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Scientific Research

Expression of the Epidermal Growth Factor Receptors and Ligands in Paired Samples of Normal Breast Tissue, Primary Breast Carcinomas and Lymph Node Metastases

Anja Brügmann[1,2*], V. Jensen[3], J. P. Garne[4], E. Nexo[2], B. S. Sorensen[2]

[1]Institute of Pathology, Aalborg Hospital, Aalborg, Denmark
[2]Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark
[3]Institute of Pathology, Aarhus University Hospital, Aarhus, Denmark
[4]Department of Breast Surgery, Aalborg Hospital, Aalborg, Denmark
Email: *ahb@rn.dk

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Abstract

Purpose: In breast cancer, the EGF receptors host an increasing number of therapeutic targets and the interactive mechanisms of actions of the receptors and their ligands justify investigation of the EGF family as an entity. Experimental design: Paired tissue samples of normal breast tissue and primary breast carcinomas were examined in a prospective study of 163 patients. A third sample was obtained from the paired ipsilateral metastatic lymph node from 58 of these patients. The mRNA expression of four EGF receptors (HER1 - HER4) and 11 activating ligands was quantified with real-time RT-PCR. Results: Expression of HER2, HER3, and HER4 mRNA was upregulated in primary carcinomas compared to normal breast tissue while HER1 was downregulated. The mRNA expression of HER3 and HER4 differed between primary breast carcinomas and lymph node metastases whereas there was no difference in the expression of HER1 and HER2. The combination of low HER3 and low HER4 expression in the primary carcinoma was significantly more frequent in lymph node-negative patients as compared to lymph node positive patients. Distinct correlation patterns of the receptors and their corresponding activating ligands appeared in both normal breast tissue and in carcinomas, notably for the HER3 and HER4 receptors and their 3 specific ligands: HB-EGF, NRG2, and NRG4. Conclusion: HER2, HER3, and HER4 showed increased mRNA expression in carcinomas and were positively correlated to each other and to specific activating ligands. Furthermore, low HER3 and HER4 expression in the carcinomas correlated to the absence of lymph node metastases.

*Corresponding author.

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Keywords

Breast Cancer, Lymph Node Metastases, EGF Receptors, EGF Ligands, RT-PCR

1. Introduction

In breast cancer, the family of epidermal growth factor (EGF) receptors is the target of an increasing number of therapeutic drugs [1] [2]. Traditionally, the biomarkers decisive for targeted treatment are evaluated in the primary breast carcinoma although the target is the metastatic cells and the minimal residual disease. Axillary lymph node metastases are the detectable clinical manifestation of metastatic cells.

The primary route for the metastatic spread of breast carcinoma is via the lymphatic system, and the axillary lymph node status remains the best prognostic factor [3] [4]. Assuming that the lymph node metastases represent a migrated fraction of the primary tumor cells, the metastatic cells would, conceivably, share an identical molecular profile [5]. Recent research has shown that the heterogeneity and clonal diversity seen in breast cancer contradict this notion [6]-[11].

In view of these findings, we analyzed the expression of all the human epidermal growth factor (EGF) receptors and their activating ligands in primary breast carcinoma and correlated it with their expression in the axillary lymph node metastasis and with normal breast tissue.

The EGF family comprises four structurally similar tyrosine kinases known as human epidermal growth factor receptor 1 to 4 (HER1-4). The receptors are abundant in numerous epithelia where their normal cell functions involve proliferation, differentiation, apoptosis, migration, and angiogenesis [1] [12]. In several epithelial cancers, including breast cancer, dysregulation of EGF receptors and their functions promotes carcinogenesis [13]-[15]. EGF receptors are transmembrane glycoproteins with an extracellular ligand-binding domain. The activating ligands are receptor specific [16]. The Epidermal growth factor (EGF), amphiregulin (AMPH), and transforming growth factor-α (TGF-α) activate HER1. Heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EPR), and betacellulin (BTC) activate both HER1 and HER4. The neuregulins (NRG) activate HER3 and HER4 [17] [18]. HER2 has no activating ligand, but possesses a constitutively active conformation activated upon dimerization. Ligand binding facilitates hetero- or homodimerization between two EGF receptors. This dimerization leads to cross phosphorylation of intracellular tyrosine kinase domains, and docking sites for signaling proteins are created.

Conceptually, the complex and interactive mechanisms of actions of the EGFR family, justifies investigation of all four receptors of the EGF family as an entity [18]-[20] as a supplement to the studies describing the receptors individually. Comparative studies exist on the HER2 expression in the primary tumors, lymph node metastases, and distant metastases [21]-[24]. In these studies semi-quantitative methods were used and the samples collected at different time points during the disease, primarily from distant metastases. Overall HER2 tends to correlate well between primary tumors and metastatic sites but significant variations in discordance rates have been reported (2% - 27%) [25]. Cardoso *et al*. studied the correlation of HER2 in primary breast carcinoma and lymph node metastases in a large archival material (n = 370) [11]. In this study the overall percentage of discordant marker status was 2%; however, for the tumors that were lymph-node positive, 15% were negative in the primary tumor. The HER2 study by Santiago *et al*. of 52 breast carcinomas and matched axillary metastasis showed an 88.5% concordance using IHC and 98% using FISH [26]. They concluded that HER2 status is stable during axillary metastatic progression.

In this study, we made a point in determining correlation using a quantitative method (real-time RT-PCR) at the time of primary surgery. At this point in the time course of the disease, the clinical decisions regarding adjuvant therapies are taken for each individual patient. We correlated the expression of the EGF receptors and their ligands in paired samples of primary breast carcinomas and corresponding lymph node metastases. Furthermore, we investigated whether their expression in the primary carcinomas could predict the metastatic status of the axillary lymph node.

2. Materials and Methods

The Regional Ethics Committee Northern Jutland, Denmark, approved this prospective cohort study, and signed informed consent was obtained from each patient (N-20070047).

2.1. Patients

One hundred and seventy-nine women with primary operable breast cancer treated at the Department of Breast Surgery, Aalborg Hospital, participated in the study. Inclusion took place during the prevalent screening phase. Patients with a medical history of cancer and patients treated with neoadjuvant therapy were not included. Patients with multicentric cancers were excluded (n = 12). Furthermore, 4 patients were excluded because they had a noninvasive lesion (ductal carcinoma in situ) or the invasive focus was less than 3 mm. Tissue specimens were successfully examined in 163 patients (**Figure 1**). The clinicopathological characteristics of the cohort are listed in **Table 1**.

2.2. Tissue Specimens

Breast tissue specimens were obtained from primary breast cancer surgical procedures. The samples were prospectively collected from November 2008 to May 2010 from unfixed mastectomy or lumpectomy specimens. All tissue specimens were transported on ice from the operating room to the Institute of Pathology, Aalborg Hospital. The normal breast tissue, tumor specimens, and lymph node samples were all frozen in liquid nitrogen within a mean period of 40 (95% c.i.: 39 to 42 min, range 20 to 79) minutes after surgical removal. A pilot study performed on samples from 10 patients showed stable mRNA quantities of HER1-4 at 15, 30, and 60 minutes after surgical removal (unpublished data), and similar results have been published by Ohashi *et al*. [27].

Normal breast tissue was sampled during macroscopic pathoanatomical examination by experienced breast pathologists. The distance between the location for tumor sampling and normal breast tissue sampling was measured in the surgical resections (n = 158). The mean distance was 48 mm (95% c.i.: 43 to 53 mm, range: 4 to 150 mm).

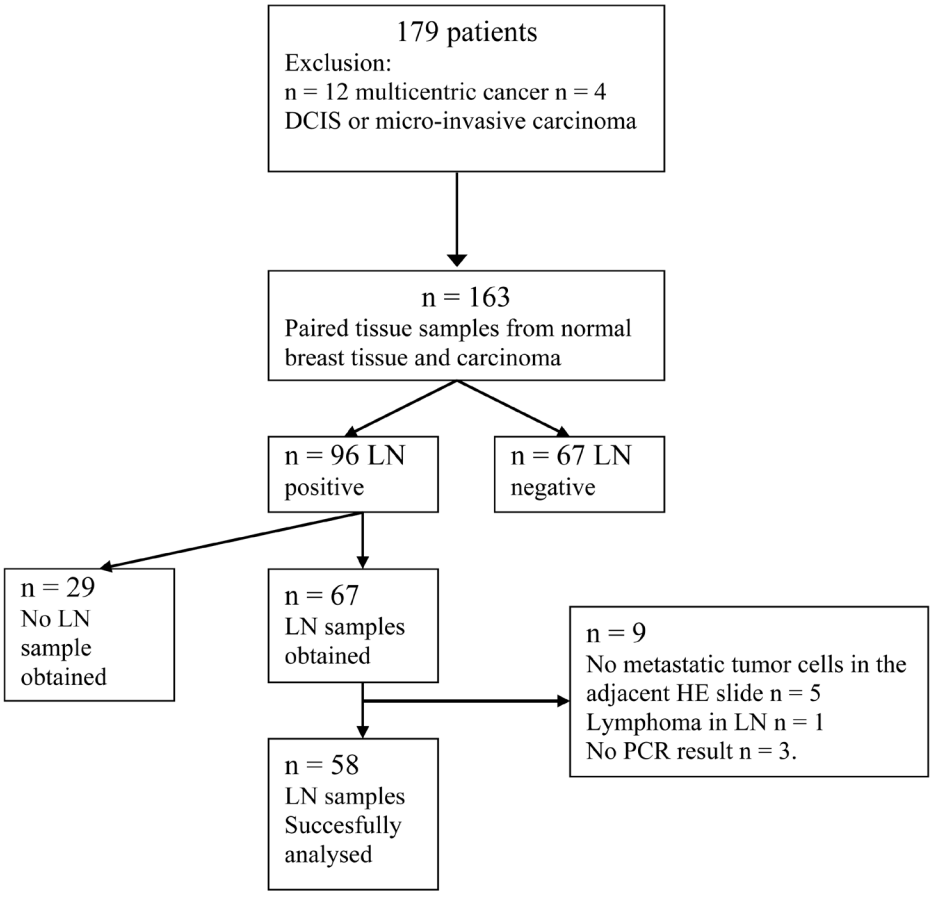

Figure 1. Flow diagram showing included and excluded patients with breast carcinoma, and lymph node status, and the tissue samples obtained. LN: Lymph node.

Table 1. Clinicopathological characteristics for 163 patients with breast carcinomas. Estrogen and HER2 status was determined by routine diagnostic immunohistochemistry/FISH (see methods section).

Total number of patients	163
Gender	Female
Age at diagnosis mean, (range) years	63 (32 - 85)
Histology, n (%)	
Invasive ductal carcinoma	129 (79%)
Invasive lobular carcinoma	23 (14%)
Other	11 (7%)
Tumor size, n (%)	
<20 mm	89 (55%)
20mm ≤ size < 50mm	70 (43%)
≥50mm	4 (2%)
Malignancy grade, n (%)	
Grade 1	53 (32%)
Grade 2	58 (36%)
Grade 3	49 (30%)
Not graded	3 (2%)
Estrogen receptor status, n (%)	
Positive	142 (87%)
Negative	21 (13%)
HER2 status, n (%)	
Normal expression	126 (77%)
Overexpression	15 (9%)
Unknown	22 (14%)
Axillary lymph node status, n (%)	
Lymph node positive	96 (59%)
Lymph node negative	67 (41%)

Tumor specimens were collected as complete 1 to 2 mm cross sectional slides and sampled at random into RNase free tubes, and immediately frozen and stored at minus 80˚C. If the tumor diameter exceeded 5 cm, the pathologist chose a representative slide of macroscopically vital tumor. To confirm the content of invasive carcinoma, the adjacent tumor cross sectional slide was immediately fixed in neutral-buffered formalin and prepared for microscopy using an in-house HE staining. The estrogen receptor was stained with the SP1 clone. The IHC HER2 immunostain was PATHWAY (4B5), Ventana, Roche and FISH was performed with HER2 FISH pharmDx, DAKO.

Lymph node samples were complete 20-µm sections collected from either sentinel lymph nodes (n = 23) or axillary dissections (n = 35). The adjacent slide was used as a control to confirm the content of metastatic carcinoma.

2.3. RNA Extraction from Tissue Samples

Total RNA was extracted from frozen tissue samples by the principles described by Chomczynski and Sacchi [28]. Due to the adipose nature of breast tissue, optimal RNA extraction was performed using a lipid tissue kit (RNeasy Lipid Tissue Kit, Qiagen). Depending on the individual tissue sample weight, we used the RNeasy midi or mini kits following the manufacturer's instructions. In brief, the tissue samples were homogenized in QIAzol Lysis Reagent on ice. After incubation for 5 minutes at room temperature, 1 ml chloroform was admixed by shaking followed by 5 min centrifugation (5000 × *g*) at 4˚C. The aqueous phase, now containing the RNA, was transferred to a fresh tube. An equal volume of 70% ethanol was added and the suspended RNA was transferred to an RNeasy spin column and centrifuged for 5 min at 5000 ×*g*. Flow-through was discarded and the membrane washed followed by centrifugation (5000 × *g*). While RNA remained bound to the RNeasy membrane, the DNA was removed by DNA digestion. The DNases were removed by buffer washings, each followed by centrifugation ensuring that no residual ethanol was carried over. In a fresh tube, the RNA was finally eluated in RNase free water with 2 centrifugations (5000 × *g*).

The yield of total RNA was determined by UV spectrophotometry (absorbance at 260 nm).

2.4. Reverse Transcription

cDNA was transcribed from the RNA extracted from the tissue samples using oligo (dT) priming. The HER2 analysis was a template specific fluorescent probe assay (taqman®).

A total RNA amount of 0.1 µg was reversely transcribed in a 20 µL reaction mixture containing 2 µL 10× PCR buffer (Applied Biosystems, Foster City, CA., USA), 5 µL $MgCl_2$ (6.3 mmol/L), 8 µL of deoxyribonucleoside triphosphates (dATP, dTTP, dGTP, and dCTP, 25 mmol/L), 2.5 mmol/L 16mer oligo dT nucleotide, 20 units RNase inhibitor (Applied Biosystems), 50 units reverse transcriptase (Applied Biosystems), and 1 µL nuclease free-water.

Reverse transcription was performed in a thermocycler (Gene Amp PCR system 9700, Applied Biosystems) at 42˚C for 30 minutes followed by 98˚C for 1 minute, and finally at 4˚C for 5 minutes. The resulting cDNA was immediately used for RT-PCR, or stored at minus 20˚C.

Analyses of all target genes were performed on the same cDNA preparation, thereby minimizing variation.

2.5. Real-Time PCR

The EGF system including HER1-4, CYT1-2, and their activating ligands and the household genes were quantified by real-time PCR with the primers and reaction conditions (**Table S1**). One µL of the cDNA was used as template and 5 µL Light Cycler 480 SYBR green I master mix (Roche Mannheim, GE) supplemented with 0.5 µL sense and antisense primers and probes (Primers and conditions as shown in **Table S1**). The volume was adjusted to 10 µL with nuclease-free water.

The samples were amplified in the Light Cycler 480 system (Roche, Light Cycler software, version 1.5.0), and PCR performed with an initial denaturation step at 95˚C, immediately followed by annealing (annealing temperatures given in **Table S1**) for 15 seconds. Quantification was done with the second derivate max method by the Light Cycler software.

2.6. The Calibration Curve

The LightCycler software constructs calibration curves based on serial dilutions of the individual calibrators in water (calibrators listed in **Table S1**). The fitted regression line of the calibrator dilution provides the read of the sample concentration. The results are expressed relative to the mRNA content in the calibrator used for generating the calibration curve.

The interassay coefficient of variation (CV) for HER1-HER4 and HMBS was 7% - 12%, calculated for 10 real-time PCR runs. For CYT1 and CYT2 and the ligands, it was 4% - 28% in 10 runs.

2.7. Normalization

In order to standardize initial RNA quantities in different samples, an endogenous reference gene was used for normalization. We used the Microsoft Excel add-in application Norm Finder [29] to rank the gene expression

stability of 5 household genes. Analyzing the 3 investigated tissue types, normal breast tissue, breast carcinoma, and lymph nodes, for 5 household genes enabled us to identify the most stable reference gene.

2.8. Identification of the Reference gene

To determine a stable expressed household gene we examined 85 samples from 35 patients comprising 35 normal specimens, 35 carcinomas, and 15 lymph node samples. The mRNA expressions of hydroxymethylbilane synthase (HMBS), *β*-Actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ) and beta-2-microglobulin (B2M) were quantified with real-time PCR. The household gene with the most stable expression was identified as HMBS with the NormFinder application [29]. The candidate genes are ranked by the NormFinder application according to their stability values. Based on the results, we employed HMBS as the reference gene for analyses of the EFG receptors and ligands.

2.9. Statistics

Data were analyzed using STATA version 10 (StataCorp LP, Texas, USA) and graphic statistic illustrations using GraphPad Prism 5 statistical software package (GraphPad Software Inc., San Diego, CA., USA). A non-parametric test was used to analyze the data. Two-sided P-values less than 0.05 were considered to be significant. Paired analyses of the paired samples were done by Wilcoxon matched-pairs signed-rank test. The correlations of the receptors and their ligands were performed by Spearman non-parametric correlation. Comparison of the lymph node-negative patients and the lymph node-positive patients was performed by Mann-Whitney U-test and Fisher's exact test.

3. Results

We examined the mRNA expressions of the four EGF receptors (HER1-HER4) including the 2 HER4 isomeric splicevariants (CYT1 and CYT2), and 11 of their activating ligands in paired samples of normal breast tissue and carcinoma specimens from 163 patients. In 58 of these patients a corresponding metastatic lymph node was obtained for analyses. The clinicopathological characteristics of the patients are described in **Table 1**.

3.1. The Receptors

There was a significant difference in mRNA expression of all four HER receptors comparing normal breast tissue with carcinoma. HER2, HER3, and HER4 (including the two isomeric splicevariants of HER4, CYT1 and CYT2 (data not shown)) were upregulated in carcinomas (**Figure 2**). In contrast, HER1 showed a significantly higher mRNA expression in the normal breast tissue specimens compared with the carcinomas.

The paired analyses of breast carcinoma versus lymph node metastases showed a significant difference regarding HER3 and HER4, whereas there was no difference in the expression of HER1 and HER2 between the 2 locations (**Figure 2**).

3.2. Receptor Correlations between the 3 Locations

The receptors were individually correlated between the 3 different locations from which the tissue samples had been obtained. Significant correlations between primary carcinoma and the lymph node metastases were found for all receptors except HER3 (**Table 2**). Between normal breast tissue and carcinoma, only HER1 showed a significant correlation.

3.3. Combinations of Receptors Were Correlated within the 3 Locations

The mRNA expressions of any combination of HER2, HER3, and HER4 were all highly significantly correlated ($P < 0.05$) in normal breast tissue as well as in carcinoma (**Table 3**), while any combinations of receptors involving HER1 did not show significant correlations.

Interestingly, in the lymph node metastases the expression of HER3-HER4 was the only receptor combination that showed a significant correlation.

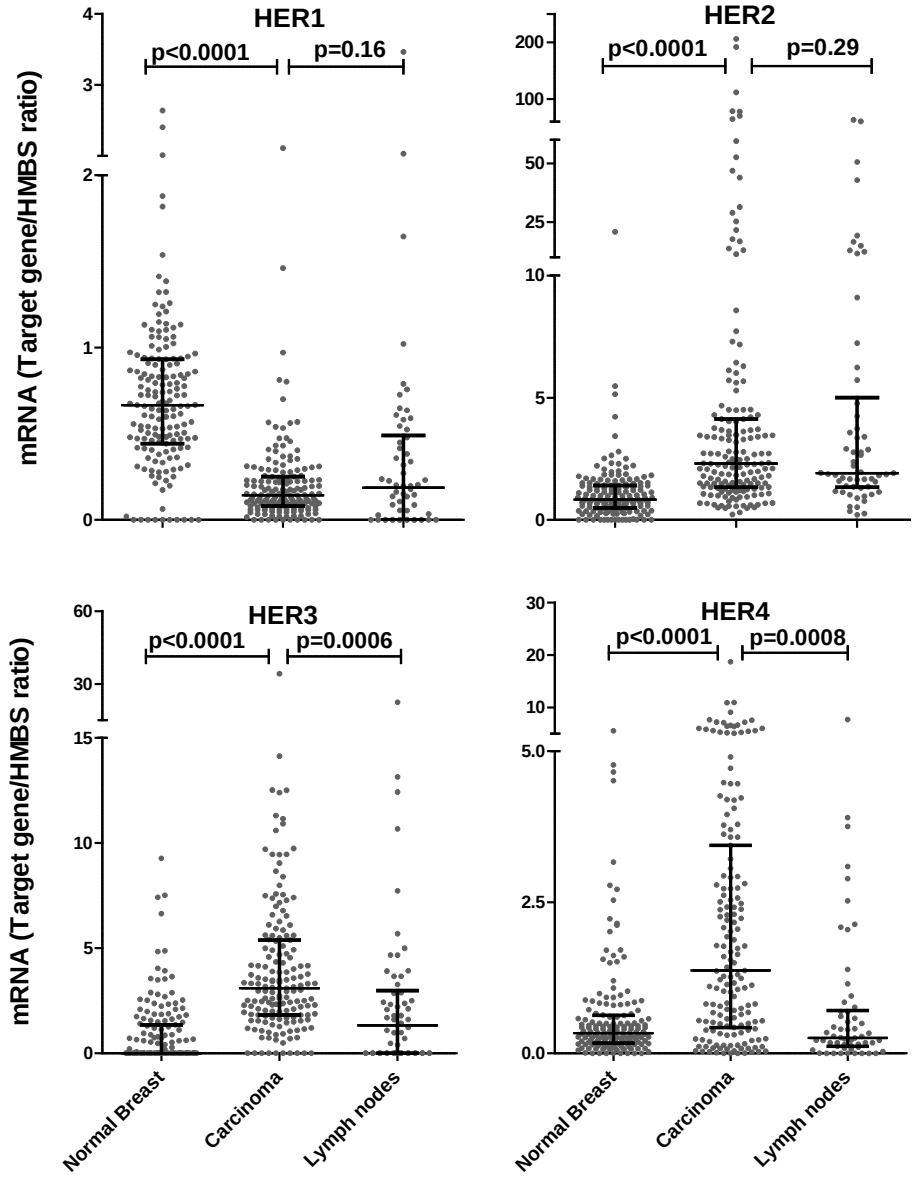

Figure 2. Paired expression of HER1–4 at 3 locations in breast cancer patients: Normal breast tissue (n = 163), breast carcinoma (n = 163), and lymph node metastases (n = 58). All data are the ratio of the target gene and the household gene, HMBS, given in arbitrary units. Medians with interquartile ranges are presented. P values were determined by Wilcoxon matched-pairs signed-rank test. Note: units are non-comparable between receptors.

Table 2. Correlations of the expression of the EGF receptors between the 3 locations by Spearman non-parametric correlation. Significant P values are marked with asterisks.

	Normal breast-carcinoma (n = 163)		Carcinoma-lymph node metastasis (n = 58)	
	Correlation	P value	Correlation	P value
HER1	0.19	0.013*	0.48	<0.0001*
HER2	0.083	0.29	0.58	<0.0001*
HER3	0.14	0.073	0.15	0.28
HER4	0.12	0.14	0.29	0.026*

Table 3. Correlations of the paired expressions of HER1-HER4 within 3 locations in breast cancer patients: Normal breast tissue (n = 163), breast carcinoma (n = 163), and lymph node metastases (n = 58). Analysed by Spearman non-parametric correlation. Significant P values are marked with asterisks.

	Normal breast tissue		Breast carcinoma		Lymph node metastases	
	Correlation	P value	Correlation	P value	Correlation	P value
HER1-HER2	0.085	0.28	−0.063	0.42	−0.12	0.36
HER1-HER3	−0.007	0.93	0.079	0.31	0.16	0.22
HER1-HER4	0.012	0.88	0.020	0.80	0.12	0.35
HER2-HER3	0.39	<0.0001*	0.28	0.0003*	0.23	0.081
HER2-HER4	0.49	<0.0001*	0.16	0.041*	0.062	0.64
HER3-HER4	0.47	<0.0001*	0.50	<0.0001*	0.54	<0.0001*

3.4. Receptors in Lymph Node-Positive and Lymph Node-Negative Patients

We explored the difference in EGF receptor expressions in the primary carcinomas for lymph node-negative patients (n = 67) as compared to lymph node-positive patients (n = 96). No difference was observed between the groups for any of the individual receptors. However, the combination of low HER3 and low HER4 expression in the primary carcinoma was significantly more frequent in lymph node-negative patients than in lymph node-positive patients, the distribution is indicated in **Table 4** (Fisher's exact test, P = 0.011).

3.5. The Ligands

We observed significant different expression levels between normal breast tissue and carcinoma, and between carcinoma and lymph node metastases, for the majority of the ligands. As compared to normal breast tissue we found breast tumors to show an upregulation for AMPH and EPI and a downregulation for HB-EGF and all neuregulins except NRG3. TGF-α and NRG3 showed no difference in expression levels between the 3 locations.

Data for all the ligands investigated are given in **Figure S1**.

3.6. Correlations of the Receptors and Their Activating Ligands

The correlation of the individual receptors and their activating ligands are listed in **Table S2**. HER1 correlated with HB-EGF in normal breast tissue, and this was also seen in carcinoma specimens in which AMPH/HER1 was also correlated.

In normal breast tissue HER3 correlated with NRG1α, NRG1β, NRG2α, and NRG3. HER4 correlated with NRG1α, NRG1β, NRG2α, NRG2β, and NRG3.

In carcinoma both HER3 and HER4 correlated with NRG2α and NRG4. Additionally, HER4 correlated with HB-EGF and NRG2β.

Notably, we could demonstrate correlation patterns for the ligands in both normal breast tissue and in the carcinoma. As a HER1 and HER4 activator, HB-EGF proved to be correlated to HER1 in both locations, even though HER1 showed a low mRNA expression in carcinomas. Furthermore, HB-EGF and HER4 correlations appeared in the carcinomas. Likewise, NRG2α and NRG4 remained correlated to both of the 2 receptors that these ligands can activate (HER3 and HER4), and for HER4 the NRG2β correlation was also retained.

The correlations of NRG1α, 1β, and NGR3 with both HER3 and HER4 were not repeated in the carcinoma specimens or in the metastatic lymph nodes.

4. Discussion

In this prospective cohort study of tissue samples from breast cancer patients, we have investigated the expression patterns of receptors and ligands of the EGF system. Comparison of normal breast tissue and carcinoma showed a significant upregulation in the mRNA expression of HER2, HER3, and HER4, whereas HER1 was downregulated in carcinomas. HER2 and HER3 mRNA overexpression in carcinomas is supported by previous

Table 4. Distribution of patients with the combination of low mRNA expression of HER3/HER4 and their lymph node status in patients with breast cancer (n=163). *Fisher's exact test (P = 0.011).

	Low HER3 and low HER4		
	Low	High	Total
+LN metastases	25	71	96*
−LN metastases	31	36	67*
Total	56	107	163

reports describing co-expression, high prevalence, and potent mitogenic signaling of this particular heterodimer in breast cancer [31]-[33]. HER3 stands out as the only EGF receptor lacking intracellular tyrosine kinase activity, but recent evidence from experimental models suggest that its non-catalytic functions are critical for cancers driven by the EGF receptor partners [34] [35]. Therefore, the importance of HER3 is being revisited [36] [37]. In a study of 278 tissue samples from breast cancer patients correlating IHC and FISH to survival, Sassen *et al*. found HER3 to have a negative impact on disease-free survival [19]. We confirm that HER2 and HER3 are overexpressed and significantly co-expressed in breast carcinomas; a prerequisite to be the most prevalent heterodimer [38]. Also, HER3 was significantly correlated to the mRNA expression of the HER3 activating ligands, NRG2α and NRG4, in breast carcinoma. Considering this to indicate a high protein expression of both receptors and ligands these findings imply that an active signaling network using these 2 ligands and HER2 and HER3 is present.

We report HER4 overexpression in primary carcinomas, in accord with previous results [33]. The functional significance of this is controversial [39]. The HER4 receptor appears to possess divergent functions demonstrated *in vitro* to depend on the isoform of the receptor [40] [41]. The HER4 response can also depend on the activating ligand [42], or the localization in the cell compartments (membrane bound, cytoplasmatic or in the nucleus) [43]. Changes in HER4 expression during the metastatic process [10] [44] lead to the conclusion that the HER4 receptor is adaptable and that the cell response can be different depending on the stimuli.

The HER1 receptor was the only of the EGF receptors in our study that showed a lower expression in the carcinomas compared with the normal breast tissue. Low HER1 mRNA expression in breast carcinoma has also been reported by Witton *et al*. [33]. Witton reports that patients with mRNA overexpression of HER1 and a HER1 positive IHC staining (16% of all cases) had reduced overall survival. The role of HER1 as a carcinogenic driver in breast cancer is well established [45] but the apparent carcinoma downregulation and presumed alternation in function has yet to be explored.

Our paired analyses between primary carcinoma and lymph node metastases showed that HER1 and HER2 were not significantly altered in expression but highly correlated. These findings provide important support for the current clinical practice for evaluating HER2 in the primary breast carcinomas on the assumptions that the protein expressions are identical to those in the minimal residual disease. In other words, the targets of the postoperative adjuvant therapy with Herceptin and Lapatinib are assumed to be present in the minimal residual disease when overexpressed in the primary carcinoma. Obviously this conclusion based on our quantitative mRNA measurements will have to be further validated in clinical trials.

Although, the mRNA expression of HER3 and HER4 are significantly lower in normal breast tissue than in the primary carcinoma, the expression of the receptors are significantly correlated at both sites. Interestingly, this correlation pattern of HER3 and HER4 was the only one to reoccur in the lymph node metastases. Combined with the mRNA expressions of the HER3 and HER4 activating ligands we describe plausible transformations of the EGF system during neoplastic progression.

The carcinomas maintain and increase several of the EGF expression patterns of normal breast tissue (**Figure 2** and **Table 2**). Only bipotent ligands (HB-EGF, NRG2α, 2β, and NRG4) capable of activating more than one receptor type and AMPH (the only ligand showing a highly significant increase in mRNA expression (**Figure S1**)) correlated to the receptors they activate in breast cancer. These findings regarding HB-EGF, AMPH, and NRG2 expression in breast cancer concur with the ligand study by Révillion *et al*. [46], but the correlation to the receptors they can activate has not been described previously.

The numerous described characteristics of normal breast tissue that are also seen in carcinoma specimens are not present in the lymph node metastases, with the exception of the HER3-HER4 correlation. The expression levels of the neuregulins cover a considerably wider range in the lymph node metastases than in the carcinomas, exemplified by NRG2α and NRG3 in **Figure S1**. We assume that in the lymph node metastases the activating ligands would either come from the tumor cells or from the blood supply because stromal cells are not always present. The alterations of the expression of the EGF system in the lymph node metastases point to the carcinoma surroundings as the most obvious physical difference between the carcinoma of the breast and the lymph node metastases. Stromal-epithelial interactions are characteristic of breast carcinomas, but juxtacrine signaling mechanisms are also a possible alternative way of receptor activation [47].

The expression of the individual EGF receptors in the primary tumor could not discriminate lymph node-positive patients from lymph node-negative patients. However, the combination of low expression of HER3 and HER4 in the primary carcinomas could distinguish the 2 groups. The combination of low HER3 and HER4 expression in the primary carcinoma was significantly more frequent in lymph node-negative patients than in lymph node-positive patients, and we interpret this as a positive prognostic indicator. On the other hand, in the primary carcinoma HER3 and HER4 could promote tumor growth by ligand specific activation of NRG2α and NRG4.

In conclusion, HER2, HER3, and HER4 showed increased mRNA expression in carcinoms and were positively correlated to each other and to specific activating ligands. The combination of low HER3 and low HER4 expression in the primary carcinoma was significantly more frequent in lymph node-negative patients as compared to lymph node positive patients.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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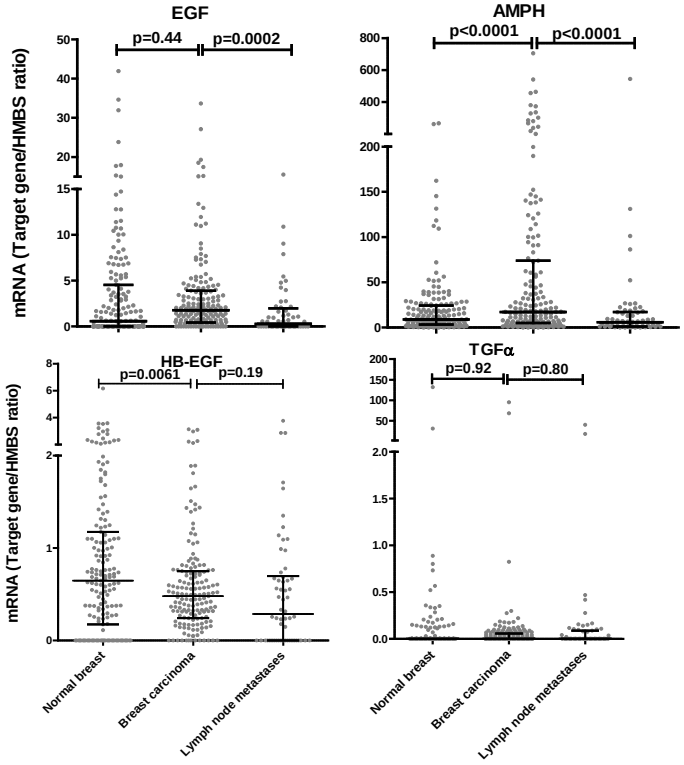
Supplementary Tables and Figures

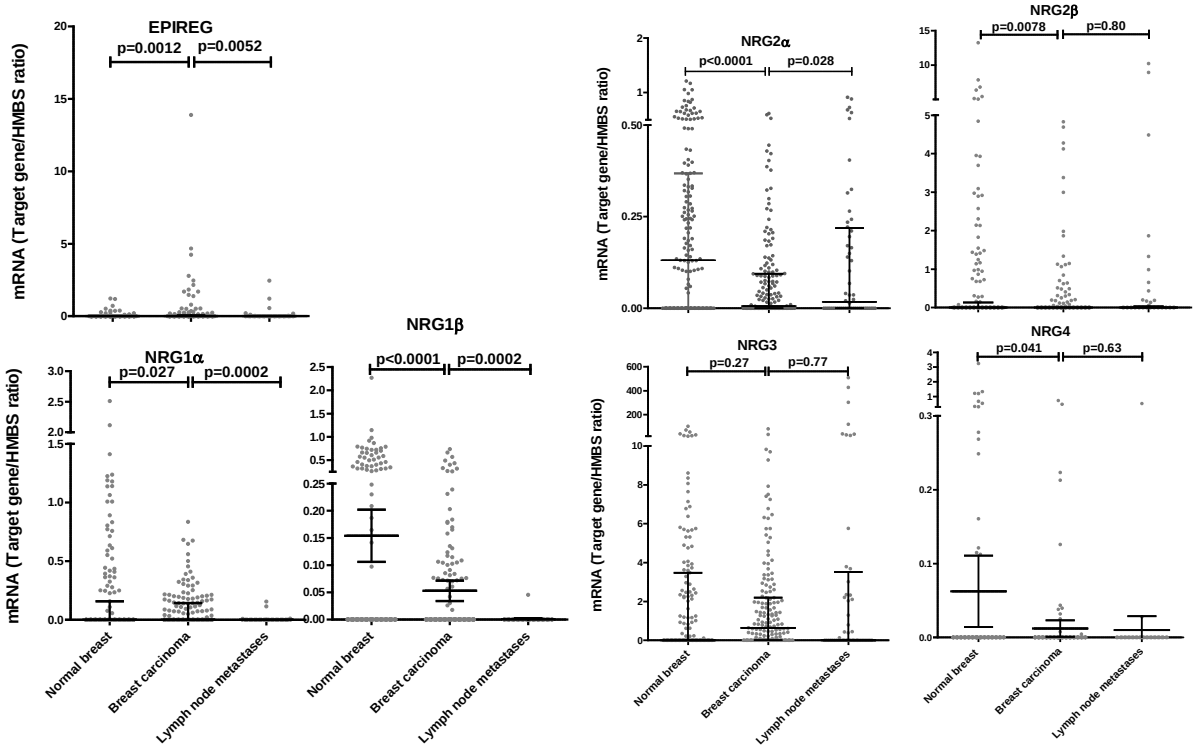
Table S1. Primers and conditions used for RT-PCR of EGF receptors and ligands.

Assay	Forward	Reverse	Primer conc./Annealing temp	Amp. size	Cali-brator
HER1	5’ -GAG AAC GCC TCC CTC A -3’	5’ -GGT ACT CGT CGG CAT C -3’	5 pmol/54℃	261 bp	HCV
HER2*	5’ -CCA GGA CCT GCT GAA CTG GT -3’	5’ -TGT ACG AGC CGC ACA TCC -3’	5 pmol/59℃	209 bp	HCV
HER3	5’ -GGT GCT GGG CTT GCT TTT -3’	5’ -CGT GGC TGG AGT TGG TGT TA -3’	5 pmol/65℃	365 bp	HEC1A
HER4	5’ -ACA GCA GTA CCG AGC CTT TGC G -3’	5’ -GCC ACT AAC ACG TAG CCT GTG AC -3’	5 pmol/64℃	141 bp	KLE
CYT1	5’ -GAT GAT CGT ATG AAG CTT CCC A -3’	5’ -AGG AGG AGG GCT GTG TC -3’	5 pmol/60℃	221 bp	CYT1
CYT2	5’ -GAT GAT CGT ATG AAG CTT CCC A -3’	5’ -CGG TAT ACA AAC TGG TTC CTA TTC -3’	5 pmol/60℃	194 bp	CYT2
EGF	5’ -GAC TTG GGA GCC TGA GCA GAA -3’	5’ -CAT GCA CAA GTG TGA CTG GAG GT -3’	5 pmol/66℃	90 bp	KLE
HB-EGF	5’ - GGT GGT GCT GAA GCT CTT TC -3’	5’ -CCC CTT GCC TTT CTT CTT TC -3’	5 pmol/61℃	282 bp	HCV
TGF-α	5’ -GCC CGC CCG TAA AAT GGT CCC CTC -3’	5’ -GTC CAC CTG GCC AAA CTC CTC CTC TGG G -3’	5 pmol/70℃	528 bp	HCV
Epiregullin	5’ -AAA GTG TAG CTC TGA CAT G -3’	5’ -CTG TAC CAT CTG CAG AAA TA -3’	10 pmol/60℃	238 bp	HCV
Amphiregullin	5’ -GGC TCA GGC CAT TAT GC -3’	5’ -ACC TGT TCA ACT CTG ACT GA -3’	10 pmol/58℃	266 bp	HCV
NRG1-α	5’ -ATC CAC CAC TGG GAC A -3’	5’ -TTT GGA TCA TGG GCA -3’	5 pmol/60℃	179 bp	KLE
NRG1-β	5’-TAG GAA ATG ACA GTG CCT C -3’	5’ -CGT AGT TTT GGC AGC GA -3’	5 pmol/65℃	321 bp	KLE
NRG2-α	5’ -AAA TAT GGC AAC GGC AG -3’	5’ -CGC AAA GGC AGT TTC T -3’	5 pmol/60℃	308 bp	RT4
NRG2-β	5’ -GTC TTA CGT CAA CAG CG -3’	5’ -CCG GTG TAT CCC ACA G -3’	5 pmol/63℃	236 bp	PANC
NRG3	5’ -ACA GTG CAA GCG AAA AC -3’	5’ -CAC TAT GAT ATG AGG GCG -3’	5 pmol/61℃	256 bp	KLE
NRG4	5’ -CTG TTG TCT GCG GTA TTC -3’	5’ -TCA TTC TTG GTC AAG AGA GT -3’	5 pmol/61℃	107 bp	RT4
HMBS	5’ -CGG TAC CCA CGC GAA TCA C -3’	5’ -GGG TAC CCA CGC GAA TCA C -3’	5 pmol/59℃	64 bp	HCV
GAPDH	5’ -TGA TGA CAT CAA GAA GGT GGT GAA G -3’	5’ -TCC TTG GAG GCC ATG TGG GCC AT -3’	5 pmol/68℃	240 bp	HCV
B2M	5’ -TGA CTT TGT CAC AGC CCA AGA TA -3’	5’ -AAT CCA AAT GCG GCA TCT TC -3’	15 pmol/64℃	84 bp	HCV
ACTB	5’ -AGG GGC CGG ACT CGT CAT ACT -3’	5’ -GGC GGC ACC ACC ATG TAC CCT -3’	10 pmol/68℃	202 bp	HCV
YWHAZ	5’ -ACT TTT GGT ACA TTG TGG CTT CAA -3’	5’ -CCG CCA GGA CAA ACC AGT AT -3’	5 pmol/59℃	71 bp	HCV

*The HER2 analysis was a template specific fluorescent probe 5’ -CAG ATT GCC AAG GGG ATG AGC TAC CTG -3’ (taqman®) 10 pmol. HCV (HCV29): Non-malignant bladder cancer cell line; HEC1A: Endometrial cancer cell line; KLE: Endometrial cancer cell line; CYT1: Human urothelial cancer cell line T24 transfected with the CYT1 variant of HER4; CYT2: Human urothelial cancer cell line T24 transfected with the CYT2 variant of HER4; RT4: Urothelial cell line; PANC (PANC1): Human pancreatic cancer cell line.



(a)



(b) (c)

Figure S1. Expression of 11 EGFR family ligands in 3 locations in breast cancer patients: Normal breast tissue (n = 163), breast carcinoma (n = 163), and lymph node metastases (n = 58). All data are the ratio of the target gene and HMBS given in arbitrary units. Medians with interquartile ranges are presented. P values determined by Wilcoxon matched-pairs signed-rank test.

Table S2. Correlations of the expression of the EGF receptors and their activating ligands in normal breast tissue (n = 163, breast carcinomas (n = 163) and lymph node metastases (n = 58) of breast cancer patients. The correlations are determined by Spearman non-parametric correlation. Significant P values are marked with asterisks.

		Normal breast tissue		Breast carcinoma		Lymph node metastases	
Receptor	Activating ligand	Correlation	P value	Correlation	P value	Correlation	P value
HER1	EGF	−0.052	0.51	0.11	0.17	0.019	0.89
	AMPH	0.092	0.25	0.27	0.0005*	0.073	0.59
	HB-EGF	0.42	<0.0001*	0.43	<0.0001*	0.23	0.09
	TGF-*α*	−0.12	0.13	0.086	0.27	0.099	0.46
	EPIREG	0.025	0.75	−0.017	0.83	0.062	0.64
HER3	NRG1*α*	0.42	<0.0001*	0.12	0.11	0.11	0.42
	NRG1*β*	0.18	0.021*	−0.086	0.28	0.16	0.23
	NRG2*α*	0.23	0.0027*	0.20	0.011*	0.12	0.38
	NRG2*β*	0.011	0.89	0.061	0.44	−0.041	0.76
	NRG3	0.23	0.0036*	0.022	0.78	−0.071	0.59
	NRG4	−0.064	0.42	−0.26	0.0010*	0.14	0.29
HER4	HB-EGF	0.099	0.21	0.22	0.0048*	0.25	0.055
	EPIREG	0.15	0.054	0.11	0.17	−0.014	0.92
	NRG1*α*	0.23	0.0037*	0.11	0.16	−0.090	0.50
	NRG1*β*	0.20	0.0093*	0.047	0.55	0.13	0.33
	NRG2*α*	0.32	<0.0001*	0.24	0.0017*	0.14	0.30
	NRG2*β*	−0.19	0.017*	0.19	0.019*	−0.07	0.60
	NRG3	0.023	0.0029*	0.14	0.083	0.013	0.92
	NRG4	−0.073	0.36	−0.22	0.0046*	−0.22	0.096