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Abstract	The levels of expression of the four receptors and eleven ligands composing the epidermal growth factor family were measured using immunohistochemical staining in one hundred cases of breast cancer. All of the family were expressed to some degree in some cases; however, individual cases showed a very wide range of expression of the family from essentially none to all the factors at high levels. The highest aggregate level of expression of a receptor was HER2 followed by HER1, then HER3, then HER4. The ligands (including two splice variants of the NRG1 and NRG2 genes) broadly fell into three groups, those with the highest aggregate expression were Epigen, Epiregulin, Neuregulin 1α, Neuregulin 2α, Neuregulin 2β, Neuregulin 4 and TGFα, moderate expression was seen with EGF, Neuregulin 1β and Neuregulin 3, and relatively low levels of expression were seen of HB-EGF, Betacellulin and Amphiregulin. Statistical analysis using Spearman's Rank Correlation showed a positive correlation of expression between each of the factors. Analysing the data using the Cox Proportional Hazards model showed that, in this dataset, the most powerful predictors of relapse free interval and overall survival were the combined measurement of only Epigen and Neuregulin 4.

Keywords (separated by '-')	ErbB - Growth factor - Growth factor receptor - Prognosis - Breast cancer
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PRECLINICAL STUDY

The complete family of epidermal growth factor receptors and their ligands are co-ordinately expressed in breast cancer

Emmet McIntyre · Edith Blackburn · Philip J. Brown · Colin G. Johnson · William J. Gullick

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Abstract The levels of expression of the four receptors and eleven ligands composing the epidermal growth factor family were measured using immunohistochemical staining in one hundred cases of breast cancer. All of the family were expressed to some degree in some cases; however, individual cases showed a very wide range of expression of the family from essentially none to all the factors at high levels. The highest aggregate level of expression of a receptor was HER2 followed by HER1, then HER3, then HER4. The ligands (including two splice variants of the NRG1 and NRG2 genes) broadly fell into three groups, those with the highest aggregate expression were Epigen, Epiregulin, Neuregulin 1α, Neuregulin 2α, Neuregulin 2β, Neuregulin 4 and TGFα, moderate expression was seen with EGF, Neuregulin 1β and Neuregulin 3, and relatively low levels of expression were seen of HB-EGF, Betacellulin and Amphiregulin. Statistical analysis using Spearman's Rank Correlation showed a positive correlation of expression between each of the factors. Analysing the data using the Cox Proportional Hazards model showed that, in this dataset, the most powerful predictors of relapse free interval and overall survival were the combined measurement of only Epigen and Neuregulin 4.

Keywords ErbB · Growth factor · Growth factor receptor · Prognosis · Breast cancer

Introduction

The epidermal growth factor family of receptors and ligands consist of four genes encoding receptors and at least eleven genes encoding ligands [1]. Four of the ligands, collectively known as the Neuregulins, are expressed as multiple splice variants [2] and the latest receptor to be discovered, HER4, is made in at least four different forms also due to mRNA splicing [3]. The receptors are stabilised in an active state as homodimers or heterodimers following ligand binding [4]. Exactly, which forms are assembled in vivo is contingent on the repertoire of ligands available in the environment and their relative affinities for each receptor type individually and possibly for preferences for binding to particular dimer pairs. We have attempted previously to construct a computer simulation of this process [5] (http://www.cs.kent.ac.uk/people/rpg/em84/CellApplet1.html) in which a patch of cell membrane can be populated with different numbers of each receptor type and each of the eleven ligands can be introduced to initiate the assembly of the various receptor pairwise combinations. This when run to equilibrium should resemble the state of the system in a simple membrane bilayer.

Overexpression of most, if not all, of the receptors and some of the ligands has been detected in breast cancer biopsies and cell lines. Antibodies or small molecule

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tyrosine kinase inhibitors have been evaluated targeted to members of the system and some of these have been introduced as clinical treatments for selected patients with some success [6]. It would be helpful, however, to understand and predict the activation state of the system in individual patients so that the choice of the available inhibitors can be most precisely made to ensure that appropriate drugs are given and that those that are used can be employed most cost-effectively.

Despite nearly 50 years of research and a long term appreciation of the potential importance of this family of molecules in breast and other cancer types as yet there has been no study published to our knowledge that described the expression patterns of the complete family of receptors and ligands in breast cancers at the protein level. Indeed some of the more recently described ligands such as Epigen [7] and Epiregulin [8] have not so far been studied in a series of clinical specimens. We report here using immunohistochemical staining a study describing the complete family in one hundred cases of unselected breast cancers.

Materials and methods

One hundred cases of breast cancer were obtained from Professor Adrian Harris and Dr Russell Leek, Cancer Research UK, Oxford, UK in the form of a tissue array. Ethical approval for use was obtained from Oxfordshire Clinical Research Ethics Committee. The patients were treated by standard protocols, which were updated regularly according to national guidelines. ER positive patients received tamoxifen for 5 years, node positive patients under 60 also received 6 cycles of intravenous CMF. Patients treated with wide local excision also received adjuvant radiation therapy. The composition of the patients is described in Supplementary Table 1 including age range, grade, tumour size, ER status, node status, menopausal status, whether treated by chemotherapy or hormonal therapy and follow up. The study was conducted and reported cohering to the guidelines published in McShane, LM, et al. Reporting recommendations for tumour marker prognostic studies. J Clin Oncol. 2005 Dec 20; 23(36): 9067–9072.

The antibodies used were mostly produced in the laboratory of Professor Gullick (Table 1). The antibody to EGF was a kind gift of the late Dr Harry Gregory. The antibodies to Epigen (Catalogue number AF1127) and to Epiregulin (Catalogue number AF1195) were purchased from R&D Systems, Minneapolis, USA and the antibody to TGFalpha (Catalogue number GF10) from Calbiochem, San Diego, USA. Immunohistochemical staining was performed using the primary antibodies described earlier and the StreptABCcomplex HRP Duet Mouse/Rabbit detection kit from Dako, Denmark. For detection of Epigen and Epiregulin rabbit anti-goat biotinylated IgG (Dako) was used with the kit. Optimisation of the concentration of each antibody was performed prior to its use on the tissue arrays. Tumours were scored for intensity of staining by inspection on an Olympus BX40 microscope with a "double head" by WJG and EM using a scale of 0 = negative, 1 = weak, 2 = moderate and 3 = strong.

Table 1 Antibodies used in this study

EGF receptor	F4	Mouse mAb	Gullick et al. [9]
HER2	21 N	Rabbit polyclonal	Gullick et al. [10]
HER3	RTJ2	Mouse mAb	Rajkumar et al. [11]
HER4	HFR1	Mouse mAb	Srininvasan et al. [12]
EGF		Rabbit polyclonal	From H Gregory
TGFalpha	GF10	Mouse mAb	CalBiochem
Amphiregulin	55AR	Rabbit polyclonal	Saeki et al. [13]
HB-EGF	111HB	Rabbit polyclonal	Chobotava et al. [14]
Epigen	AF1127	Goat polyclonal	R&D Systems
Epiregulin	AF1195	Goat Polyclonal	R&D Systems
Betacellulin	97BTC	Rabbit polyclonal	Srinivasan et al. [15]
NRG1α	76HRG	Rabbit polyclonal	Normanno et al. [16]
NRG1β	102HRG	Rabbit polyclonal	Srinivasan et al. (15)
NRG2α	121NRG	Rabbit polyclonal	Dunn et al. [17]
NRG2β	120NRG	Rabbit polyclonal	Dunn et al. [17]
NRG3	122NRG3	Rabbit polyclonal	Dunn et al. [17]
NRG4	123NRG4	Rabbit polyclonal	Dunn et al. [17]

Results

Each antibody detected specifically its cognate protein in a proportion of cases. Results with antibodies to Epigen and Epiregulin, which have not previously been measured in breast cancer, are shown in Fig. 1a and b. In order to assess the overall expression levels for each protein we summed the scores for the hundred cases. The highest aggregate score for the four receptors was for HER2. It should be noted that this does not reveal heterogeneity of expression between cases, for instance many previous studies have reported that about 20% of breast cancers score 3+ for HER2 but this would not be apparent in this analysis. However, it does demonstrate, in particular with the ligands, some of which have not previously been studied, that there are broad categories of expression present. Highest scoring ligands included Epigen, Epiregulin, Neuregulin 1α, Neuregulin 2α, Neuregulin 2β, Neuregulin 4 and TGFα, moderate expression was seen with EGF, Neuregulin 1β and Neuregulin 3 and low levels of expression were seen of HB-EGF, Betacellulin and Amphiregulin.

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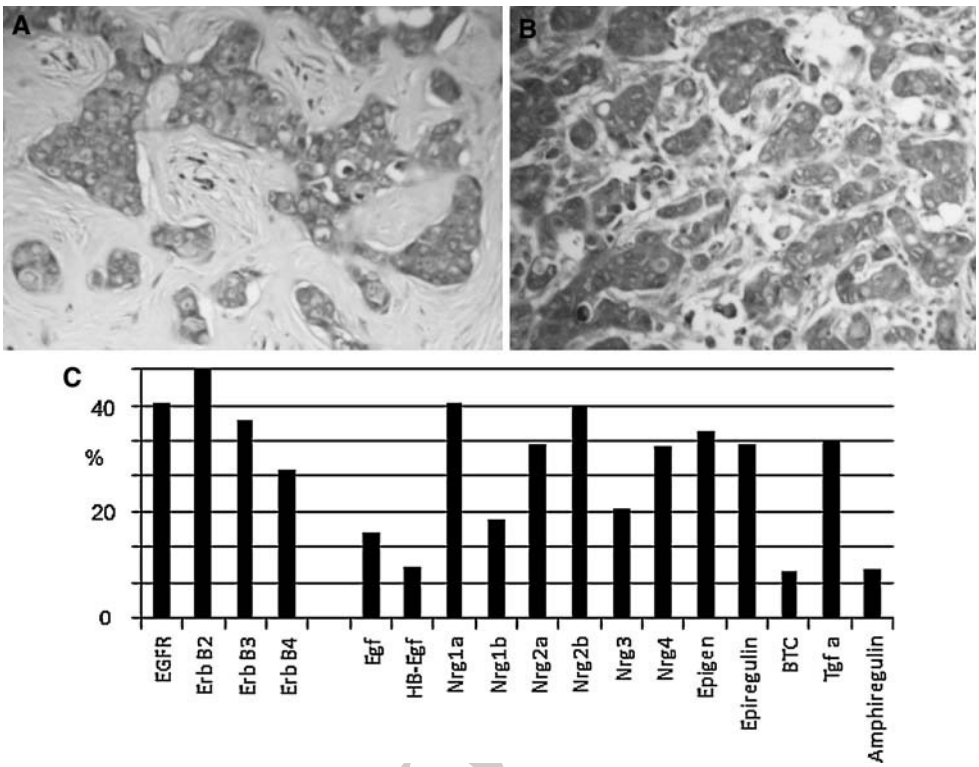

Fig. 1 Example of immunostaining of a case of breast cancer with the antibody to Epiregulin (**a**) and Epigen (**b**). **c** Aggregate scores of the ligands and receptors

The data obtained was analysed for any associations between expression of each ligand and receptor with each of the others using Spearman's Rank Correlation. From the data in Fig. 2a, it can be seen that all the ligands and receptors were positively associated. To provide a visual representation of this large dataset, we have shown the cases ordered on the ordinate in ascending score for total ligands (Fig. 2b, left axis, range 0–33) and shown the total receptor score (range 0–12, right axis). The data reveal a strong association between increasing total ligand score and increasing total receptor score. It is also apparent that there are some cases that essentially lack any receptor or ligand expression at the cut of value scored while other cases showed high levels of almost all the ligands and receptors suggesting very great heterogeneity in the presence of this highly interactive family of signalling molecules between individual cases.

In order to assess the relationship between the expression of the ligands and receptors and clinical and molecular variables, the tumours were divided in three ways. First, they were dichotomised by low and high ligand levels; second, by low and high receptor levels and finally, by low and high aggregate ligand and receptor levels. No significant associations were found although the strongest relationship was between receptor levels and tumour size ($P = 0.06$) (Supplementary Table 2).

Kaplan–Meier curves for overall survival (OS) were generated for all the receptors and ligands based on lack of expression (0) or any level of expression (1–3) (Fig. 3). Several of the factors have not previously been studied in breast cancer and thus the dichotomisation of the data was chosen to ensure as far as possible similar numbers of cases in each category. HER2 expression would normally be divided into low (0–2) versus high (3) as this has been shown previously to give the best discrimination between good and poor survival but it was considered more appropriate in this study to maintain consistency within the analysis. HER2 was separately analysed as a single factor as low (0–2) versus high (3) and, as expected, high expression was associated with reduced OS. Analysis of the survival data using Cox's Proportional Hazards model identified Epigen and Neuregulin 4 as the factors most strongly associated with OS.

Interestingly, expression of Epigen was positively associated with improved survival, and NRG expression was associated with worse OS. Various laboratory studies have shown that different activation states of the EGF family may induce either growth or differentiation and thus in the light of our still imperfect knowledge of the system it is not unexpected that some factors may have opposite effects. In further analysis using the model omitting sequentially the weakest factor (backwards elimination dropping the factor with the smallest positive or negative coefficient), the combination of Epigen ($P = 0.003$) and NRG4 ($P = 0.01$) retained the strongest association with OS (Table 2). In order to assess the influence of these

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A

	AR	Tgf α	BTC	Epireg	Epigen	Nrg4	Nrg3	Nrg2 β	Nrg2 α	Nrg1 β	Nrg1α	HB-Egf	Egf	ErbB4	ErbB3	ErbB2
EGFR	0.42	0.70	0.38	0.28	0.40	0.50	0.64	0.59	0.50	0.67	0.64	0.51	0.67	0.60	0.59	0.66
p-value	5.52E-06	2.20E-16	4.23E-05	0.00244	1.57E-05	3.53E-08	1.76E-13	4.10E-11	5.23E-08	6.25E-15	2.16E-13	2.31E-08	5.24E-15	7.70E-12	3.85E-11	1.78E-14
ErbB2	0.49	0.58	0.42	0.34	0.46	0.62	0.59	0.64	0.62	0.64	0.69	0.44	0.59	0.56	0.51	
p-value	8.24E-08	4.40E-11	6.24E-06	0.0002	6.28E-07	2.15E-12	3.70E-11	2.90E-13	1.63E-12	2.38E-13	< 2.2e-16	1.74E-06	3.13E-11	4.24E-10	1.62E-08	
ErbB3	0.41	0.54	0.48	0.33	0.34	0.59	0.51	0.57	0.55	0.59	0.60	0.42	0.59	0.56		
p-value	6.89E-06	1.76E-09	1.20E-07	0.00038	0.00023	3.84E-11	1.72E-08	1.44E-10	7.81E-10	2.01E-11	7.85E-12	4.61E-06	3.08E-11	4.65E-10		
ErbB4	0.37	0.68	0.38	0.30	0.42	0.63	0.54	0.49	0.65	0.56	0.67	0.37	0.51			
p-value	7.41E-05	1.55E-15	3.30E-05	0.00106	4.20E-06	7.32E-13	1.36E-09	5.44E-08	4.84E-14	3.36E-10	4.36E	6.14E-05	1.59E-08			
Egf	0.57	0.56	0.56	0.18	0.50	0.54	0.63	0.53	0.57	0.60	0.65	0.61				
p-value	1.28E-10	3.38E-10	3.92E-10	0.03666	4.79E-08	1.75E-09	7.38E-13	3.19E-09	2.02E-10	1.21E-11	3.20E-14	5.42E-12				
HBEfg	0.59	0.39	0.65	0.32	0.36	0.53	0.55	0.52	0.47	0.56	0.51					
p-value	2.82E-11	1.92E-05	6.35E-14	0.00049	0.00011	4.40E-09	9.64E-10	7.57E-09	2.62E-07	5.27E-10	1.80E-08					
Nrg1α	0.57	0.68	0.54	0.30	0.45	0.67	0.66	0.75	0.65	0.70						
p-value	2.40E-10	2.21E-15	2.11E-09	0.00112	6.99E-07	4.77E-16	2.78E-14	2.20E-16	3.31E-14	2.20E-16						
Nrg1β	0.52	0.57	0.53	0.22	0.32	0.60	0.65	0.63	0.54							
p-value	1.09E-08	2.12E-10	6.08E-09	0.01355	0.00048	1.11E-11	64304.6	3.90E-13	2.29E-09							
Nrg2α	0.43	0.66	0.51	0.37	0.59	0.70	0.62	0.65								
p-value	2.61E-06	1.78E-14	2.44E-08	5.43E-05	4.15E-11	2.20E-16	9.32E-13	7.60E-14								
Nrg2β	0.61	0.59	0.64	0.36	0.51	0.66	0.60									
p-value	3.90E-12	2.21E-11	1.28E-13	7.97E-05	1.50E-08	1.18E-14	9.14E-12									
Nrg3	0.58	0.58	0.58	0.24	0.50	0.56										
p-value	4.72E-11	7.77E-11	8.05E-11	0.0083	5.21E-08	5.01E-10										
Nrg4	0.45	0.52	0.51	0.35	0.37											
p-value	9.19E-07	8.46E-09	1.62E-08	0.00013	7.14E-05											
Epigen	0.32	0.46	0.39	0.46												
p-value	0.00056	6.78E-07	1.85E-05	3.75E-07												
Epireg	0.26	0.38	0.22													
p-value	0.004	4.45E-05	0.01399													
BTC	0.66	0.37														
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Tgfα	0.35															
p-value	0.00014															

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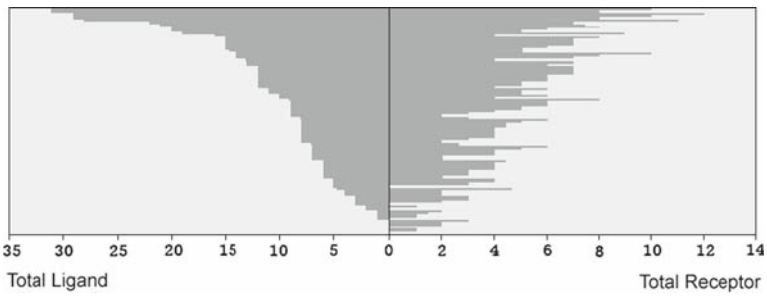

Fig. 2 **a** Spearman's rank correlation analysis of all ligands and receptors. **b** Cases were ordered on the ordinate in increasing ligand score (*left*) and associated receptor scores (*right*)

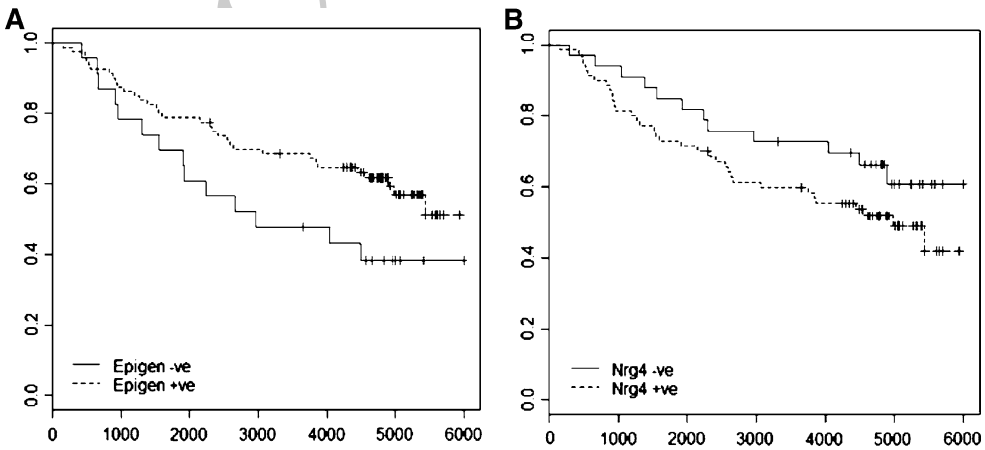

Fig. 3 Kaplan Meier charts showing the survival (days) of the patients based on the level of expression (0 vs. 1–3) of Epigen (*left*) and NRG4 (*right*)

factors in a more molecularly homogeneous group of cases and to see if there were any major effects of treatment, the oestrogen receptor positive cases were analysed separately. Again positive expression of Epigen was associated with good OS ($P = 0.0092$), but NRG2α became the other predictive factor ($P = 0.0057$). The dataset was only hundred cases (although 1,700 data points were acquired for the 17 factors measured) and further studies on larger datasets would be required to confirm or refute these apparent relationships.

Discussion

Each ligand and each receptor were expressed at a range of levels in a proportion of cases of breast cancer in this study.

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Table 2 Cox proportional hazard results for overall survival

	Coeff.	*P*
EGFR	−0.6397	0.240
ErbB2	−0.0235	0.970
ErbB3	0.0812	0.870
ErbB4	0.2826	0.600
Egf	−0.1251	0.800
HBEgf	0.2006	0.720
Nrg1a	−0.7294	0.200
Nrg1b	0.2555	0.570
Nrg2a	0.2613	0.660
Nrg2b	0.8110	0.260
Nrg3	−0.5034	0.270
Nrg4	1.0819	0.062
Epigen	−1.1589	0.019
Epiregulin	0.7059	0.230
BTC	0.3027	0.600
Tgfa	−0.3076	0.570
Amphiregulin	−0.6340	0.230

Statistical analysis of the data revealed a strong associate between the expression of any member of the family and all other members. Although breast cancer is acknowledged, both clinically and by analysis of molecular factors, to be a heterogeneous disease it is still perhaps surprising how different the composition of the factors between cases were. In some individuals (at the precision of measurement available from simple immunostaining), there were essentially no ligands or receptors present. In other individuals, all the receptors and essentially all the ligands were present at the highest quartile of measurement. This suggests that the family may be, in some cases, relatively unimportant whereas in others it clearly has the potential to be an important influence on cell activity. This may also reflect a sensitivity or lack of sensitivity to drugs designed to inhibit this system.

Individual receptors and ligands were, in some cases, associated negatively or positively with shorter relapse free interval or survival. This was not unexpected as some ligands are known to provoke increased rates of cell growth while others appear to stimulate differentiation. Using the Cox's Proportional Hazards model, we show that a combination of Epigen and Neuregulin 4 in this series of cases together gives the greatest separation of aggressive from indolent disease. This result could not be predicted as we are currently unaware of their individual activities in any detail nor their effect on the balance between growth on the one hand and differentiation on the other. It is likely, however, that measuring a subset of the family may allow prediction of the natural history of the disease in some cases. Here two factors emerged, but further test datasets would be required to determine whether this was generalisable. The Neuregulins are produced as multiple splice variants for instance, five have so far been identified as products of the NRG4 gene [18] and these have very different destinations within or without the cell and as such may also have different functions. The antibodies used here to the ligands (where known) are directed to the EGF-like sequence which is shared by all the so far reported splice variants and should thus detect the sum of the expressed gene products. The use of reagents which can discriminate between the splice variants may give a better ability to predict their involvement and influence in the disease.

The use of computer simulations of the EGF system has been an area of considerable study as we have a reasonable knowledge of its constituents and some understanding of how they function. It may be in the future that a "reading" of the family of receptors and ligands (or a subset of them) may be able to more accurately predict prognosis and, more importantly, select patients for treatment with particular combinations of signal transduction inhibitor drugs.

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