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ORIGINAL

Assessment of c-erbB 3 and c-erbB 4 expression in breast cancer patients and correlation with clinicopathological parameters

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ABSTRACT

Objetive: ErbB Receptor Tyrosine Kinases possess an established role in mammary gland development and breast tumorigenesis. We aimed to assess the expression of c-erbB3 and c-erbB4 RTKs in early breast carcinomas and investigate its possible correlation with Estrogen or Progesterone Receptors, tumor stage and grade, disease recurrence and patient's outcome.

Patients and methods: Forty-nine specimens of early breast carcinomas deriving from patients that had sustained partial or total mastectomy with axillary lymph node resection were studied retrospectively. Expression of RTKs was detected implementing: a) an anti-HER-3 mouse polyclonal antibody; and b) an anti-HER-4 mouse polyclonal antibody. For both c-erbB3 and c-erbB4, either a cytoplasmic or a nuclear staining pattern of tumor cells was considered positive.

Results: Expression of c-erbB4 exhibited statistically significant association with tumor grade and unfavorable patient's outcome. C-erbB3 expression did not correlate with tumor recurrence or patient's outcome.

No association was established between the expression of both RTKs and that of Estrogen or Progesterone Receptors. C-erbB4 expression did not posess statistically significant association with patient's death or disease recurrence. C-erbB3 expression did not correlate with tumor grade or recurrence and patient's death.

Conclusions: In the context of compound molecular mechanisms, alterations in c-erbB3 and c-erbB4 expression merit appraisal as future interventions in breast cancer treatment.

Key words: Breast cancer. Immunohistochemistry. C-erbB 3. C-erbB 4. Survival. Tumor grade. Tumor progression.

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INTRODUCTION

Breast represents a challenging tissue for molecular cancer research because of a unique property to substain significant changes during puberty and child-bearing age and involute in menopause. These physiological alterations of the mammary gland share similarities with aberrations occurring during malignant transformation process.

The ErbB (HER) family of Receptor Tyrosine Kinases (RTKs) belong to a growth factor's receptor superfamily which comprises of c-erbB1 (also known as epidermal growth factor receptor-EGFR or HER1), c-erbB2 (HER2), c-erbB3 (HER3), and c-erbB4 (HER4) receptor proteins (1). All four are cellular membrane proteins with three characteristic regions: a cysteine-rich extracellular domain where the ligand is bound, a phylogenetically highly-conserved intracellular segment with tyrosine-kinase activity, and a single hydrophobic transmembrane domain that anchors the protein to the cell (1). Following their ligand-binding induced activation, these proteins form homo- and heterodimers. This procedure results in enhancement of their intrinsic tyrosine kinase activity and the initiation of a complex cascade of intracellular signaling, that finally results in diverse biologic responses, including proliferation, programmed cell death, cell motility and adhesion and cell survival (1-3). The outcome of these proteins's activation depends upon the conjunction of signaling pathways induced following their activation; while the magnitude and duration of the observed cellular processes are depended upon the composition of the receptor pair and the identity of the ligand (1).

RTKs are involved in mammary gland development during puberty and pregnancy, as well as in the maintenance of tissue homeostasis (1). At least eleven ligands for the HER family have been described. C-erbB3 and c-erbB4 are being activated by the neuregulin family of ligands which includes a number of different isoforms of

NEU (named upon their ligand neuregulin) differantiation factor/ HRG (heregulins), that bind to c-erbB3 or c-erbB4 preferably forming heterodimers with the orphan receptor c-erbB3 (1). Ligand's binding to the ectodomain results in the allosteric transition, dimerisation and activation of the protein kinase thus resulting in the autophosphorylation and the initiation of the signaling cascade. Despite both homo- and heterodmerization result in activation of the ErbB network, heterodimers are more potent and mitogenic (1).

RTKs mediate their functions through several cellular pathways like the MAP-kinase pathway, inducing cyclin D1 (a key cell cycle regulator downstream of c-erbBs) overexpression (1,4,5) as well as by mechanisms involving induction of several antiapoptotic proteins (bcl-2, bcl-x and caspase inhibitors) (6) and decrease of proapoptotic molecules (bax, bad). In parallel, RTKs are involved in other pathways such as the Phosphatidiloinositole 3` kinase-Protein kinase B (P13K- PKB) and that of the Phospholipase C – protein kinase C (PLC-PKC) (7-10).

C-erbB2 is overexpressed due to gene amplification in 20-30% of breast and ovarian tumors (1), and is the sole receptor not possessing a high affinity ligand; however it is commonly found in activated form since it represents the preferred heterodimerisation partner among the other c-erbB receptors (11).

Both c-erbB3 and c-erbB4 are being actively expressed in numerous adult and fetal tissue lining epithelia of the gastrointestinal, urinary and reproductive tract, skin, in mesenchymal and neuronal cells, as well as in skin, lung, ovary, and gastrointestinal tract tumors (1,12). C-erbB3 receptor's individual intracellular function remains unclarified; of note is that c-erbB3, containing substitutions in critical amino acids within its intracellular portion lacks kinase activity of this domain (1). C-erbB4 represents the most recently identified member of the family and shares extensive homology with c-erbB3.

Overexpression of c-erbB3 and c-erbB4 receptors has been documented in breast cancer cell lines and might correlate with tumor progression (1,13). The aim of this study was the assessment of the expression of c-erbB3 and c-erbB4 proteins in "early" breast carcinomas and the evaluation of possible correlations between these molecules with the expression of ER and PR (Estrogen and Progesterone Receptor) and other clinicopathological parameters, like tumor stage and grade, disease recurrence and the patient's outcome.

PATIENTS AND METHODS

Specimens and clinicopathological data

Forty-nine specimens of "early" breast carcinomas (defined as T1 and T2 tumors with negative axillary lymph nodes) were studied retrospectively. All specimens derived from women that had sustained partial or total mastectomy with resection of axillary lymph nodes in Patras University Hospital. Patient's mean age was 59 years (range between 26 and 80 years). Each patient was followed-up for a mean period of 103 months (range from 23 to 145 months) during which, 8 patients had recurrence of their disease and 5 of them finally died from metastatic breast cancer.

A mean number of thirteen lymph nodes was pathologically examined (range 6 to 24). Regarding histologic type, 45 ductal carcinomas of classic type were identified, 3 mucinous and 1 medullary. Fifteen patients (30.6% of total) were staged as having T1 tumors, and thirty-four (69.4% respectively) as T2. Moreover, according to the Nottingham modification of the Bloom-Richardson system, twelve specimens (24.5% of total) were Grade I, twenty-four (49.0%) were Grade II and thirteen (26.5% respectively) were Grade III.

Histologic examination revealed infiltrative ductal breast carcinoma in all forty-nine specimens; while in eleven of them a concomitant intraepithelial (in situ) carcinoma was also documented, representing more than 25% of the whole tumor.

All specimens were preserved in 10% formaldehyde and were constitutively embedded in paraffin.

Immunohistochemistry

De-waxed and hydrated 5mm sections were quenched with H_2O_2 (0.6%) in 100% methanol for 20 minutes to inhibit endogenous peroxidase activity. For antigenic retrieval the slides were steamed within a pressure cooker. Non specific binding was blocked by incubating the sections in TBS solution containing 3% BSA and were then incubated with each of the following antibodies, for 25 minutes in room temperature: a) anti-HER-3 mouse polyclonal antibody (Santa Cruz Biothechnology Inc, UK) in a dilution 1:100; and b) anti-HER-4 mouse polyclonal antibody (Santa Cruz Biothechnology Inc, UK) in a dilution 1:200 Labeling was detected using the streptavidine-biotin complex method, while 3,3' diaminovenzidine (DAB) was used as a chromogen. Negative staining controls were included in which no primary antibody had been added. As positive controls the peritumoral nonneoplastic breast tissue was used.

Immunohistochemical staining was graded on a scale of 0 to 1 according to the following assessment: 0 accounted for < 5% positive cells; 1 accounted for > 6% positive cells. For both c-erbB3 and c-erbB4, either a cytoplasmic or a nuclear staining pattern of tumor cells was considered positive (Fig. 1).

Statistical analysis

The associations between c-erbB-3 and c-erbB-4 expression as well as the associations between these mole-

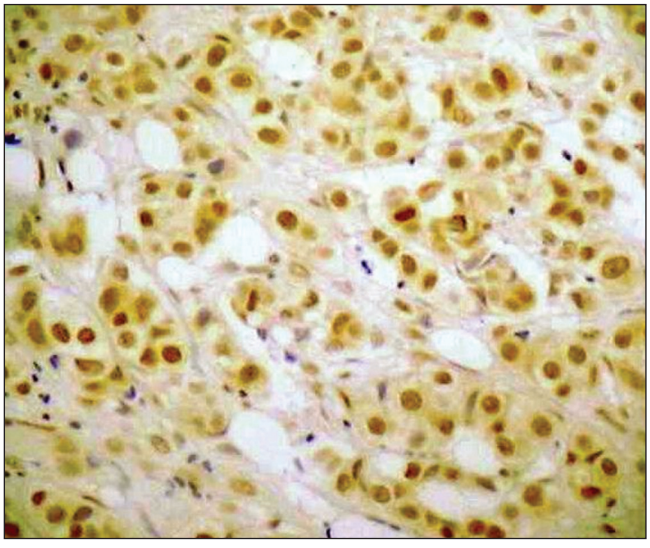

Fig. 1. Photomicrograph illustrating positive c-erbB4 immunostaining in breast carcinoma cells.

cules with the expression of the hormone receptors ER and PR and other clinicopathological markers were analyzed using the chi-square test. Life tables were calculated according to the Kaplan-Meier method. Multivariate survival analyses were performed with the Cox proportional hazards model, entering the following covariates: a) c-erbB-3 expression (negative *versus* positive); b) c-erbB-4 (negative *versus* positive); c) tumor size in centimetres (< 2 cm, 2-5 cm, > 5 cm); d) histological grade (I, II, and III); e) ER (negative *versus* positive); and f) PR (negative *versus* positive); Cox regression was performed using a backward stepwise selection of variables, and a p of 0.05 was adopted as the limit for inclusion of a covariate. All statistical tests were two sided. All analyses were performed using SPSS 13.0 for Windows statistical package (SPSS Inc., Chicago, IL, USA).

RESULTS

Twelve specimens (24.5% of total) exhibited positive immunostaining for c-erbB3 protein, while the remainder thirty-seven tumor specimens (75.5% respectively) did not illustrate immunoreactivity for the c-erbB3 receptor. Accordingly, regarding c-erbB4, twent-eight cases (57.1% of total) showed positive immunoreaction, while the remaining twenty-one (42.9% respectively) were negative.

The expression of both proteins exhibited strong interdependence (Pearson's correlation 0.301 and p = 0.035). more precisely, among the 12 c-erbB-3 positive tumors, there were only 2 that exhibited negative c-erbB-4 immunoreactivity, while from the 28 c-erbB-4 positive tumors, only 10 were negative for the c-erbB-3 receptor. Expression of c-erbB4 was found to have a statistically significant association with tumor grade (Table I) (Spearman's R = 0.372 and p = 0.009). Moreover, c-erbB4 receptor's expression exhibited a strong correlation with patient's death, (Pearson's correlation 0.292 and p = 0.042), as illustrated in the Kaplan–Meier curve (Fig. 2). The association between the protein's expression and tumor recurrence approached borderline (Pearson's correlation 0.271 and p = 0.06) as graphically illustrated in the Kaplan-Meier curve for tumor recurrence (Fig. 3).

Table I. Crosstabulation between tumor grade and c-erbB4 and c-erbB4 expression

Degree	*c-erbB-3 positive*	*c-erbB-3 negative*	*c-erbB-4 positive*	*c-erbB-4 negative*	*Total*
1	3	9	4	8	12
2	3	21	13	11	24
3	6	7	11	2	13
Total	12	37	28	21	49

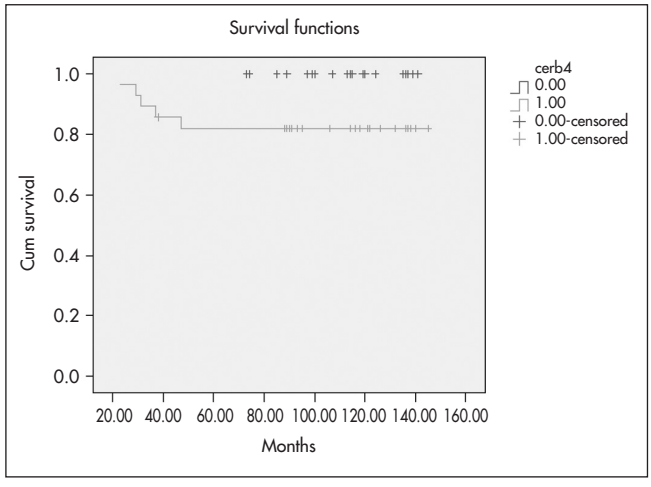

Fig. 2. Plot 1: Kaplan- Meier plot showing the decrease in patient's survival in the cases with positive c-erbB4 expression.

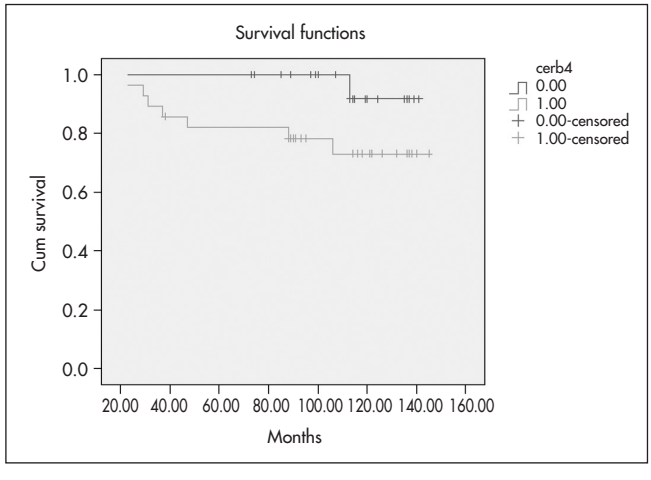

Fig. 3. Plot 2: Kaplan-Meier plot showing an increase in disease recurrence (shorter time interval till the recurrence occurred) in correlation to positive c-erbB4 expression.

No significant association was documented between c-erbB3 expression and tumor recurrence or patients' death ($p = 0.261$).

Additionally, no correlation was established between the expression of both RTKs with the expression of either Estrogen Receptor (Tables II) or Progesterone Receptor. In univariate analysis, the only statistically significant association that emerged was that between tumor grade and ER expression (Table III) ($p = 0.046$). In multivariate Cox regression survival analysis, c-erbB4 expression did not posess statistically significant association either with patient's death or disease recurrence. Finally, c-erbB3 expression did not exhibit strong association with tumor grade (Table I), tumor recurrence or with patient's death, as expected from the aforementioned results.

Table II. Crosstabulation between c-erbB3 and c-erbB4 expression and ER receptor status

	ER negativo	*ER positivo*	*Total*
c-erbB-3 negativo	12 (24,5%)	25 (51%)	37 (75,5%)
c-erbB-3 positivo	5 (10,2%)	7 (14,3%)	12 (24,5%)
c-erbB-4 negativo	6 (12,2%)	15 (30,6%)	21 (42,9%)
c-erbB- 4 positivo	11 (22,4%)	17 (34,7%)	28 (57,1%)

Table III. Crosstabulation between tumor grade and ER receptor status

			ER		
			Negative	*Positive*	*Total*
Grade	1.00	Count	2	10	12
		% of total	4,1%	20,4%	24,5%
	2.00	Count	7	17	24
		% of total	14,3%	34,7%	49,0%
	3.00	Count	8	5	13
		% of total	16,3%	10,2%	26,5%
Total	1.00	Count	17	32	49
		% of total	34,7%	65,3%	100,0%

DISCUSSION

Our results illustrate a strong inter-association between the expression of c-erbB3 and c-erbB4 receptors; this observation is in line with the existing in the literature data regarding RTKs intracellular interaction (1). Our findings of simultaneous increased expression of the c-erbB3 and c-erbB4 proteins in the studied breast cancer specimens might be attributed to crossreaction of the receptors and formation of heterodimers, via combinational protein interactions. Following ligand's binding to the proteins and the subsequent activation of c-erbB3 and c-erbB4, it is c-erbB2 protein that acts as the preferred heterodimerization partner for both proteins and subsequently generates the optimal signaling complexes for the initiation of the intracellular cascade (14,15). Both homo- and hetero- dimerisation can activate the c-erbB network, yet heterodimers are considered as more potent and mitogenic (1). A combinational interaction of ligands, receptors, effectors and transcriptional factors permits a high degree of adaptability and signal diversification (16).

Furthermore, c-erbB4 receptor's expression exhibited a substantive correlation both with tumor grade and patient's outcome; particularly with patient's death. A possible correlation with disease recurrence might have emerged if a bigger sample with more recurrent patients was studied.

Previous studies have demonstrated that c-erbB4 protein's overexpression is correlated with cell proliferation (1); our data corroborate these observations. Positive immunostaining for c-erbB4 proteins has been documented in 49.3% of cervix uteri squamous cell carcinomas specimens (17). In parallel, c-erbB4 protein is being expressed in ovarian tumors (9,18) and is often activated in children's brain tumors (19), colorectal adenocarcinomas (20) and gastric tumors (21). However, other authors have reported reduction in c-erbB4 expression in non-metastatic pancreatic carcinomas (22).

Moreover, our results did not exhibit a correlation between c-erbB3 overexpression with tumor grade, or patient's outcome. This observation is congruent to other studies implying that c-erbB3 has no individual role in malignant transformation process (1); the kinase-deficient receptor requires heterodimerisation with a kinase active partner. The latter could be attributed to the absence of intracellular tyrosine kinase activity, thus requiring simultaneous activation of other c-erbB family members for the triggering of cellular pathways. It might be hypothesized that each receptor induces diverse cellular functions; if both c-erbB3 and c-erbB4 are present, then each receptor's relative expression together with that of c-erbB2 to which they heterodimerise determines the eventual cellular response.

Disruption of complex intracellular pathways, including the function of RTKs and their signals can contribute in malignant processes as this study also indicates. While both c-erbB2-specific and c-erbB1-specific chimeric or humanized monoclonal antibodies represent established modalities in current breast cancer treatment (1), the role of c-erbB3 and c-erbB4 expression within elaborate molecular mechanisms merit appraisal in the context of future therapeutic perspectives.

REFERENCES

1. Marmor MD, Skaria KB, Yarden Y. Signal transduction and oncogenesis by ErbB/HER receptors. Int J Radiat Oncol Biol Phys 2004; 58: 903-13.
2. Bosch FX, Lorincz A, Munoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. J Clin Pathol 2002; 55: 244-65.
3. Munoz N, Bosch FX, de Sanjosé S, et al. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 2003; 348: 518-27.
4. Hynes NE, Horsch K, Olayioye MA, Badache A. The ErbB receptor tyrosine family as signal integrators. Endocr Relat Cancer 2001; 8: 151-9.
5. Harari D, Yarden Y. Molecular mechanisms underlying ErbB2/HER2 action in breast cancer Oncogene 2000; 19: 6102-14.
6. Danielsen AJ, Maihle NJ. The EGF/ErbB receptor family and apoptosis. Growth Factors 2002; 20: 1-15.
7. Roskoski R Jr. The ErbB/HER receptor protein-tyrosine kinases and cancer. Biochem Biophys Res Commun 2004; 319: 1-11.
8. Prenzel N, Fischer OM, Streit S, Hart S, Ullrich A. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. Endocr Relat Cancer 2001; 8: 11-31.
9. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. Nature 2001; 411: 355-65.
10. Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell 2000; 103: 211-25.
11. Schaefer G, Akita RW, Sliwkowski MX. A discrete three-amino acid segment (LVI) at the C-terminal end of kinase-impaired ErbB3 is required for transactivation of ErbB2. J Biol Chem 1999; 274: 859-66.
12. Srinivasan R, Poulsom R, Hurst HC, Gullick WJ. Expression of the c-erbB-4/HER4 protein and mRNA in normal human fetal and adult tissues and in a survey of nine solid tumour types. J Pathol 1998; 185: 236-45.
13. Allen LF, Lenehan PF, Eiseman IA, Elliott WL, Fry DW. Potential benefits of the irreversible pan-erbB inhibitor, CI-1033, in the treatment of breast cancer. Semin Oncol 2002; 29: 11-21.
14. Ferguson KM, Darling PJ, Mohan MJ, Macatee TL, Lemmon MA. Extracellular domains drive homo- but not hatarodimerization of erbB receptors. EMBO J 2000; 19: 4632-43.
15. Brennan PJ, Kumagai T, Berezov A, Murali R, Greene MI. HER2/Neu mechanisms of dimerization/oligomerization. Oncogene 2002; 21: 328.
16. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol 2001; 2: 127-37.
17. Ravazoula P, Androutsopoulos G, Koumoundourou D, Michail G, Kourounis G. Immunohistochemical detection of HPV proteins and c-erbB receptors in cervical lesion specimens from young women. Eur J Gynaecol Oncol 2006; 27: 69-73.
18. Leibl S, Bodo K, Gogg-Kammerer M, et al. Ovarian granulosa cell tumors frequently express EGFR (Her-1), Her-3, and Her-4: Anim-munohistochemicalstudy. Gynecol Oncol 2006; 101 (1): 18-23. Epub 2005 Dec.
19. Bodey B, Kaiser HE, Siegel SE. Epidermal growth factor receptor (EGFR) expression in childhood brain tumors. In Vivo 2005; 19 (5): 931-41.
20. Kountourakis P, Pavlakis K, Psyrri A, et al. Prognostic significance of HER3 and HER4 protein expression in colorectal adenocarcinomas. BMC Cancer 2006; 6: 46.
21. Uchida T, Wada K, Akamatsu T, et al. A novel epidermal growth factor-like molecule containing two follistatin modules stimulates tyrosine phosphorylation of erbB-4 in MKN28 gastric cancer cells. Biochem Biophys Res Commun 1999; 266: 593-602.
22. Graber HU, Friess H, Kaufmann B, et al. ErbB-4 mRNA expression is decreased in non-metastatic pancreatic cancer. Int J Cancer 1999; 84: 24-7.