Immunoexpression of HER family, neuregulin, MAPK and AKT in invasive ductal carcinomas of the breast

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Summary

Background: The purpose of this study was to investigate the frequency of expression of the erbB/HER family of growth factor receptors, their ligand neuregulinα (NRGα) and the most important pathways activated by HER receptors that are mitogen-activated protein kinase (MAPK) and serine/threonine kinase (AKT) in invasive ductal carcinomas of the breast, not otherwise specified (IDC-NOS). *Methods*: 59 of the IDC-NOS of the breast were studied for ER, PR, EGFR, c-erbB-2, c-erbB-3, c-erbB-4, neuregulin Ab-3, phospho-AKT, and phospho-p44/42 map kinase using the streptavidin-biotin horseradish method. *Results*: Of the 59 tumours, 44 (75%) were ER+, 37 (63%) PR+, four (7%) EGFR+, seven (12%) c-erbB-2+, seven (12%) c-erbB-3+ and 14 (24%) c-erbB-4+α Strong cytoplasmic and/or nuclear immunoexpression was revealed in 17 (29%) cases for NRGα, 13 (22%) cases for p-AKT, and nuclear immunoexpression with p-MAPK was found in 17 (29%) cases. *Conclusion*: The results suggest that high-grade breast carcinomas are not only associated with ER/PR- negativity, but seem to be activated by receptor tyrosine kinase growth factors.

Key words: Breast Carcinoma; HER family; Immunohistochemistry.

Introduction

The HER family of receptor tyrosine kinases (RTKs) consists of four receptors: Epidermal growth factor receptor (EGFR) (also called HER-1 or erbB-1), HER-2 (erbB-2), HER-3 (erbB-3), and HER-4 (erbB-4). The family is associated with extensive receptor-receptor interactions and diversity of ligands. HER ligands can be divided into three groups: The first group includes EGF, amphiregulin (AR), and transforming growth factor α (TGFα) that bind specifically to HER-1. The second group consists of betacellulin (BTC), heparin binding EGF, and epiregulin, which exhibit dual specificity for HER-1 and HER-4. The third group, composed of the neuregulins (also known as neu differentiation factors or heregulins), bind to HER-3 and HER-4 or only HER-4 [1]. Multiple erbB-receptor homo- or heterodimers trigger intracellular signalling leading to specific cellular responses, e.g., stimulation or inhibition of proliferation [2]. The EGF family of RTKs and ligands play an important role in the pathogenesis of breast cancer (BCa) [3-5].

c-erbB-2 is the best studied member of the type 1 growth factor receptor (T1GFR) family and its amplification occurs in 15-25% of BCa cases [6]. This oncogene activates the phospho-inositol-3-kinase (PI-3K) pathway that inhibits apoptosis. The survival signal is also normally coupled to the MAPK pathway. Increased Her-2 expression in cancer enhances and prolongs signalling from both the PI-3K and MAPK pathways [1].

The main purpose of this study was to determine the immunoexpression of the following: erbB/Her family of growth factor receptors and their ligand neuregulin· (NRGα), and the most important pathways activated by HER, MAPK and the serine/threonine kinase AKT in BCa [invasive ductal carcinomas of the breast, not otherwise specified (IDC-NOS)]. A further aim was to investigate coexpressions and correlations with the well-known histopathological prognostic parameters including tumour stage, grade, lymph node status, oestrogen receptor (ER), progesterone receptor (PR) status and clinical outcome.

Materials and Methods

The study included 59 cases of IDC-NOS (mean age 59 years) from modified radical mastectomies or lumpectomies from patients whose complete clinical records and follow-up information were available from Marmara University Hospital, Turkey during the period 1998-2004. All surgical material was fixed in 4% formalin and embedded in paraffin. The tumours were classified according to the pTNM system (sixth edition) and were graded according to Elston & Ellis [7]. The study was approved by the Ethics Committee of the Marmara University Hospital.

Immunohistochemistry

The primary antibodies used are summarised in Table 1. Immunohistochemistry was performed using the streptavidin-biotin immunoperoxidase method (UltraVision Detection System Anti-Polyvalent, HRP; Fremont, CA). Four-micrometer sections were cut on to Menzel SuperFrost® Plus glass slides. The slides were dewaxed overnight in an incubator at 37ºC,

Revised manuscript accepted for publication November 22, 2007

deparaffinized in three changes of xylene and rehydrated in two changes of 95% ethanol. Sections were covered with 3% H2O2 in methanol for 20 min to block endogenous peroxidase activity of the tissue, followed by a washing procedure with distilled water. Antigen retrieval for ER, PR, c-erbB-2, c-erbB-3, c-erbB-4, NRG·, phospho-p44/42 MAP kinase and phospho-AKT antibodies was performed by incubating the slides in a microwave oven (160 W) with 0.01 M citrate buffer (pH 6.0) for 15 min, followed by cooling for 20 min. at room temperature. The slides were incubated with Protease XXV at 37°C for 20 min for EGFR. The slides were washed twice with tris-buffered saline (TBS), and then blocked with normal serum for 5 min. Slides were then placed in a humid chamber and incubated for 30 min with the primary antibody as indicated in Table 1. After two rinses in TBS, the slides were incubated with biotin-conjugated secondary antibody for 10 min at room temperature. The slides were rinsed again and treated with horseradish peroxidase-conjugated streptavidin for 10 min at room temperature. Tissue staining was visualized with a DAB substrate chromogen solution. Slides were counterstained with hematoxylin, dehydrated, and mounted.

Table 1 — *Primary antibodies used for immunohistochemistry*

Antibody	Clone	Dilution	Incubation period
ER	SP1; Lab Vision	1/400	RT 30 min
PR	SP2; Lab Vision	1/400	RT 30 min
EGFR	111,6; Lab Vision	RTU	ON at 4º C
c-erbB-2	e2-4001+3B5; Lab Vision	RTU	RT 30 min
c-erbB-3	HER-3; Lab Vision	RTU	RT 60 min
c-erbB-4	HER-4; Lab Vision	RTU	RT 60 min
Neuregulin Ab-3	NDF/GGF/Neuregulin; Lab Vision	1/150	RT 30 min
Phospho-p44/42 Map Kinase	Thr 202/Tyr 204; Cell Signalling	1/100	RT 60 min
Phospho-AKT	Ser 473; Cell Signalling	1/50	ON at 4º C

Abbreviations: RTU: ready to use, RT: room temperature, ON: overnight

Positive and negative control slides were included in each staining series. No significant staining was observed in the negative controls using serum replacing the primary antibody. The results of the immunohistochemistry procedures were assessed by two pathologists independently and the cases were discussed for resolution where discrepancies occurred.

Evaluation of immunostaining

ER and PR: Staining was scored as positive using a cut-off value of ›10% of the tumour cell nuclei.

EGFR, c-erbB-2, c-erbB-3 and c-erbB-4: no staining or weak incomplete membrane staining in any proportion of tumour cells was scored as 0 or 1+, complete membrane staining that was either non-uniform or weak in intensity but with obvious circumferential distribution in at least 10% of cells was scored as 2+, complete intense uniform membrane staining of > 30% of invasive tumour cells was scored as 3+ and regarded as positive for HER family proteins [8].

EGFR staining was weak or moderately positive on cytoplasmic membranes of myoepithelial cells of all benign ducts whenever they were found around the tumours in the same sections.

p-AKT and NRGα: The same criteria as specified for the HER family above were used. Strong cytoplasmic and/or nuclear staining of > 30% of the tumour cells compared to weak staining of the normal breast tissue as a positive control was evaluated as positive immunostaining for p-AKT and NRGα [9]. Cytoplasmic staining of ductal cells with NRGα in all benign ducts whenever they were found around tumours in the same sections was also noted.

Table 2 — *Results of immunohistochemistry and clinical follow-up of the cases*

Cas	G	pT	N	ER	PR	EGFR	erbB-2	erbB-3	erbB-4	NRG	MAPK	AKT	Clin
1	3	2	0	+	+	–	–	–	+	+	++	–	NED
2	2	3	1	+	+	–	++	–	++	–	–	–	NED
3	2	3	1	–	–	–	++	–	–	–	–	–	NED
4	3	2	1	–	–	–	–	–	–	–	+	–	EX
5	2	1	0	+	+	–	++	+++	+++	++	++	+	NED
6	2	1	0	+	+	–	+	–	–	–	+++	–	NED
7	2	1	1	+	–	–	+++	–	+	++	–	–	NED
8	3	2	1	+	–	–	+++	+	++	–	+	+	NED
9	3	2	1	–	–	+++	+	–	–	++	+	+++	MET
10	3	2	1	–	–	++	+++	–	–	++	–	++	NED
11	3	2	1	+	–	–	–	–	–	–	–	+	NED
12	1	2	0	+	+	–	–	–	–	–	++	+	MET
13	2	2	0	+	+	–	–	–	–	++	–	–	NED
14	1	1	0	+	+	–	–	–	–	+++	–	–	NED
15	2	2	1	+	+	–	++	–	+	–	++	–	NED
16	2	1	3	+	+	–	–	+	++	–	+++	++	NED
17	3	2	1	+	–	–	+++	+	–	–	–	–	NED
18	2	2	X	+	+	–	+	++	++	+	–	++	NED
19	3	2	1	+	+	–	–	–	+	+	+	+	NED
20	3	3	x	+	+	–	–	–	–	–	+	–	NED
21	2	1	1	+	+	–	–	–	+	–	–	–	NED
22	2	2	1	–	–	–	–	–	–	–	+	+	EX
23	3	3	1	–	–	–	–	++	+	–	–	–	NED
24	2	2	2	+	+	–	++	–	–	+	–	–	EX
25	1	1	0	+	+	–	–	–	–	–	–	++	NED
26	2	1	0	+	+	–	–	–	+	–	++	–	NED
27	2	1	0	+	+	–	–	–	+	–	+	–	NED
28	2	2	x	+	+	–	+	++	+	++	+	–	EX
29	1	2	x	+	+	–	–	–	+	–	+++	+++	EX
30	2	2	2	–	–	–	+++	+	–	+++	–	+	NED
31	2	2	2	+	+	–	++	+	–	+	+	+	MET
32	3	2	x	+	–	–	–	–	+	–	–	–	EX
33	3	2	1	+	+	–	–	–	–	++	++	+++	NED
34	2	2	x	+	+	–	++	+	+++	++	–	–	NED
35	2	1	0	+	–	–	++	–	++	+	–	++	NED
36	2	1	0	–	+	–	–	–	–	–	+++	–	NED
37	2	2	2	+	+	–	+	–	+	–	–	–	NED
38	3	3	2	+	+	++	–	–	+	++	++	+++	MET
39	1	1	0	+	–	–	–	–	–	–	–	–	NED
40	2	2	1	+	–	–	++	–	+++	+	–	–	NED
41	1	2	1	+	+	–	–	+	–	++	+++	–	NED
42	2	2	x	+	+	–	–	–	+	++	–	–	NED
43	2	2	0	–	–	+++	–	–	+	–	–	–	NED
44	1	2	1	+	+	–	–	++	++	++	–	+++	NED
45	1	2	1	+	+	–	–	–	+	+	+	–	MET
46	2	2	0	+	+	–	–	+++	+++	++	++	++	NED
47	2	1	0	–	–	–	–	–	+	++	–	–	NED
48	2	2	0	–	–	–	+++	–	–	++	+	+++	EX
49	3	2	1	+	+	–	–	–	++	–	–	+	NED
50	1	2	1	–	–	–	–	–	+	–	–	+	EX
51	3	2	1	–	+	–	–	–	–	–	–	–	NED
52	3	2	1	–	–	+	+	+	+++	–	–	–	NED
53	3	2	0	–	–	–	–	–	+	–	+++	–	EX
54	2	2	2	+	+	–	–	–	+	–	+	+++	EX
55	2	1	0	+	+	–	++	+	++	–	+	–	NED
56	1	1	1	+	+	–	–	–	–	–	++	–	NED
57	2	2	2	+	+	–	–	–	–	+	++	–	NED
58	3	2	x	+	+	–	–	+	+	–	++	+	EX
59	2	2	1	+	–	–	+++	+++	++	+	+	+	NED

Abbreviations: G: grade, pT: stage, N: lymph node, ER: oestrogen receptor, PR: progesterone receptor, NRG: neuregulinα, Clin: clinical follow-up, NED: no evidence of disease, MET: metastatic disease, EX: exitus.

Table 3A — *Results of statistical analysis*

	χ^2	Degrees of freedom	χ^2 p value
G & pT	0.355	4	0.0001
G & N	12,079	6	0.06
G & erbB-2	16,568	6	0.011
pT & N	18.44	6	0.005
N & AKT	15,982	9	0.067
ER& PR	20,972	1	0.0001
ER & EGFR	10,164	3	0.017
PR & erbB-2	13,432	3	0.004
PR & MAPK	10.992	3	0.012
erbB-2 & EGFR	16.146	9	0.064
erbB-2 & erbB-3	17.238	9	0.045
erbB-2 & erbB-4	16.237	9	0.062
erbB-3 & erbB-4	29.89	9	0.0001
Clin & pT	10.703	4	0.030
Clin & N	11.971	6	0.063
Clin & ER	5.486	2	0.064
Clin & erbB-4	14.074	6	0.029
Clin & AKT	12.151	6	0.059

Abbreviations: G: grade, pT: stage, N: lymph node, ER: oestrogen receptor, PR: progesterone receptor, NRG: neuregulinα, Clin: clinical follow-up.

Table 3B — *Results of statistical analysis*

	G	pT	N	ER	PR	EGFR	erbB-2	erbB-3	erbB-4	NRG	MAPK	AKT	Clin
G		+	±	–	–	–	+	–	–	–	–	–	–
pT	+		+	–	–	–	–	–	–	–	–	–	+
N	±	+		–	–	–	–	–	–	–	–	±	±
ER	–	–	–		+	+	–	–	–	–	–	–	±
PR	–	–	–	+		–	+	–	–	–	+	–	–
EGFR	–	–	–	+	–		±	–	–	–	–	–	–
erbB-2	+	–	–	–	+	±		+	±	–	–	–	–
erbB-3	–	–	–	–	–	–	–		+	–	–	–	–
erbB-4	–	–	–	–	–	–	±	+		–	–	–	+
NRG	–	–	–	–	–	–	–	–	–		–	–	–
MAPK	–	–	–	–	+	–	–	–	–	–		–	–
Akt	–	–	±	–	–	–	–	–	–	–	–		±
Clin	–	+	±	±	–	–	–	–	+	–	–	±	

Abbrevations: G: grade, pT: stage, N: lymph node, ER: oestrogen receptor, PR: progesterone receptor, NRG: neuregulin-, Clin: clinical follow-up, +: statistically significant correlation, –: no statistical significance, ±: tendency of correlation.

p-MAPK: < 10% nuclear immunostaining of the tumour cells was scored as 0, between 10-30% was scored as 1+, 30-60% was scored as 2+, and > 60% was scored as 3+. Nuclear immunoexpression of the tumour cells > 30% was scored as 2 + or 3+ positive for p-MAPK.

Statistical analysis: Statistical analysis of possible associations between categorical variables was performed by means of chi-square while coefficient associations were assessed using Somers' D tests. P values of less than 0.05 were considered statistically significant.

Results

Among the 59 IDC-NOS cases studied, 75% (44/59) were ER positive and 63% (37/59) were PR positive (Table 2). There was a statistically significant correlation between tumour stage and lymph node status, and between ER and PR status ($p < 0.05$). With regard to clinical follow-up, 73% (43/59) of the patients were well with no evidence of disease, 8.5% (5/59) had metastatic disease and 18.6% (11/59) died of breast carcinoma. There was a tendency of correlation between ER negativity and poor clinical outcome ($p = 0.064$) (Table 3).

EGFR

EGFR protein expression occurred in 7% (4/59) of the IDC-NOS cases (Table 2) (Figure 1A). There was a statistically significant negative correlation between the presence of ER and EGFR overexpression ($p < 0.05$). There was also a tendency for positive correlation with EGFR and c-erbB-2 ($p = 0.064$) (Table 3). However we could not reveal any statistical significant association between EGFR overexpression and cerbB-3, cerbB-4, NRGα, p-MAPK or p-AKT immunoexpression or the clinical outcome of the patients.

c-erbB-2 protein expression

c-erbB-2 protein overexpression was found in 12% (7/59) of the cases (Table 2) (Figure 1B). There was a statistically significant correlation between c-erbB-2 overexpression and tumour high grade, PR negativity, and c-erbB-3 immunoexpression ($p < 0.05$). There was a positive tendency for correlation between c-erbB-2 overexpression and EGFR immunoexpression ($p = 0.064$) and c-erbB-4 expression ($p = 0.062$) (Table 3). We could not find any significant association between c-erbB-2 overexpression and occurrence of NRGα, p-MAPK or p-AKT immunoexpressions and the patients' clinical outcome.

c-erbB-3

c-erbB-3 protein expression was found in 12% (7/59) of the BCa cases (Table 2) (Figure 1C). There was a statistically significant correlation between c-erbB-3 and c-erbB-2 or c-erbB-4 expression ($p < 0.05$) (Table 3). In contrast, we could not find any significant association with EGFR, NRGα, p-MAPK or p-AKT immunoexpression or the clinical outcome.

c-erbB-4

c-erbB-4 protein expression was found in 24% (14/59) of the BCa cases (Table 2) (Figure 1D). A significant association was found with c-erbB-3 expression ($p < 0.05$) and a positive tendency for correlation with c-erbB-2 overexpression was revealed ($p = 0.062$) (Table 4). There was no correlation with EGFR, NRGα, p-MAPK or p-AKT immunoexpression. There was a significant statistical correlation between c-erbB-4 expression and good clinical outcome ($p < 0.05$) (Table 3).

Neuregulin Ab-3

NRGα expression was found in 29% (17/59) of the BCa cases (Table 2A & 2B) (Figure 1E). We could not demonstrate any significant association with the histopathological prognostic parameters or the proteins that were studied.

Phospho-p44/42 MAP

p-MAPK protein expression was found in 29% (17/59) of the cases (Table 2A & 2B) (Figure 1F). A statistically significant correlation was revealed between p-MAPK

Fig. 1A

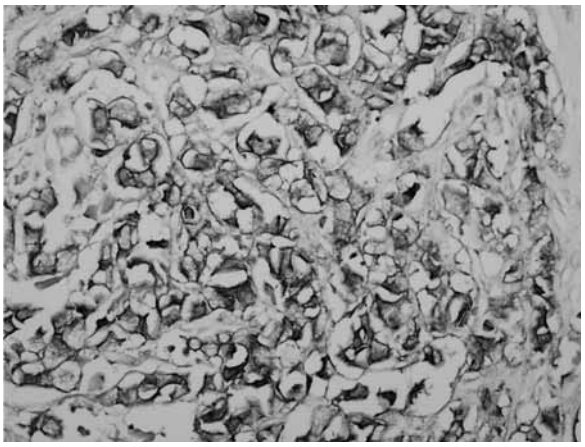

Fig. 1B

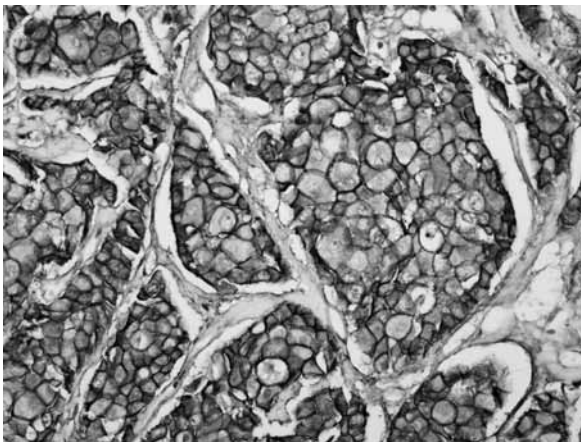

Fig. 1C

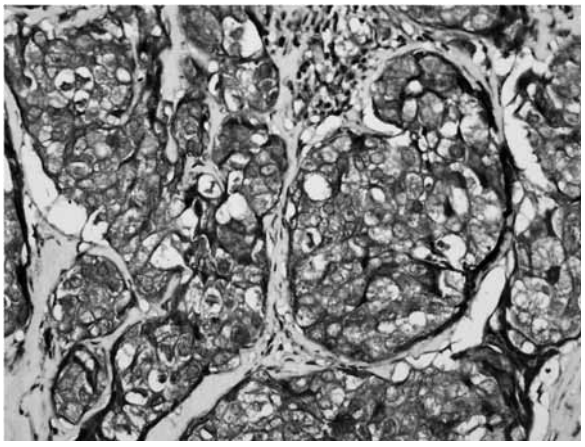

Fig. 1D

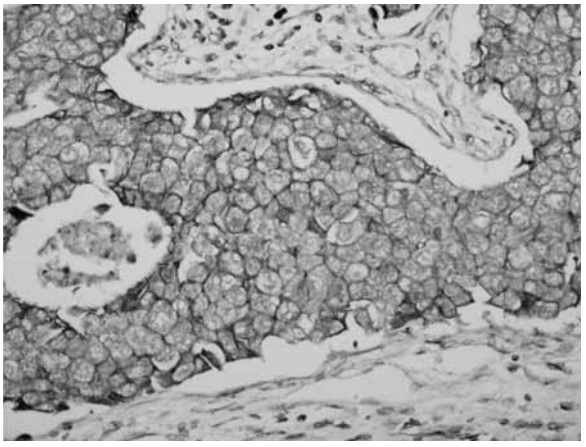

Fig. 1E

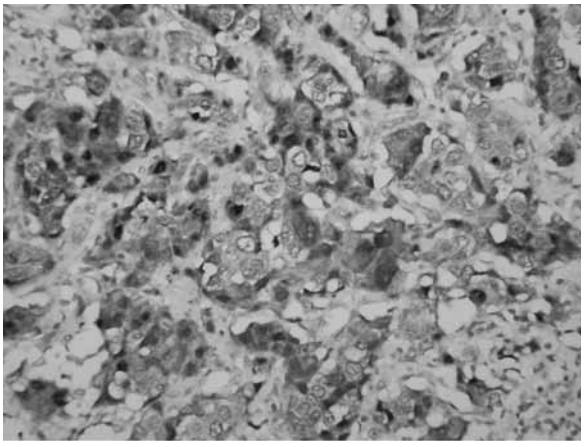

Fig. 1F

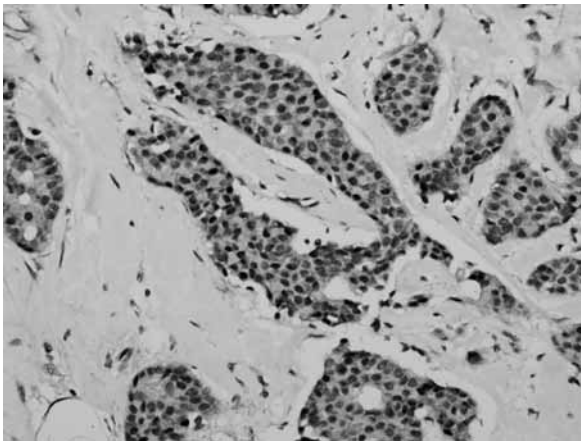

Fig. 1G

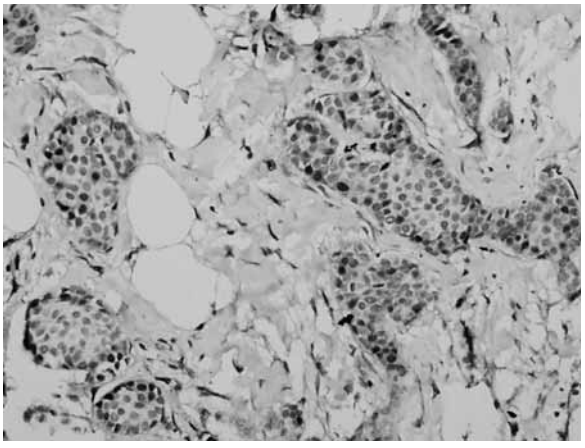

Figure 1A — Obvious circumferential distribution of EGFR.

Figure 1B — Complete intense uniform membrane staining of c-erb-2.

Figure 1C — Strong membranous staining with c-erb-3.

Figure 1D — Obvious circumferential staining of c-erb-4.

Figure 1E — Cytoplasmic and/or nuclear expression with NRGα

Figure 1F — Strong nuclear staining with p-AKT

Figure 1G — Strong nuclear staining with p-MAPK.

expression and PR immunoexpression ($p < 0.05$) (Table 3). There was no significant correlation with ER, EGFR, NRGα, p-MAPK or p-AKT immunoexpression.

Phospho-AKT

p-AKT protein expression was assessed in 22% (13/59) of the cases (Table 2) (Figure 1G). A tendency for a positive correlation was found with lymph node status ($p = 0.067$) and poor clinical outcome ($p = 0.059$) (Table 3).

Coexpression profile of the proteins

Five (9%) of the cases demonstrated coexpression with c-erbB-3+c-erbB-4, p-MAPK+p-AKT, NRG·+p-MAPK while four (7%) of the cases were positive for both c-erbB-2 and NRGα. There were only three (5%) cases coexpressing NRGα+p-MAPK+p-AKT. A statistically significant correlation was revealed between c-erbB-2 and c-erbB-3 overexpression and between c-erbB-3 and c-erbB-4 ($p < 0.05$). There was no statistical significance but there was a tendency of positive correlation of coexpression between c-erbB-2 and c-erbB-4 ($p = 0.062$) and between c-erbB-2 and EGFR ($p = 0.064$). Tumour high grade was found to be correlated with c-erbB-2 overexpression ($p < 0.05$) but we could not find any significant correlation between coexpression profiles and histopathological prognostic parameters such as tumour stage, lymph node or hormonal status, and clinical outcome of the patients.

Discussion

c-erbB-2 is a member of the T1GFR family and its amplification occurs in 15-25% of BCa, predicting a poor prognosis [6,10,11]. Although c-erbB-2 is the best-studied member of the T1GFR family, the other family members and their coexpression profile and correlations with NRG·, p-MAPK and p-AKT and with the well-known histopathological prognostic parameters such as tumour stage, grade, lymph node status, ER, PR status, and clinical outcome in IDC-NOS of the breast are not well established.

EGFR: EGFR occurs in 15-36% of BCas [12,13,14]. EGFR has been demonstrated in breast cancer cell growth and its overexpression was found to be indicative of poor prognosis. No relationship between EGFR expression and steroid receptor status was observed [15]. It has also been demonstrated that erbB1 overexpression in malignancies besides tumour proliferation results in increased tumour cell motility in vivo together with enhanced intravasation and metastasis and ErbB3-dependent motility and intravasation in BCa metastasis [16]. EGFR expression may have prognostic significance in patients with locally advanced BCa who are treated with anthracycline chemotherapy [17]. In our study group, EGFR overexpression was found in four cases (7%). Our rate of frequency appears to be low compared to other reports (15%-36%). This discrepancy could be due to the small number of patients (59 cases) studied and/or the antibodies that were used for the evaluation of the immunohistochemistry or the study group that the results were based on. It is well known that some histological types of BCa such as basal-like carcinomas, metaplastic carcinomas and squamous carcinomas of the breast have a high rate of EGFR overexpression [18-20]. Our study group consisted entirely of IDC (NOS) and the majority of the cases (91.5%) were Stage I or II. Three of the four EGFR positive cases had lymph node metastasis and two had metastatic disease. A statistically significant association with ER negativity and EGFR overexpression ($p < 0.05$) and a borderline positive correlation with EGFR and c-erbB-2 overexpression ($p = 0.064$) were revealed.

Triple-negative BCa (ER-negative, PR-negative, and HER2-negative) is a high risk breast cancer that lacks the benefit of any specific therapy that targets these proteins. Those tumours have positive expression of basal cytokeratins (basal phenotype), P-cadherin, p53, and EGFR [21]. Among our cases there were only five cases that were triple negative for immunohistochemistry and while 60% died of BCa, the only case which demonstrated EGFR overexpression had a good clinical outcome with no evidence of disease.

HER4: There are reports supporting the hypothesis that HER1-3 is associated with driving tumour proliferation, whereas HER4 is involved in a non-proliferative or even a protective role [22]. Low nuclear grade, low proliferation rate and presence of HER4 expression in ductal carcinoma in situ (DCIS) were found to be independent predictors of nonrecurrence. HER4 expression has been suggested to identify women who could avoid radiotherapy after breast-conserving surgery for DCIS [23]. We also studied two DCIS cases that were not included in the study group and both cases were only positive for c-erbB-4*;* c-erbB-4 was found to antagonise the c-erbB-2 effect during the clinical course of BCa and its expression was associated with a more favourable outcome. It is therefore suggested that clarifying the status of c-erbB-4 expression could be significant to achieve the best results with immunotherapy against the c-erbB-2 receptor [14]. Our clinical follow-up results of the patients also demonstrate a statistically significant correlation between c-erbB-4 overexpression and good clinical outcome ($p < 0.05$).

TGFR family member coexpression: TGFR family member coexpression (HER1, HER2, HER3 but not HER4) was found to have a negative synergistic effect on patient outcome, independent of tumour size or lymph node status [24]. ErbB3 functions as an indispensable ErbB2 dimerisation partner and is required for proliferation of ErbB2-overexpressing tumour cells [25]. Strong cytoplasmic c-erbB-3 immunoexpression has been found to be significantly correlated with local recurrence in BCa but there were no significant associations with survival, Union International Contre Cancer (UICC) criteria, age, menopausal status, ER status, histological grade, c-erbB-2 status or the presence of vascular invasion [26]. On the other hand, combined ErbB-2 and ErbB-3 expression has been found to be associated with nodal involvement and reduced overall survival [13]. The strongest overall corre-

lation in nodal positive tumours was found between Her-2/neu and Her-3/neu and also between Her-2/neu and Her-4/neu [27]. In our study group we have also revealed a statistically significant correlation between c-erbB-2 and c-erbB-3 overexpression and between c-erbB-3 and c-erbB-4 ($p < 0.05$). There was no statistical significance but there was a borderline positive correlation of coexpression between c-erbB-2 and c-erbB-4 ($p = 0.062$) and between c-erbB-2 and EGFR ($p = 0.064$). While high-grade tumour was found to be correlated with c-erbB-2 overexpression ($p < 0.05$), we could not find any significant correlation between coexpression profiles and histopathological prognostic parameters such as tumour stage, lymph node and patient hormonal status. This lack of correlation could be due to the limited number of cases or the tumour stage of the cases (91.5% were Stage I or II) studied.

A study based on tissue microarray technology of 324 patients with lymph node negative BCa has demonstrated that HER-2 and EGFR show similar expression patterns while RTK family receptors did not show a correlation with the clinical outcome [28]. In the recent study, we could not also find any significant correlation between the coexpression profiles of the TGFR family and the clinical outcome of the patients.

NRGα: Absent or low levels of NRG1-α were found to be associated with a poorer prognosis compared to tumours that had moderate to high levels of the protein [29]. It has been shown that the downregulation of ErbB3 by Nrdp1 overexpression is accompanied by decreased growth factor-mediated cellular proliferation and motility due in part to attenuation of ERK and PI3K signalling [30,31]. Nerve growth factor receptor (NGFR) is a useful marker for breast myoepithelial cells and can be used to rule out invasive disease [32]. NGFR was found to be restricted to the myoepithelial layer. Positivity for NGFR was observed in 11 out of 245 (4.5%) BCa [33]. We revealed cytoplasmic and nuclear NRGα immunoexpression in 29% of IDC cases and weak/moderate staining in the cytoplasm of the ductal cells of normal breast tissue whenever they were found around the tumour in the same sections. We could not find any statistically significant correlation between NRGα immunoexpression and the other parameters that were studied. Cytoplasmic staining of NRG· appears to be a marker for ductal cells of the breast.

MAPK: Strong p-ERK staining in tumour cells was associated with early stages, negative nodal status and long recurrence-free survival [34]. Nuclear ERK2 expression was found to be an independent prognostic factor of shortened overall survival of patients, while cytoplasmic ERK2 had an independent, favourable effect on both disease-free and overall survival [35]. In this study we have also found a statistically significant correlation between nuclear p-MAPK overexpression and PR positivity ($p < 0.05$). We could not find any statistically significant correlation between p-MAPK overexpression and the clinical outcome of the patients. This lack of association could be due to the small number of cases studied. Although we could not find any statistically significant correlation between p-AKT, NRGα and p-MAPK, immunoexpressing tumour cells were similar. If nuclear staining occurs with p-AKT or NRGα it is most commonly revealed in the ductal carcinoma cells which are at the outer section (basally located) of the tumour group and this is also a common finding for p-MAPK.

AKT: Activation of AKT and its prognostic value in BCa have been reported [36,37]. The AKT pathway was found to be activated in early breast cancer during the in situ stages [38]. Our two DCIS cases also demonstrated p-AKT overexpression (unreported data). p-AKT has been found to be significantly associated with c-erbB-2 overexpression and with a poor prognosis [39]. The results of our study support the notion that p-AKT overexpression has a borderline positive correlation with lymph node metastasis ($p = 0.067$) and poor clinical outcome ($p = 0.059$). Five of the seven cases who had strong p-AKT, immunoexpression either had metastatic disease or died as a result of BCa.

Conclusion: This study was entirely based on IDC-NOS and majority of the cases were tumour Stage I or II. The results indicate that high-grade histomorphology of BCas is not only associated with ER-/PR-negativity, but seems to be activated by receptor tyrosine kinase growth factors such as EGFR and c-erbB-2 overexpression. While c-erbB-4 overexpression may predict a good clinical outcome for patients, p-AKT expression seems to predict a poor clinical outcome. The expression and coexpression profile of receptor thyrosine kinase growth factors are usually not characterised in the pathological diagnosis. However information about tumourigenesis in diagnostic pathology reports targeting multiple erbB-receptors may provide an exceptional strategy for an effective cancer therapy.

Acknowledgement

This study has been supported by the Marmara University Research Foundation with project numbers: SAĞ-TUS-290906-0205 and SAG-YYT-200407-0068.

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