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ErbB/HER ligands in human breast cancer, and relationships with their receptors, the bio-pathological features and prognosis

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Background: The aim of this study is to provide an expression profile of *ErbB/HER* ligands in breast cancer. We analysed the relationships with their receptors, the bio-pathological features and prognosis.
Patients and methods: Epidermal growth factor (*EGF*), transforming growth factor-α (*TGFα*), amphiregulin (*AREG*), betacellulin (*BTC*), heparin-binding *EGF*-like growth factor (*HB-EGF*), epiregulin (*EREG*) and neuregulins1–4 (*NRG1–4*) were quantified in 363 tumours by real-time reverse transcription–polymerase chain reaction using TaqMan probes.
Results: Ligands were detected in 80%–96% of the cases, except *NRG3* (42%) and *EREG* (45.5%). At least one ligand was expressed in 304 cases (cut-off: upper quartile). Almost all combinations of receptor and ligand co-expressions were observed, but *TGFα* is preferentially expressed in tumours co-expressing *EGFR/HER3*, *NRG3* in those co-expressing *EGFR/HER4*, *AREG* and *EREG* in those co-expressing *HER2/HER4*. *EGF* and *AREG* were associated with estradiol receptors, small tumour size, low histoprognostic grading, high HER4 levels. *TGFα*, *HB-EGF* and *NRG2* were negatively related to these parameters. In Cox univariate analyses, *EGF* was a prognostic factor.
Conclusion: Our study demonstrates that (i) *ErbB/HER* ligands, including *BTC* and *EREG*, are expressed in most breast cancers; and (ii) *TGFα*, *HB-EGF* and *NRG2* high expressions are related to the biological aggressiveness of the tumours.
Key words: breast cancer, *EGF*-related ligands, *ErbB/HER* receptors, expression profile, prognosis

introduction

The *ErbB/HER* receptors, also called type I growth factor receptors, are Epidermal growth factor (*EGF*)-receptor (EGFR/c-*erbB1*/*HER1*), c-*erbB2*/*HER2*/neu, c-*erbB3*/*HER3* and c-*erbB4*/*HER4* [1]. Their activation, resulting from the formation of homo- and heterodimers, depends on their ligands. Some of these ligands bind exclusively to *EGFR*, such as *EGF*, transforming growth factor-α (*TGFα*) and amphiregulin (*AREG*), or bind exclusively to *HER4*, such as *NRG* 3 and 4 (*NRG3* and *NRG4*). Others have a dual specificity and bind either both EGFR and *HER4*, such as betacellulin (*BTC*), heparin-binding *EGF*-like growth factor (*HB-EGF*) and epiregulin (*EREG*), or bind both *HER3* and *HER4*, such as *NRG1* and *NRG2*. Putative ligands of *HER2* have been characterized [2] but, as yet, no ligand binding directly *HER2* has been identified. However, it is demonstrated that *HER2* is the preferred heterodimerization partner of the three other receptors [3].

The *ErbB/HER* receptors are involved in development and progression of a variety of human cancers. Therapeutic approaches, based on monoclonal antibodies (mAbs) and tyrosine kinase inhibitors, have therefore been developed to target these receptors. In breast cancer, amplification and/or overexpression of *HER2* are observed in ∼25% of the cases [4], while they does not occur in normal tissue. Trastuzumab, a recombinant humanized mAb directed against *HER2*, is routinely used for treatment of metastatic breast cancer in patients with *HER2*-positive tumours. It also improves outcomes among women with surgically removed *HER2*-positive breast cancer, when combined with adjuvant chemotherapy [5–7]. Despite the selection of the *HER2*-positive metastatic breast cancer patients, the response rate to trastuzumab used as a single agent does not exceed 35% [8], and ranges from 50% to 84% when combined with first-line chemotherapy [9–11]. This suggests that additional biomarkers could be useful to better predict the response to trastuzumab.

Several lines of evidence indicate that the *ErbB/HER* ligands could be implicated in the unresponsiveness to the treatments targeting *HER2*. The anti-*HER2* murine mAb 4D5 does not inhibit the proliferation of the *HER2* overexpressing breast cancer cells BT474, if treated with *EGF*, *BTC* and *HRG* [12]. A tissue microarray analysis of breast cancer patients treated with combination of chemotherapy and trastuzumab demonstrated that response to treatment depends on the

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expression of *HER2*, and also of the other receptors of the family, their ligands and the activation of downstream signalling proteins [13]. Unresponsiveness to trastuzumab also seems to be associated with a *TGFα*-related mechanism of escape to trastuzumab-induced *HER2* endocytosis and down-regulation [14]. Interestingly, Menendez et al. [15] recently reported that patients with breast cancer overexpressing heregulin and activated *HER2*, but without *HER2* overexpression, could benefit from therapy combining trastuzumab and chemotherapy. All these observations indicate that a better characterization of the *ErbB*/*HER* ligand/receptor network in breast cancers could be useful to predict responsiveness or unresponsiveness to the drugs targeting the *ErbB*/*HER* receptors.

We already reported the messenger RNA (mRNA) expression of the four *ErbB*/*HER* receptors in a large series of human primary breast cancers [16]. In the present paper, we assess the mRNA expression of their 10 ligands in the same tumour samples providing for the first time an expression profile of the whole *ErbB*/*HER* ligand/receptor network in a large sample set of breast cancers. The relationships with *ErbB*/*HER* receptors, the classical biological, clinico-pathological factors and the prognosis are presented.

patients and methods

patients

With the agreement of the investigator's Institutional Review Board, 363 breast cancer samples were obtained from patients undergoing surgery for locoregional disease in the Centre Oscar Lambret (anticancer centre of the North of France) from May 1989 to December 1991. The population and the treatments have been already detailed [16]. The median duration follow-up of living patients was 77.6 months; there were 94 deaths and 126 relapses.

isolation of total RNA

The total RNA was isolated (RNeasy Mini Kit; Qiagen, Courtabœuf, France) from 40 mg of each tumour sample [16]. The purity of the RNA was checked by the ratio between the absorbance values at 260 nm and 280 nm and ranged between 1.8 and 2.1 demonstrating the high quality of the RNA. This was confirmed in 52 (16%) randomly selected samples either by electrophoresis in 1.5% agarose gel containing ethidium bromide or using an Agilent 2100 Bioanalyzer.

PCR primers and TaqMan fluorogenic probes

The polymerase chain reaction (PCR) primers (Qbiogene, Illkirch, France) and the TaqMan fluorogenic probes (Eurogentec, Seraing, Belgium) were designed using the Primer Express software program (Applied Biosystems, Courtaboeuf, France). Their sequences were as follows: sense 5′-caggtaatggagcgaagctttca-3′, antisense 5′-gtgcatcgacatagttcattcttcttg-3′, probe 5′-tctcctatcagctaacccattatggcaaca-3′ for *EGF* (199 bp); sense 5′-ttaatgactgcccagattccca-3′, antisense 5′-ggaggtccgcatgctcaca-3′, probe 5′-cgtgcaccaacgtacccagaatgg-3′ for *TGFα* (133 bp); sense 5′-ccaaaacaagacggaaagtga-3′, antisense 5′-tgttactgcttccaggtgctc-3′, probe 5′-ttgcctccctttttctttctttttgg-3′ for *AREG* (175 bp); sense 5′-ttcactgtgtggtggcagatgg-3′, antisense 5′-tccgctttgattgtgtggtgg-3′, probe 5′-tgcacagttttcctcagggtctccacaga-3′ for *BTC* (111 bp); sense 5′-cctcctctcggtgcggg-3′, antisense 5′-agtcaccagtgccgagagaactg-3′, probe 5′-ccaccgacggcagcagcttcatg-3′ for *HB-EGF* (86 bp); sense 5′-atcatgtatcccaggagagtccag-3′, antisense 5′-aagtgttcacatcggacaccagt-3′, probe 5′-tccatgcaaacaatagccattcatgtcaga-3′ for *EREG* (207 bp); sense 5′-ccattagaatatcagtatccacagaagg-3′, antisense 5′-ccttctccgcacattttacaaga-3′, probe 5′-tctacatccaccactgggacaagcc-3′ for *NRG1* (99 bp); sense 5′-tccccagccttcctaccgtt-3′, antisense 5′-tgtagtcgtgagttctttctgccg-3′, probe 5′-cggttgagctccttgccatccttg-3′ for *NRG2* (102 bp); sense 5′-cgaggacagtgcaagcgaaa-3′, antisense 5′-ttggtcaatgcagagtctctttgtatt-3′, probe 5′-cacagcctttctccccctgagtcccac-3′ for *NRG3* (117 bp); and sense 5′-aacagatcacgaagagcccтgt-3′, antisense 5′-tgggaatagtaggtatcacataacaaagc-3′, probe 5′-cccattcaggcaaaacgacttgtgactg-3′ for *NRG4* (86 bp).

Total gene specificity of the sequences was confirmed carrying out BLASTn searches against the non-redundant set of GenBank, European Molecular Biology Laboratory and DNA Data Bank of Japan database sequences. The PCR products were checked by automated sequencing (ABI PRISM 3100-Avant Genetic Analyzer System; Applied Biosystems).

RT–PCR conditions

Fifty nanogram of total RNA were reverse transcribed in a one-step methodology [16]. Optimal concentration of $MgCl_2$ was 2mM for *HB-EGF*; 4 mM for *EGF*, *NRG1* and *NRG2* and 5 mM for *TGFα*, *AREG*, *BTC*, *EREG*, *NRG3* and *NRG4*. During PCR, the annealing/extension temperature was 60°C for *AREG*, *EREG*, *NRG1*, *NRG3* and *NRG4*; 61°C for *EGF*; 62°C for *TGFα*; 63°C for *BTC* and 65°C for *HB-EGF* and *NRG2*. A non-template control was included in each experiment.

production of the RNA standards

For each ligand, a PCR was carried out with its specific primers modified at their 5′ end as follows: T7 RNA polymerase promoter sequence (sense primer) and a polyadenylic acid [poly(A)] tail (antisense primer). Each standard was obtained after *in vitro* transcription (RiboMAX™ Large scale RNA Production System T7, Promega, Charbonnières, France) of the PCR product followed by purification (Oligotex mRNA mini kit; Qiagen) of the poly(A) *in vitro* transcript. Its concentration was estimated by measuring the absorbance at 260 nm. The absolute number of standard RNA templates was calculated using the molecular weight of the poly(A) *in vitro* transcript and Avogadro's number. The stock solution was aliquoted and stored at −80°C.

Relative quantification of *ErbB*/*HER* ligands. For each ligand, the quantification of its mRNA level (in copies per microgram total RNA) was carried out by preparing a standard curve using known dilutions of its corresponding standard RNA. Its mRNA level was then normalized to the mRNA level (in copies per microgram total RNA) of the TATA Box Binding Protein gene. This ratio is referred as ligand expression (in arbitrary units).

statistical analyses

The statistical analyses were done using the SPSS software (Version 13.0.1). Correlations between parameters were assessed according to the Spearman non-parametric test. Relationships between qualitative variables were determined using the χ^2 test (with Yates' correction when necessary). Non-parametric Mann–Whitney and Kruskal–Wallis tests were used to compare expression of ligands in subgroups of patients and tumours. Overall survival (OS) and relapse-free survival (RFS) were studied by Kaplan–Meier method analysis. Comparison between curves was carried out by the log-rank test. The proportional hazard regression method of Cox was used to assess the prognostic significance of parameters taken in association.

results

expression of the *ErbB*/*HER* ligands

The distributions of the *ErbB*/*HER* ligands mRNA expression in the tumours are not Gaussian (Figure 1). *EGF*, *TGFα*, *AREG*, *BTC*, *HB-EGF*, *NRG2* and *NRG4* were detected in 96% of the

cases, while *NRG1*, *EREG* and *NRG3* were detected in 80%, 45.5% and 42%, respectively.

co-expression of the *ErbB*/*HER* ligands

Number of *ErbB*/*HER* ligands positively correlate to each other (Table 1). At least one of the ten ligands was expressed at a level higher than its upper quartile value in 84% (304 of 363) of the cases. This percentage was similar in the *HER2*-positive (85.7%, 78 of 91) and in the *HER2*-negative tumours (83.8%, 228 of 272). The incidence of *ErbB*/*HER* ligands positivity ranged from ~40% to 75% (Table 2).

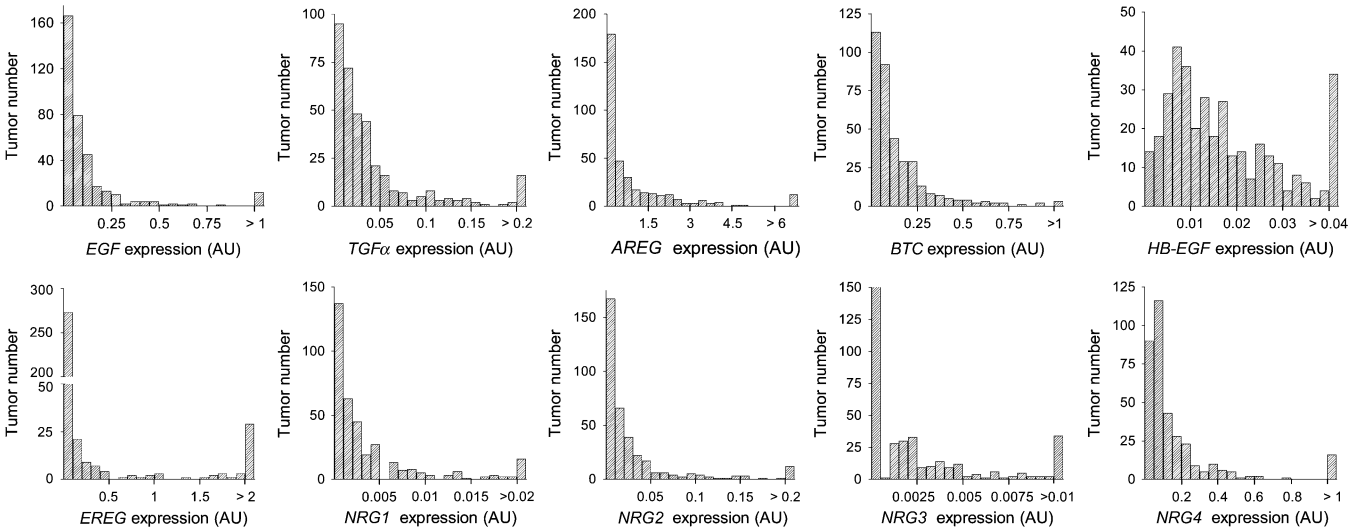


Figure 1. Distribution of 363 breast cancer samples as a function of their mRNA expression of *ErbB*/*HER* ligands, in arbitrary units (AU).

Table 1. Correlations (Spearman test, $N = 363$) between the *ErbB*/*HER* ligands

	TGFα	*AREG*	*BTC*	*HB-EGF*	*EREG*	*NRG1*	*NRG2*	*NRG3*	*NRG4*
EGF									
r	NS	0.23	NS	0.15	−0.18	NS	NS	0.37	0.38
P		<0.001		0.008	<0.001			<0.001	<0.001
NRG4									
r	NS	0.15	NS	NS	NS	NS	NS	0.61	
P		0.005						<0.001	
NRG3									
r	NS	0.17	NS	NS	NS	NS	NS		
P		0.002							
NRG2									
r	0.51	NS	0.24	0.40	0.21	0.39			
P	<0.001		<0.001	<0.001	<0.001	<0.001			
NRG1									
r	0.20	0.14	NS	0.41	NS				
P	<0.001	0.008		<0.001					
EREG									
r	0.17	0.26	0.17	NS					
P	<0.001	<0.001	<0.001						
HB-EGF									
r	0.35	NS	0.21						
P	<0.001		<0.001						
BTC									
r	0.39	NS							
P	<0.001								
AREG									
r	NS								
P									

TGFα, transforming growth factor-α; *AREG*, amphiregulin; *BTC*, betacellulin; *HB-EGF*, heparin-binding epidermal growth factor-like growth factor; *EREG*, epiregulin; *NRG*, neuregulins; *EGF*, epidermal growth factor; *NS*, not statistically significant.

co-expression and relationships with their receptors

The co-expression of the *ErbB*/*HER* ligands and receptors is presented in Table 3. Almost all combinations of receptor and ligand co-expressions were observed. The most frequent co-expressions were *TGFα* in tumours co-expressing *EGFR* and *HER3*, *NRG3* in tumours co-expressing *EGFR* and *HER4*, *AREG* and *EREG* in tumours co-expressing *HER2* and *HER4*.

Several highly statistically significant correlations (Spearman test, $P < 0.001$) were found between the *ErbB*/*HER* ligands and their receptors. They correlated positively with *EGFR*, except *AREG*, *NRG3* and *NRG4*. Considering *HER3* and *HER4*, a positive correlation was observed with *EGF*, *AREG*, *NRG3* and *NRG4* while an inverse correlation was found with *TGFα*, *EREG* and *NRG2*.

Additionally, *EGF* was positively associated to *HER3* (χ^2 test, $P = 0.003$) and to *HER4* ($P = 0.002$). Similarly, *AREG*, *NRG3* and *NRG4* were associated with *HER4* ($P = 0.005$, $P = 0.04$ and $P = 0.01$, respectively). In contrast, *TGFα* was inversely related to *HER3* ($P < 0.001$) and *EREG* to *HER4* ($P = 0.04$). A positive relationship was found between *EGFR* and *TGFα*, *HB-EGF*, *NRG1*, *NRG2* (all, $P < 0.001$) and *BTC* ($P = 0.045$).

relationships with ER and PR

Among the breast cancer samples studied, 73.7% of the cases were estrogen receptor (ER) positive and 72.5% were progesteron receptor (PR) positive. ER and PR were closely correlated ($P < 0.001$), as well as ER and age ($P < 0.001$) and PR and age ($P = 0.019$).

EGF and *AREG* correlated positively to ER ($P = 0.001$ for both) and PR ($P < 0.001$ for *AREG*). A negative correlation was observed between hormone receptors and *TGFα* (ER and PR, $P < 0.001$), *HB-EGF* (ER, $P = 0.008$; PR, $P = 0.018$), *EREG* (ER, $P < 0.001$; PR, $P = 0.01$), *NRG1* (ER, $P = 0.002$) and *NRG2* (ER, $P < 0.001$; PR, $P < 0.001$). Additionally, the χ^2 test revealed that *EGF* and *AREG* were positively associated with the presence of ER ($P = 0.04$ and $P = <0.001$, respectively). In contrast, ER and PR positivity were inversely related to *TGFα*, *HB-EGF* (both, $P < 0.001$) and *NRG2* (ER, $P = 0.007$; PR, $P = 0.004$).

Accordingly, median expression level of *EGF* and *AREG* were 2–4 times higher in ER and PR positive than in ER and PR negative tumours (Figure 2). Inversely, median expression of *NRG2*, *TGFα* and *HB-EGF* was 1.5–3 times higher in ER and PR negative than in ER- and PR-positive tumours (Figure 2). These differences were highly significant for *TGFα*, *AREG*, *NRG2* and *EGF* ($P < 0.001$).

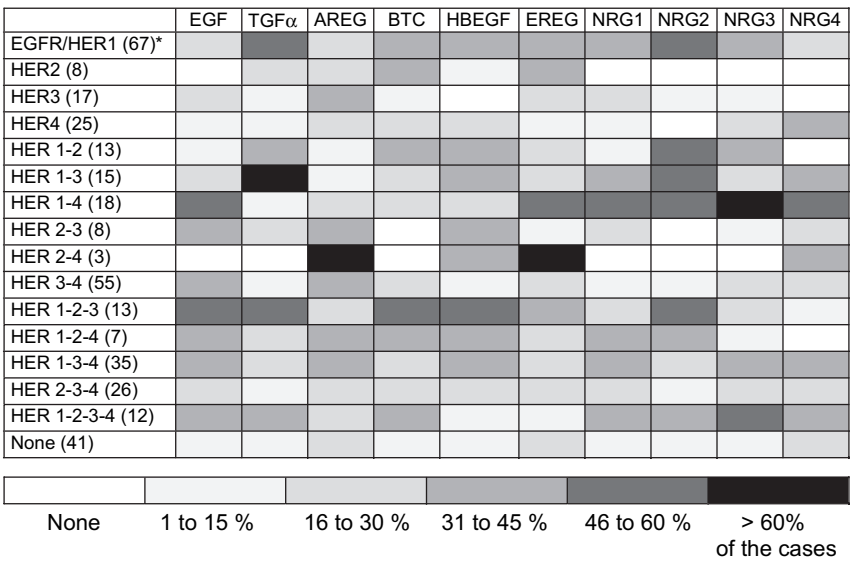
Table 2. *ErbB*/*HER* ligand positivity according to their receptor binding

(%)	[a]Number of positive cases	Positivity
Ligands binding to *EGFR* (*EGF*, *TGFα*, *AREG*, *BTC*, *HB-EGF*, *EREG*)	266	73.3
Ligands binding to *HER3* (*NRG1*, *NRG2*)	139	39.3
Ligands binding to *HER4* (*BTC*, *HB-EGF*, *EREG*, *NRG1*, *NRG2*, *NRG3*, *NRG4*)	269	74.1

[a]Cut-off : upper quartile.

EGFR, epidermal growth factor-receptor; *EGF*, epidermal growth factor; *TGFα*, transforming growth factor-α; *AREG*, amphiregulin; *BTC*, betacellulin; *HB-EGF*, heparin-binding EGF-like growth factor; *EREG*, epiregulin; *NRG1*–4, neuregulins.

Table 3. Co-expression of *ErbB*/*HER* ligands and receptors



*Number in parentheses: number of cases [cut-off for epidermal growth factor-receptor (*EGFR*)/*HER1*, *HER3* and *HER4*: median (16); cut-off for *HER2*: upper quartile].

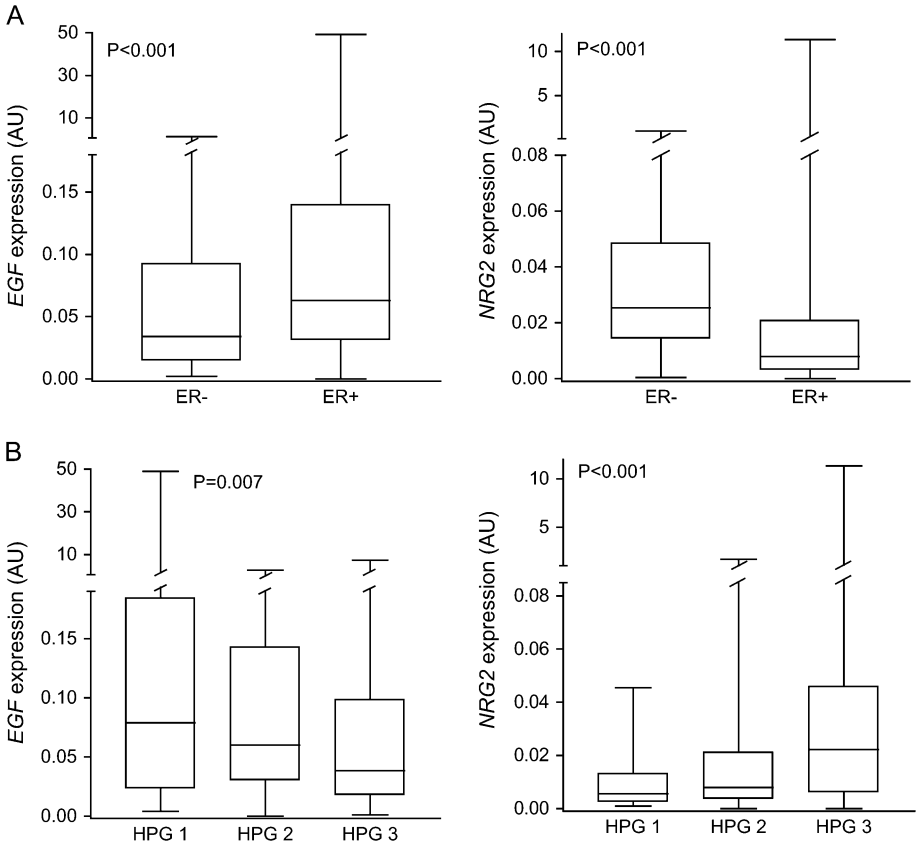


Figure 2. Expression of *EGF* and *NRG2* in breast cancer samples according to ER status (A) and HPG (B).

relationships with the clinico-pathological parameters

EGF was negatively associated with tumour size (χ^2 test, $P = 0.05$) and Histopronostic grading (HPG) ($P = 0.02$), whereas *TGFα*, *HB-EGF* and *NRG2* were related positively to HPG ($P < 0.001$, $P = 0.003$ and $P < 0.001$, respectively).

The median expression level of *EGF* and *AREG* was significantly enhanced in low HPG tumours as compared with high HPG tumours ($P = 0.007$ and $P = 0.001$, respectively), while it was the contrary for *NRG2*, *TGFα* (both, $P < 0.001$), *HB-EGF* ($P = 0.01$) and *BTC* ($P = 0.04$) (Figure 2). Considering the histological tumour type, *EGF* positivity rate was lower in ductal breast cancers ($P < 0.001$) and *BTC* positivity rate was lower in lobular breast cancers.

prognosis studies

For each ligand, the median value of expression and both the lower and upper quartiles were tested for their ability to distinguish between two populations of patients with different prognoses. Regardless of the threshold tested, they were not prognostic factors except *EGF*. Its best cut-off for prognosis was 0.131, corresponding to the upper quartile. Patients with *EGF*-positive tumours exhibited longer survival (Figure 3). In Cox univariate analyses, histoprognostic grading, node involvement, tumour diameter, ER, PR, *HER3*, *HER4*, *EGFR* and *EGF* were prognostic factor for OS, while histoprognostic grading, node involvement, tumour diameter *HER4* and *EGF* were prognostic factor for RFS. In multivariate analyses, *EGF* did not retain its prognostic value. The independent prognostic factors for OS were both tumour size [$P = 0.03$; risk ratio (RR), 1.64] and PR ($P = 0.01$; RR, 0.44), and both tumour size ($P = 0.019$; RR, 1.54) and node involvement ($P = 0.05$; RR, 1.48) considering RFS.

discussion

In this study, we demonstrate that transcripts of *EGF*, *TGFα*, *AREG*, *BTC*, *HB-EGF*, *NRG2* and *NRG4* are present in almost all the human breast cancers, while 42%, 45.5% and 80% of these tumours express *NRG3*, *EREG* and *NRG1* mRNA. *BTC* and *EREG* expressions have never been reported as yet in breast cancer, and this is therefore the first time that the transcripts of the 10 *ErbB*/*HER* ligands are simultaneously analysed.

Our results are in line with studies reporting that *ErbB*/*HER* ligands are expressed at mRNA and protein levels in breast malignant tumours. *EGF* transcripts have been detected in 83% of breast cancers, and EGF protein in ∼15%–80% of the cases [17–19]. About 70% of the breast cancers expressed *TGFα* mRNA [17], and TGFα and AREG proteins have been detected in 30%–80% of these tumours [17, 20]. The expression of the other ligands is largely less documented. HB-EGF has been seen by immunohistochemistry in 53%–78% of breast cancers [18, 21]. Dunn et al. [22] reported that the four NRG were expressed in breast cancers at both the protein and mRNA levels.

It is well established that mRNA expression does not necessarily fit with protein expression. Indeed, gene expression

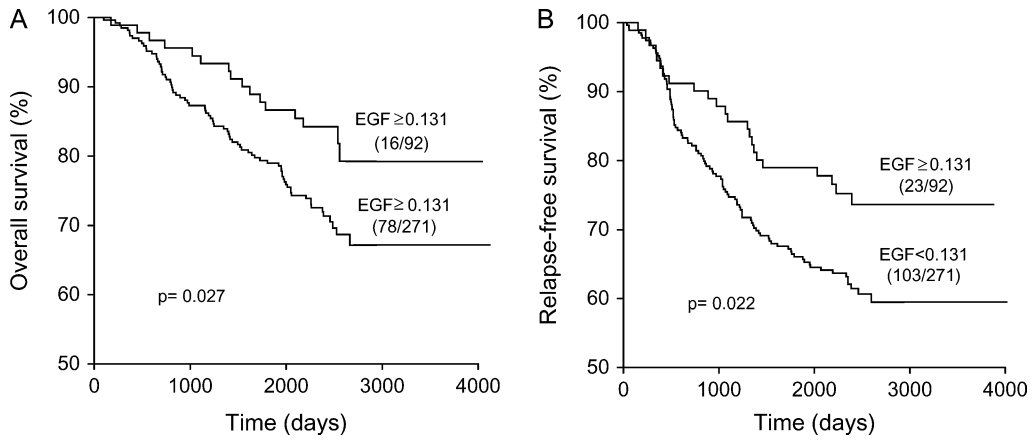


Figure 3. Overall survival (OS) (A) and relapse-free survival (RFS) (B) according to the expression of epidermal growth factor (*EGF*) (cut-off, upper quartile). Numbers in parentheses indicate failures/total number of patients in each group.

is regulated at many levels, including the post-transcriptional down-regulation by microRNAs [23]. However, we demonstrated a close correlation ($P = 0.0067$) between the *HER2* gene expression assessed by reverse transcription (RT)–PCR and oncoprotein expression determined by an enzyme immuno assay [24]. Moreover, several lines of evidence indicate that the mRNA expression of the *ErbB*/*HER* ligands in breast cancer cell lines or breast cancer samples correlate with their amount of protein. Number of studies reported that *EGF* and *TGFα* mRNA expressions were in concordance with the expression of their corresponding proteins [17, 25]. Additionally, *AREG* expression assessed by Northern and/or dot blots was significantly associated with *AREG* expression detected by immunohistochemistry [26]. Moreover, the NRG 1–4 protein expression was related with expression of mRNA, as revealed by RT–PCR and *in situ* hybridization [22]. These observations led us to assume that the *ErbB*/*HER* ligands mRNA quantified in the breast cancer samples might probably reflect their protein expression.

EGF, TGFα and HB-EGF are also produced by normal mammary cells [27–29]. The amount of EGF in serum ranged from 0.2 to 1.14 ng/ml, while it was largely higher in mammary fluids (111–548 ng/ml) or milk (65–140 ng/ml), demonstrating an active production by epithelial cells [27]. Interestingly, high intracystic EGF levels seem associated with an increased breast cancer risk in women with gross cystic disease of the breast [28].

The above results and the fact that the mammary epithelial tumour cells are the major tissue component in primary breast cancers, indicate that the mRNA ligands quantified in the present study are probably expressed by tumour cells. This is supported by numerous *in vitro* studies demonstrating that human breast cancer cell lines express and produce EGF and TGFα [17, 25]. In breast cancer samples, their expression has been visualized on tumour cells [17, 18]. Similarly, HB-EGF and NRG1–4 expression seems localized in the epithelial breast cancer cells [18, 21, 22]. *BTC* mRNA is expressed by the MCF7 breast cancer cells [30]. Although its localization in breast cancer samples has never been reported, this observation provides indirect evidence that the *BTC* mRNA quantified in the present study might indeed arise from the tumour cells. However, we cannot exclude the possibility that the *ErbB*/*HER* ligands mRNA would be also expressed by the other cell types of the tumour. For example, even if Lejeune et al. [26] reported that the immunological staining of AREG was restricted to the tumour epithelium, Ma et al. [20] found expression of AREG in both invasive epithelial tumour cells and stromal cells.

We observed that the mRNA levels of the different ligands are positively correlated to each other. These results indicate that the ErbB/HER ligands share some common mechanisms of regulation, as already reported in the literature, when these growth factors were considered separately [17]. We found that ligands binding *HER3* were expressed in ∼40% of the tumours, and ligands binding *EGFR* or *HER4* in ∼75% of the cases. Our study also revealed that at least one of the 10 *ErbB*/*HER* ligands was expressed at high levels in ∼85% of the breast cancers, whatever its *HER2* status. This finding suggests that *HER2* might be activated in a large number of *HER2*-positive and negative tumours via heterodimerization.

In the present series of biopsies, we previously reported the expression of the *ErbB*/*HER* receptors [16]: here we demonstrate co-expression with their ligands. These observations provide additional evidence that the ErbB/HER ligands might act on breast cancer cells via autocrine or paracrine pathways. We also demonstrate that *EGF* and *AREG* correlated positively to the expression of the four *ErbB*/*HER* receptors. These results corroborate *in vitro* data, demonstrating that EGF increases both protein and mRNA levels of EGFR [31]. Concerning the expression of the other ligands, we observed a similar positive correlation with EGFR while a negative correlation was observed with *HER3* and *HER4*. In a same way, an increase in *EGFR* mRNA induced by TGFα has been described [25]. Almost all combinations of receptors and ligands co-expressions were observed, demonstrating that the expression profile of the ErbB/HER ligand/receptor network is complex. This finding indicates that it would be helpful to evaluate this expression profile before administration of drugs targeting the ErbB/HER receptors, instead of looking at the ErbB/HER ligand/receptor separately.

We demonstrated that the mRNA expressions of both *EGF* and *AREG* correlated positively with ER or PR levels, ER- and PR-positive tumours expressing high levels of *EGF* and *AREG* as previously reported [17, 32]. Conversely, we found negative

correlations between the other ligands and steroid receptors. These results were confirmed by our observation that ER- and PR-negative tumours expressed higher levels of *TGFα*, *HB-EGF* and *NRG2* than their positive counterparts. Similarly, the levels of TGFα are higher in ER-negative than in ER-positive human breast cancer cell lines [17, 25]. The relationships observed in the present study between *ErbB*/*HER* ligands and steroid receptors are in agreement with previous reports demonstrating that some of these growth factors are regulated by estradiol at either transcriptional and/or translational levels [17]. Interestingly, we previously reported such strong correlations between the four *ErbB*/*HER* receptors and the steroid receptors in breast cancer samples [16], and demonstrated that the mRNA expressions of the four *ErbB*/*HER* receptors were down-regulated by estradiol and up-regulated by 4-OH tamoxifen in MCF-7 human breast cancer cells [33].

Our results pointed out that elevated expressions of *EGF* and *AREG* are associated with small tumour diameter and low histoprognostic grading. Conversely, *TGFα*, *HB-EGF* and *NRG2* high expressions are associated with large tumour diameter and high histoprognostic grading. In contrast with our results, it has been reported that *HB-EGF* expression was inversely related to biological aggressiveness of the breast carcinoma [21]. These observations evidence a tumour population with a differentiated phenotype expressing EGF and AREG, HER3 and HER4, steroid receptors, with a small diameter, low histoprognostic grading and node negative. In agreement with Pirinen et al. [34], *EGF* was found to be associated with a more favourable prognosis, in terms of both overall and RFS in univariate analyses.

In conclusion, our study indicates that the 10 *ErbB*/*HER* ligands are co-expressed in a large number of breast cancers and might bind their ErbB/HER receptors, leading to activation of either their own receptors (via homodimerization) or of the other receptors of the family, including *HER2* (via heterodimerization).

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