and autologous reference tissue, but no correlation was found. Correlation between the receptors in serum were studied; significant correlations were found between HER1-3, HER2-4 and HER3-4 (Table 3).

ER status

Of the 118 patient with breast cancer, 97 (82%) were classified as ER positive and 21 (18%) as ER negative. The HER1 concentration was significantly higher in ER negative tissue compared to ER positive tissue (Figure 2, Table 4). No difference was observed between ER negative and positive tissues with respect to HER2 concentrations, while the median values of HER3 and HER4 were significantly higher in ER positive tissues

Table 2 HER1-4 protein concentrations in different groups.

	HER1	HER2	HER3	HER4
Normal tissue				
Range, pg/mg protein	(1195–11,997.7)	(208.5–13,783.4)	(25.3–1574.2)	(4.0–165.7)
Median, pg/mg protein	6479	1293.8	232.9	50.1
Autologous reference tissue				
Range, pg/mg protein	(453–22,518)	(660–60,000)	(133–14,426)	(20–4272)
Median, pg/mg protein	5166	6091	1960	516
p-Value	0.122	10^{-6}	$<10^{-7}$	$<10^{-7}$
Cancer tissue				
Range, pg/mg protein	(267–45,803)	(3498–2,034,610)	(373–26,240)	(26–4133)
Median, pg/mg protein	3309	31,656	3213	421
p-Value	8×10^{-6}	$<10^{-7}$	3×10^{-5}	0.720
Serum				
Range, pg/mL	(39,100–88,000)	(6100–23,300)	(1000–45,100)	(0.49–129.3)
Median, pg/mL	59,500	11,400	1900	5.6

p-Values in the autologous reference tissue group refer to differences in medians between normal tissue and autologous reference tissue (Mann-Whitney U-test). p-Values in the cancer tissue group refer to differences in medians between autologous reference tissue and cancer tissue (Wilcoxon test).
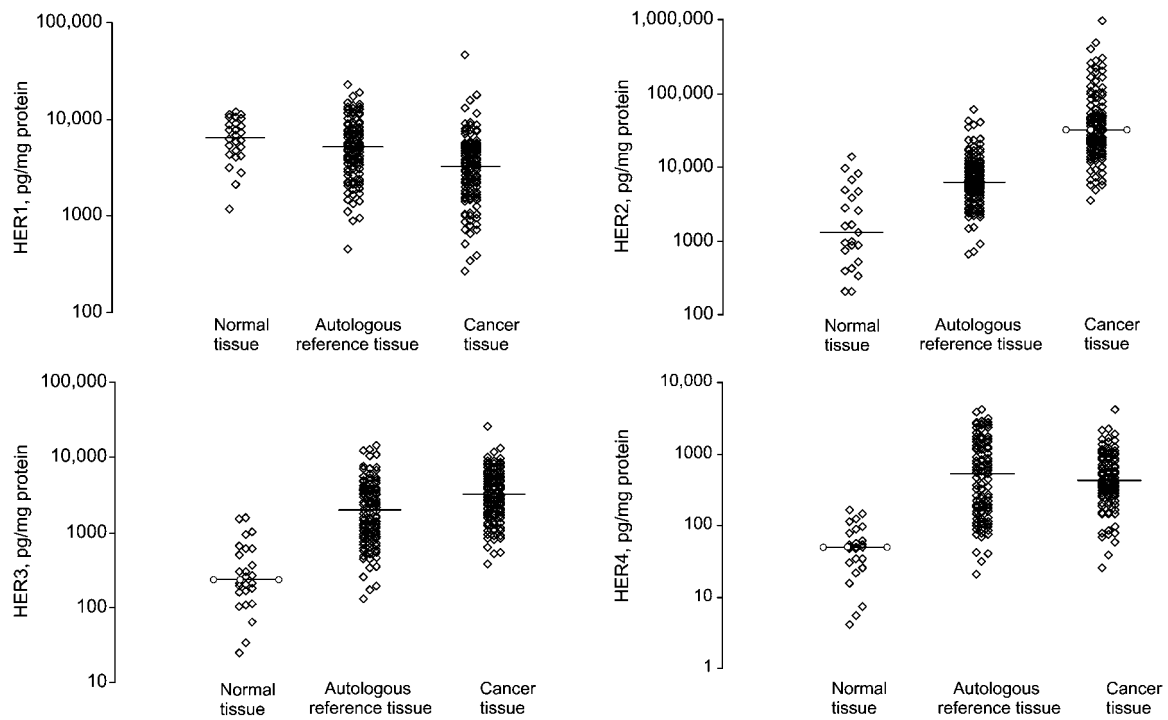
Figure 1 HER1-4 protein concentrations in cancer tissue, autologous reference tissue and in normal tissue quantified using ELISA.

as compared to ER negative tissues (Figure 2, Table 4).

Clinical data

HER1-4 protein concentrations in breast cancer tissue were compared with tumor grade, axillary node status (Table 4) and tumor size (data not shown). No significant correlations were found. The median follow up was 943 days (range 119–1323 days). During this period relapses were observed in 15 patients. Patients with high HER2 concentrations in cancer tissue using a cut-off value of either 50,000 (Figure 3A), 100,000 or 200,000 pg/mg (data not shown) or HER3 concentrations below the median had increased risk of recurrence (Figure 3B). Neither HER1 nor HER4 individual expression showed significant association with disease free survival.

The impact of expression of HER1 and HER2 in combination with HER3 and HER4 was evaluated by categorizing patients into two groups; one with high concentration [at or above the median $(+)$] and another with low concentration [below the median $(-)$]. The

patients were divided into the following groups: Group (a) patients with high HER2, HER3 and HER4, (b) low HER2 and high HER3 and HER4, (c) low HER2, HER3 and HER4 (d) high HER2 and low HER3 and HER4, (e) high HER2, high HER3 and low HER4 or high HER2, low HER3 and high HER4, (f) low HER2, high HER3 and low HER4 or low HER2, low HER3 and high HER4. The same grouping was performed for HER1, HER3 and HER4 receptor combinations (Figure 3C and D). Disease free survival between all groups was not significant. However, patients with high HER2 combined with high HER3 and HER4 had longer disease free survival compared to patients with high HER2 combined with low HER3 and HER4 (p=0.038) (Figure 3C, a compared with d).

Discussion

To our knowledge, we are the first to apply a quantitative method to determine HER1-4 protein concentrations in autologous reference tissue and compare

Table 3 Correlations between the HER1-4 receptors.

	Normal tissue		Autologous reference tissue		Cancer tissue		Serum	
	Correlation	p-Value	Correlation	p-Value	Correlation	p-Value	Correlation	p-Value
HER1-2	0.558	0.0056	0.417	5×10^{-6}	0.169	0.075	0.126	0.180
HER1-3	0.537	0.0047	0.427	5×10^{-6}	0.011	0.911	0.232	0.013
HER1-4	0.570	0.0029	0.267	0.007	0.02	0.837	0.055	0.572
HER2-3	0.705	0.00017	0.294	0.002	0.379	3×10^{-5}	0.058	0.541
HER2-4	0.254	0.243	0.217	0.029	0.079	0.417	0.192	0.044
HER3-4	0.164	0.434	0.824	$<10^{-6}$	0.581	$<10^{-6}$	0.212	0.025

The correlations were determined using Spearman's ρ.
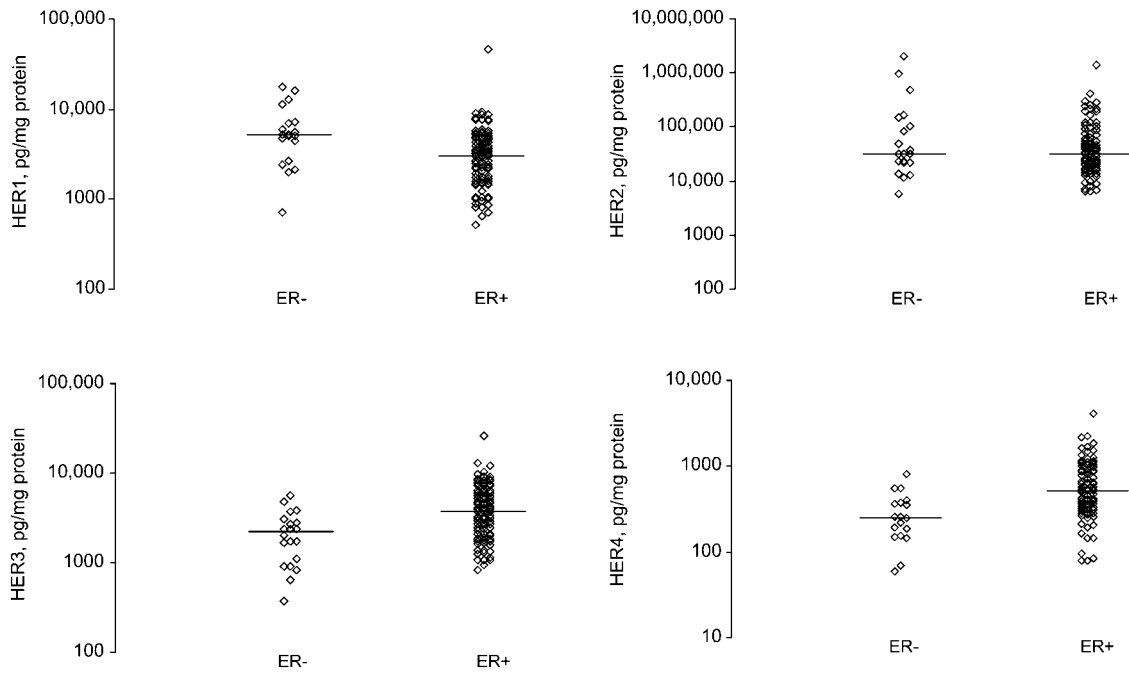
Figure 2 HER1-4 protein concentrations in breast cancer tissue divided according to ER positivity.

these concentrations to concentrations in cancer tissue and normal breast tissue. We have previously shown that HER2 is up regulated in autologous reference tissue compared to normal breast tissue (8). Therefore, we hypothesized that HER1, 3 and 4 in autologous reference tissue might follow a similar pattern, and change in a way that is characteristic for cancer tissue and that differs from normal breast tissue.

The results showed down regulation of HER1 and up regulation of HER2-4 in autologous reference tissue and cancer tissue compared to normal breast tissue. Other authors have also examined HER1-4 using other methods including IHC, FISH, real-time reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting (9–12). Previous reports have shown an increased content of HER2 and HER3 in breast cancer tissue as compared to normal tissues (13–15) which are in agreement with our results. Furthermore,

the observation that HER1 is decreased in cancer tissue as compared to normal tissue has been described previously in the literature for breast cancer (15, 16) as well as other malignancies such as ovarian cancer (17). The reason for the decreased concentration of HER1 in cancer tissue is unknown, however, it has been suggested that it could be due to a system in which HER1 is constantly activated followed by internalization, ubiquination and degradation resulting in a decreased HER1 concentration (17). This suggestion is based on the observation that *HER1* gene expression is similar in normal and cancerous tissue. In addition, it has been shown that most HER1 is internalized following ligand binding and ultimately degraded in lysosomes; HER2-4 receptors are endocytosis impaired, and are more often recycled back to the cell surface (18, 19).

Significant correlations were found between all the receptor combinations in the autologous reference

Table 4 Relationships between HER1-4 in cancer tissue and pathological parameters.

	n	HER1, pg/mg Median (range)	HER2, pg/mg Median (range)	HER3, pg/mg Median (range)	HER4, pg/mg Median (range)
Axillary node status					
–	49	3244 (267–9308)	33,141 (3498–2,034,610)	3101 (811–26,242)	497 (79–4133)
+	69	3240 (341–45,803)	31,656 (5814–964,220)	3255 (373–12,000)	406 (26–2225)
p-Value		0.978	0.593	0.783	0.322
Tumor grade					
1	20	3863 (719–8729)	31,656 (6241–295,610)	3213 (948–9799)	531 (280–2166)
2	51	3560 (267–45,803)	25,619 (3498–402,690)	3749 (516–13,112)	555 (26–4133)
3	41	2991 (392–17,774)	34,354 (4833–2,034,610)	2947 (373–26,242)	351 (59–1519)
p-Value		0.552	0.892	0.575	0.130
ER					
–	21	5166 (719–17,774)	31,482 (5814–2,034,610)	2215 (373–5561)	250 (59–815)
+	97	3039 (267–45,803)	33,141 (3498–1,358,800)	3758 (516–26,242)	521 (26–4133)
p-Value		0.0025	0.582	0.0013	0.00059

Statistical analysis applied was Wilcoxon test (axillary node status and ER status) and Kruskal-Wallis test (tumor grade). ER, estrogen receptor.
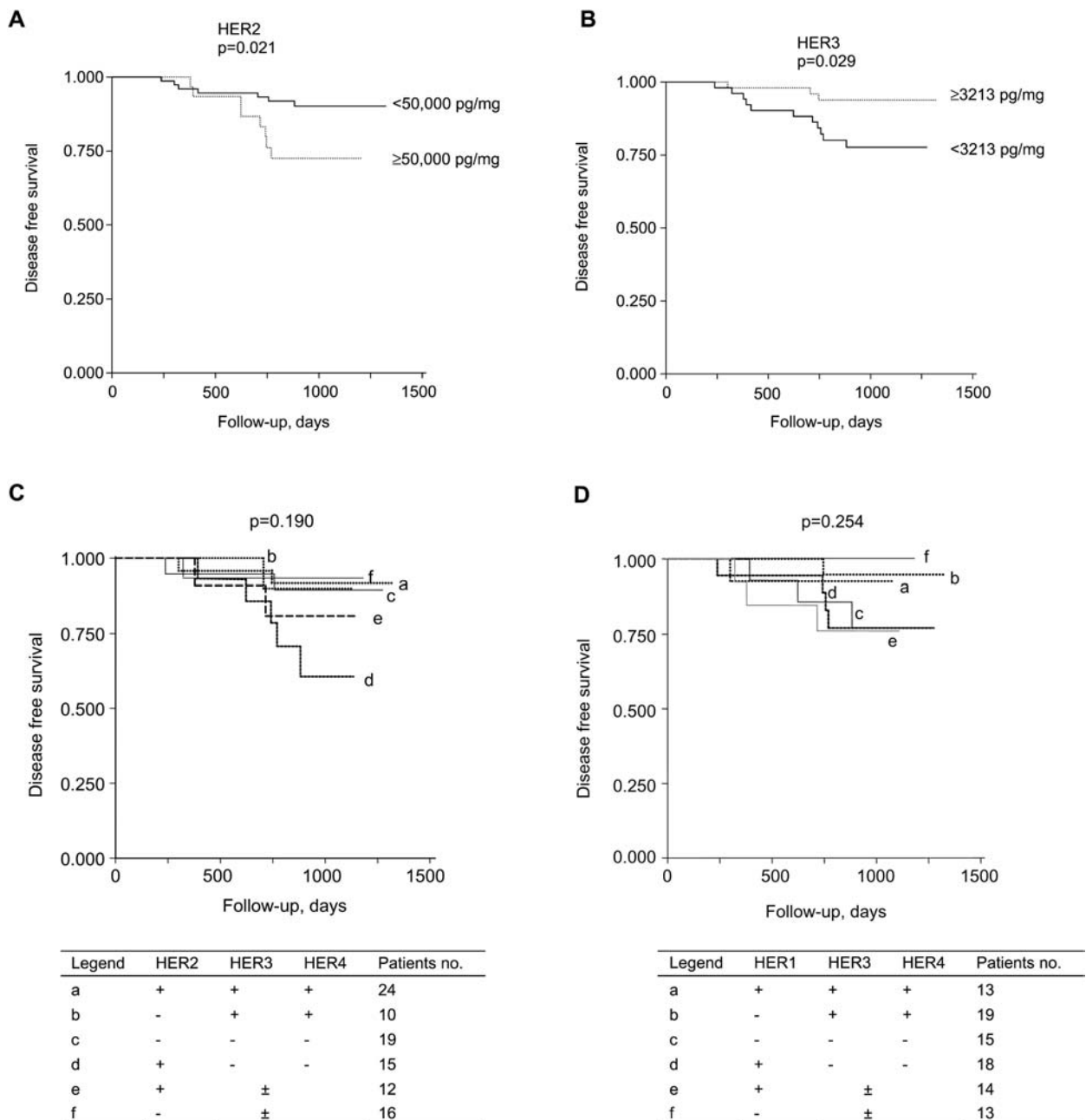
Figure 3   Kaplan-Meier curves showing disease free survival of HER2 and HER3 expressed individually (A and B) and of various HER1-4 receptor combinations (C and D).
p-Value represents log-rank difference in disease free survival between two groups (A and B) and all groups (C and D).

tissue. However, in cancer tissue the correlations between HER1-2, HER1-3, HER1-4 and HER2-4 were lost. Since these patients had not yet received treatment, and the changes in quantitative measurements of the receptors are reliable, we believe that this reflects the changes occurring during the development of cancer. These results are in agreement with a report by Bianchi et al. (20), but not with the findings of Abd El-Rahim et al. (21). Reasons for these discrepancies could be due to the detection methods used, the antibodies used or patient material. The significant correlation found between HER2-3 and HER3-4 in breast cancer tissue is in agreement with previous studies that have suggested a significant role of HER2 and HER3 heterodimers in breast tumorigenesis (9, 10, 13, 20).

We demonstrate that patients with high concentrations of HER2 or low concentrations of HER3 in breast cancer tissue had increased risk for relapse, while HER1 and HER4 by themselves showed no prognostic value. In addition, combining high expression of HER2 in combination with high concentrations of HER3 and HER4 showed increased disease free survival time compared to high concentrations of HER2 in combination with low concentrations of HER3 and HER4. This shows that expression of HER3 and HER4 can influence the effect of HER2 on disease free survival.

The majority of authors describe increased expression of HER1 or HER2 to be associated with unfavorable prognosis (3, 4, 12, 21). However, some authors found no prognostic value of HER1 (22, 23) or HER2

(10, 24). The follow up time, number of patients and the detection method may explain these differences. Most studies show high concentrations of HER3 or HER4 to be associated with improved disease free and overall survival (10–13, 23), which are in agreement with our results. The counteracting effect of high HER3 and HER4 on disease free survival that we observed is comparable to a previous study performed on patients with bladder cancer (25).

The primary focus of our study was to investigate whether the pronounced changes seen in the receptor concentrations HER1-4 in breast cancer tissue, compared to normal breast tissue from healthy women, could be found also in the autologous reference tissue. This was found to be correct and might be due to carcinoma cells in the reference tissue. As the tissue pathology was examined, it was shown to be free of invasive growth, but as micro DCIS cells were not specifically looked for, DCIS could be the cause. Other authors have shown that a free margin to the primary tumor of 1 cm reduces the risk of local recurrence in adjacent tissue to a minimum (26, 27). However, previous work showed a risk of multifocality (28). We performed an extensive literature search and were unable to find systematic sectional investigations of breast tissue after mastectomy to document this. Whatever the explanation, DCIS or general activation of normal cells, our findings show that the disease is not limited to the tumor itself, but also to the surrounding cells. Therefore, we suggest that the characteristic changes in cancer tissue are mirrored in the autologous reference tissue.

In conclusion, these results point to a continuum in the process of cancer development. Step 1 being a change in stimulation of growth factor receptors as indicated by changes in the concentration of receptors, even in autologous reference tissue. In step 2, deregulated expression occurs where the normal correlations between receptor concentrations are lost. To further investigate this, a new study is planned for a cohort of patients with measurement of HER1-4 concentrations in tissue, relevant ligands and receptor activation as evidenced by phosphorylation.

Statement

Acknowledgements

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