

HER Family Expression in Egyptian Breast Cancer Patients

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ABSTRACT

Objective: This study aimed to study the expression of HER 2, HER 3 and HER 4 members of HER family (c-erbB-2, c-erbB-3 and c-erbB-4, respectively) in Egyptian breast cancer patients, to correlate them to different prognostic markers: age, menopausal status, tumor size, histological grade, presence of intraduct component, lymph node (LN) involvement, estrogen and progesteron receptors (ER and PR) status as well as to each other and to evaluate their clinical impact.

Material and Methods: This study included 49 female cancer breast patients with invasive duct carcinoma (IDC). Their age ranged from 25 to 70 years with a mean of 48.4 years. HER 2 and HER 3 were examined by both immunohistochemistry (IH) and reverse transcriptase polymerase chain reaction (RT-PCR) and HER 4 by RT-PCR only. Patients were followed up for at least one year after the date of diagnosis for the development of either local recurrence or distant metastases.

Results: HER 2 expression was encountered in 79.5% (39/49) by IH vs. 81.6% (40/49) by RT-PCR, HER 3 in 46.9% (23/49) by IH vs. 67.3% (33/49) by RT-PCR and HER 4 in 55.1% (27/49) by RT-PCR. No significant association was found between any HER family member on one side and neither age, menopausal status, tumor size nor histological grade on the other side. A significant association was detected between: HER 2 expression and LN ($p = 0.01$) and HER 3 expression and presence of intraduct component ($p = 0.02$). A significant association was detected between HER 4 expression and both ER and PR ($p = 0.001$, $p = 0.02$). An inverse association was encountered between HER 4 over expression and both LN and HER 2 over expression ($p = 0.009$, $p = 0.003$, respectively). Follow up of 40 patients revealed that 32.5% (13/40) developed recurrence. In the 13 cases who developed recurrences, HER 2 was positive in 11, HER 3 in 10 and HER 4 in 9 vs. 23, 17 and 15, respectively in the 27 cases who didn't develop recurrence.

Conclusions: Immunohistochemistry proved to be an efficient screening test for HER 2 and HER 3; molecular techniques (RT-PCR) should be restricted to negative IH cases. HER 2 and HER 3 were associated with bad prognostic parameters while HER 4 could not be considered as one of the favorable factors till proved otherwise.

Key Words: HER expression - Cancer breast - RT - PCR.

INTRODUCTION

Four distinct trans membranous glycoprotein receptors have been identified as members of the type I growth factor receptor family, which posses ligand regulated tyrosine kinase activity, a class of oncogenes prevalent in solid tumors especially those of breast [1]. They are EGFR, c-erbB-2, c-erbB-3 and c-erbB-4 (alternatively, authors use the HER terminology instead; HER 1-4). They play important roles in normal growth regulation/cell differentiation and in the genesis and prognosis of human neoplasia [2,3]. Receptor heterodimerization in various combinations, a unique feature of the type I receptor, enables multifunctional intracellular responses to be generated with differing strengths and specificities depending on the ligand type, specific receptor combination or relative level of receptor expression. Various ligands have been identified that activate individual HER receptors. Although no ligand has been identified that binds directly to HER 2 receptors, HER 2 is the preferred partner within the family [4,5]. Heterodimers containing HER 2 appear to show high signaling potency, which may explain the particularly significant role of HER 2 in the oncogenic phenotype.

Normal epithelial cells possess 2 copies of HER 2 gene and express low level of HER 2 protein on the surface equivalent to some tens of thousands of copies/cell. With oncogenic transformation, HER 2 gene amplification and/or over expression of the receptor may raise 10-100 folds with over expression of receptors up to millions. This occurs in breast, stomach, ovarian and bladder cancers. In breast cancer; it is the most frequently studied member; it is encountered in almost all cases of high-grade

comido intra duct carcinoma, 10-40% in invasive duct carcinoma and rarely in intralobular carcinoma. HER 2 over expression is inversely associated with established good prognostic factors such as ER status and directly associated with poorer prognosis with shorter DFS and OS [6,7].

Expression of HER 3 has been detected in normal human adult and fetal tissues, nearly most developing tissues except hemopoietic ones [8]. Over expression of HER 3 protein was demonstrated in: breast, pancreatic, gastric, cervical, prostatic and bladder cancers [9]. Controversy still exists as regards the prognostic significance of HER 3 in breast cancer.

HER 4 is the most recently identified member cloned in 1993 [10]. Its expression is found in a range of normal human fetal and adult tissues notably in the brain, heart and more specifically post-synaptic membrane of neuromuscular synapses [11]. HER 4 may be under-expressed or even absent as in squamous cell carcinoma of head and neck and cancer prostate [12]. It is over expressed in medulloblastoma in children [13]. In cancer breast it is either under or over expressed. Signal transduction of HER 4 receptor may play an important role in cell growth and differentiation. In human breast cancer cell lines, cells expressing HER 4 exhibited herregulin dependant antiproliferative and differentiation responses correlated with HER 4 activation abolished by stable expression of a kinase inactive HER4.

Most studies on HER family members in cancer breast are related to expression of single member. Since heterodimerization between its members is necessary for activation, studies of the three receptors together may shed light on some new clinical implications.

In this work we have studied the expression of 3 members of the HER family (HER 2, 3, 4) in 49 Egyptian cancer breast patients with invasive duct carcinoma (IDC) in order to evaluate their frequency in our cases, to verify their association with different prognostic parameters as well as their short-term impact on prognosis.

MATERIAL AND METHODS

This study was performed on 49 female cancer breast patients with invasive duct carcinoma (IDC) presenting to the National Cancer

Institute, Cairo University, during the period from January 2000 to December 2002. Their age ranged from 25 to 70 years with a mean of 48.4 years.

All patients were subjected to full clinical, laboratory, pathological and radiological investigations in the form of chest X-ray, abdominal ultrasonography and isotopic bone scan to exclude the presence of metastasis. They underwent surgery either in the form of conservative wide local excision and axillary lymph node dissection or modified radical mastectomy.

Surgically removed tissue samples were subjected to the following:

- 1- RNA extraction: Total RNA was isolated by QIAamp RNA isolation kit (Qiagen, West Sussex, UK). The integrity of RNA was checked by agarose gel electrophoresis and ethidium bromide staining. RNA was stored in -80°C till used.
- 2- RT-PCR: Qiagen one-step RT-PCR was performed using a thermal cycler (Perkin Elmer Cetus). β -actin primers were used in RT-PCR reaction as RNA loading control. The primer sequences used for amplification are shown in Table (1). Coamplification of β -actin was performed for HER 2 and HER 4. HER 3 was separately amplified since coamplification with β -actin was not satisfactory.

RT-PCR was performed in a final volume of 50 μ l reaction in the presence of 10 pM of each primer, 0.4 mM of each dNTP, 10 μ l of 5x one step RT-PCR buffer, 10 μ l of 5x Q-Solution and 2 μ l one step RT-PCR enzyme mix. cDNA synthesis was done at 50°C for 30 min, 95°C for 15 min.

Amplification cycles were optimized at 25 cycles for HER 2 and 30 cycles for HER 3 and 4. Cycle conditions for HER 2, 3 and 4 were denaturation at 94°C for 45s, annealing and extension at 52°C and 68°C, respectively for 1 min [14]. RT-PCR products were separated on 2% agarose gel stained with ethidium bromide and photographed. Two types of hundred base pair marker were used (Gibco-BRL and Promega).

- 3- Immunohistochemical staining: Formalin-fixed, paraffin-embedded tumor samples were used and 4-5 m sections were stained immunohistochemically by using Biotin-

Streptavidin Amplified (B-SA) System [15]. Clone TAB 250 rabbit antihuman c-erbB-2 oncoprotein and RTJ1 monoclonal antibody for c-erbB3 were used (Santa Cruz Biotech., USA). Sp1 and Sp2 antibodies were used for ER and PR (Dako cytomation, Denmark).

Positive staining appears as brown black positivity while negative cells appear blue or green. For HER 2, the staining is located at the cell membrane and occasionally in the cytoplasm of the neoplastic cells. In case of cytoplasmic staining alone, it is considered negative. For HER 3, the staining is predominantly cytoplasmic. For ERs and PRs, the staining is nuclear [15].

Treatment and clinical outcome:

Most of the patients received adjuvant treatment after surgery whether local in the form of postoperative radiotherapy (4500 cGy/20 fr to chest wall in cases after modified radical mastectomy with T3-4 or LN +ve ≥ 4 and 5000 cGy/25 fr with boost 1000 cGy/5 fr in cases after conservative surgery) and/or systemic therapy in the form of chemotherapy (FAC or CMF) and/or hormonal therapy (Tamoxifen) depending on the stage of the disease and the hormonal receptors status.

Patients were followed up for at least 1 year from the date of diagnosis for the development of either local recurrence or distant metastases. Because of the small number of patients both were categorized in one group as a failure.

Statistical analysis [16]:

Statistical package for social sciences (SPSS) version 9 was used. Quantitative variables were summarized using mean and SD, median minimum & maximum values. Qualitative data were summarized using frequencies and percentages.

The relation between quantitative variables was tested by Spearman Correlation. Chi or Fisher's exact tests were used whenever appropriate to test the association between the different qualitative variables. Differences were considered significant when p was ≤ 0.05 and highly significant when $p \leq 0.01$.

RESULTS

This study included 49 female breast cancer cases with IDC. Nineteen cases were premenopausal and 30 were postmenopausal. Tumor

size ranged from 0.5-7 cm. In two cases tumor size was less than 2cm (T1), between 2-5 cm (T3) in 27 cases and more than 5 cm (T3) in 20 cases. Two cases were grade I, 36 were grade II and 11 were grade III; during evaluation grade I was added to grade II due to small number of cases. Twenty cases had intraduct component and 29 were purely invasive. The different prognostic parameters of the 49 cases are detailed in Table (2).

All the cases that were immunohistochemically reactive for HER 2 and HER 3 were also positive with RT-PCR, except for one case for HER 2 and 10 cases for HER 3, which were negative by IH but revealed weak bands at the level of mRNA by RT-PCR.

HER 2: It was expressed in 100% (5/5) of cases with age < 35 years, 67% (14/21) in cases with age between 35-50 years and 91% (21/23) in cases with ages > 50 years ($p = 0.05$).

Fig. (1) shows a positive and a negative case of IDC with immunohistochemical staining of HER 2. Fig. (2) shows RT-PCR products of HER 2.

A direct significant association was found between HER 2 and the presence of positive lymph node metastasis ($p = 0.01$) and an inverse association with both ER and PR status ($p = 0.01$ and 0.02 , respectively). Table (3) shows the relation between HER 2 and some prognostic parameters. No significant association was found between HER 2 expression and menopausal status, tumor size, tumor grade and presence of intraduct component.

HER 3: No significant association was found between HER 3 expression and any of the clinicopathological parameters except for the presence of intraduct component ($p = 0.02$); 51.5% (17/33) of HER 3 positive cases had intraduct component vs. 18.8% (3/16) of negative cases. Fig. (3) shows a case of IDC with HER 3 positivity by IH. Fig. (4) shows RT-PCR products of HER 3.

HER 4: The significant association between HER 4 expression and the prognostic parameters is shown in Table (4). Fig. (5) shows the RT-PCR products of HER 4.

Out of the 49 patients included in this study, 40 patients were evaluable, i.e. followed up for

at least 1 year from the date of diagnosis; the remaining 9 were non-evaluable (lost follow up). Of these 40 patients, 13 (32.5%) developed recurrences either local or distal; 2 local recurrences, 9 distant metastases and 2 with both local recurrence and distant metastases and all were considered as failure.

In the 13 cases who developed recurrences,

Table (1): RT-PCR primer pairs used in co-amplification of β -actin and HER2, 3 and 4.

Gene	Primer pairs	Sequence	PCR product length (bp)
HER2	Sense	5'-AGCTCTGCTAC CAGGACACG-3'	168
	Antisense	5'-TCAGGCTCTGA CAATCCTCA-3'	
β -actin	Sense	5'-CCCTGGAGAAG AGCTACGAG-3'	223
	Antisense	5'-ATGCCAGGGTA CATGGTGGT-3'	
HER3	Sense	5'-AAAAATGGGCT CAAGATGTG-3'	224
	Antisense	5'-CGGAAGACATT GAGCTTCTC-3'	
HER4	Sense	5'-CTCTGGTGGTC TTCCTTCTACC-3'	232
	Antisense	5'-TGATAGTAGGC AGCATTGCC-3'	
β -actin	Sense	5'-CTTTGATTGCA CATTGTGT-3'	160
	Antisense	5'-GAAAGCAATGC TATCACCTC-3'	

Table (3): Significant association between HER 2 and prognostic parameters.

Parameter	HER 2				<i>p</i> value
	+ve		-ve		
	No. of cases (40)	%	No. of cases (9)	%	
<i>LN:</i>					
+ve	26	65	2	22.2	0.01*
-ve	14	35	7	77.8	
<i>ER:</i>					
+ve	19	47.5	7	77.7	0.01
-ve	21	52.5	2	22.3	
<i>PR:</i>					
+ve	16	65	6	66.6	0.02
-ve	24	35	3	33.4	
<i>HER 2 (IHC):</i>					
+ve	38	95	1	11.1	0.001
-ve	2	5	8	88.9	
<i>HER 4 (RT-PCR):</i>					
+ve	19	47.5	8	88.9	0.02
-ve	21	52.5	1	11.1	

+ve = Positive. -ve = Negative.
* *p*-value ≤ 0.05 is considered significant.

HER 2 was expressed in 11 cases (85%), HER 3 in 10 cases (77%) and HER 4 in 9 cases (69%), compared to 85%, 63% and 56% positivity for Her2, Her3 and Her4, respectively in the 27 cases who did not develop recurrence. There was no significant association between development of recurrence and the expression of any of the HER family members.

Table (2): The different prognostic parameters in 49 cancer breast cases with IDC.

Parameter	No. of cases (49)	%
<i>HER 2 (IHC):</i>		
+ve	39	79.6
-ve	10	20.4
<i>HER 2 (RT-PCR):</i>		
+ve	40	81.6
-ve	9	18.4
<i>HER 3 (IHC):</i>		
+ve	23	46.9
-ve	26	53.1
<i>HER 3 (RT-PCR):</i>		
+ve	33	67.3
-ve	16	32.7
<i>HER 4 (RT-PCR):</i>		
+ve	27	55.1
-ve	22	44.9
<i>ER:</i>		
+ve	26	53.1
-ve	23	46.9
<i>PR:</i>		
+ve	22	44.9
-ve	27	53.1

+ve = Positive. -ve = Negative.

Table (4): Significant association between c-erb-B4 and prognostic parameters.

Parameter	HER 4				<i>p</i> value
	+ve		-ve		
	No. of cases (27)	%	No. of cases (22)	%	
<i>LN:</i>					
+ve	11	40.7	17	77.8	0.09
-ve	16	59.3	5	22.2	
<i>ER:</i>					
+ve	19	70.3	7	31.8	0.001
-ve	8	29.7	15	68.2	
<i>PR:</i>					
+ve	15	55.5	7	31.8	0.02
-ve	12	44.5	15	68.2	
<i>HER 2 (RT-PCR):</i>					
+ve	19	70.4	21	95.5	0.03
-ve	8	29.6	1	4.5	

+ve = Positive. -ve = Negative.
p-value ≤ 0.05 is considered significant.

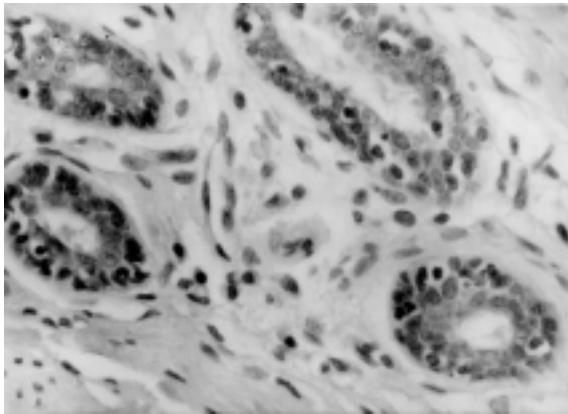


Fig. (1-A): IH staining of a case of cancer breast with IDC negative for HER 2 expression, (X100).

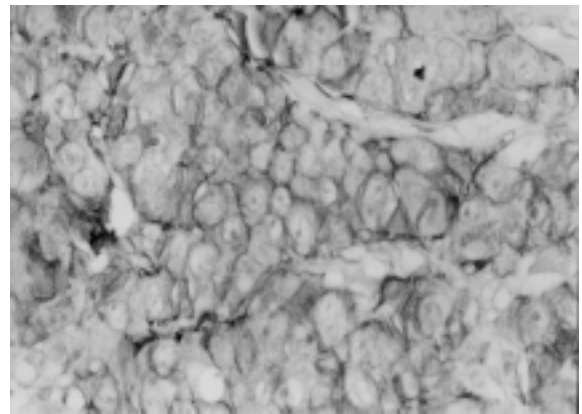


Fig. (1-B): IH staining of a case of cancer breast with IDC positive for HER 2 expression, (X400).

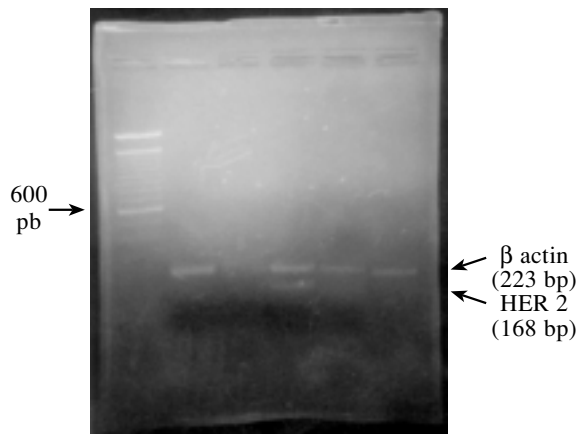


Fig. (2): RT-PCR of HER 2 in cancer breast cases.
Lane 1: 100 bp ladder (Gibco-BRL).
Lane 2 and 4: -ve cases. Lane 3: +ve case.

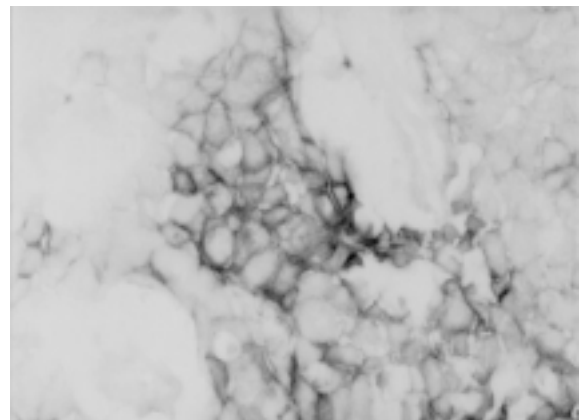


Fig. (3): IH staining of a case of cancer breast with IDC positive for HER 3 expression, (X400).

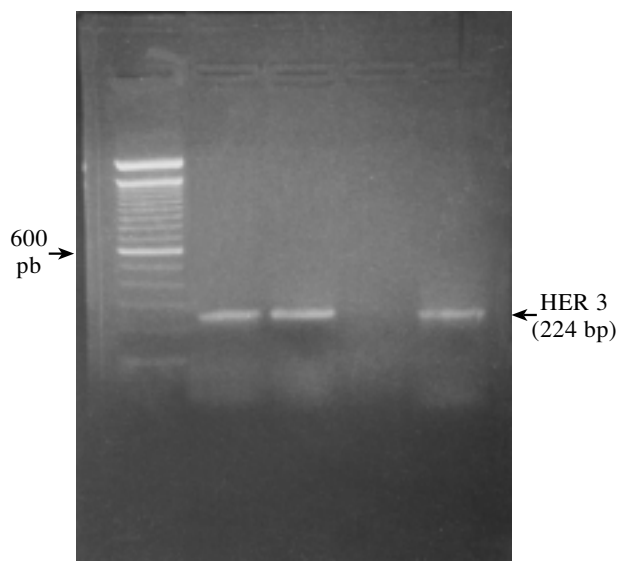


Fig. (4): RT-PCR of HER 3 in cancer breast cases.
Lane 1: 100 bp ladder (Gibco-BRL).
Lane 2 ,3,5: +ve cases. Lane 4: -ve case.

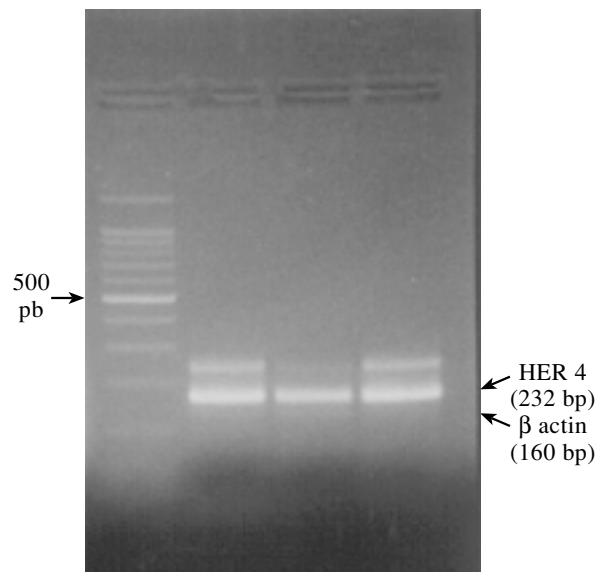


Fig. (5): RT-PCR of HER 4 in cancer breast cases.
Lane 1: 100 bp ladder (Promega).
Lane 2 ,4: +ve cases. Lane 3: -ve case.

DISCUSSION

Breast carcinoma is one of the most life threatening diseases in women and it is the main cause of death from cancer. It ranks as the first malignancy affecting females, contributing 30% of all female cancers [17]. In Egypt, it constitutes 35% of all female cancer cases at the NCI [18].

Breast tumor development is driven by multiple risk factors including genetic, environmental and hormonal factors. It is believed that the combined effect of these factors is associated with activation of genes of importance for breast cancer development [19]. HER family members may play a complex and ultimately more flexible role in cellular signaling by forming heterodimers between its members. Such heterodimerization, causes cross talk between HER family members and creates a more complex system for signal transduction [14,20].

In the present study, the expression of HER family members (HER 2, 3, 4) was determined in a group of 49 cancer breast patients with IDC and correlated to clinicopathological parameters and to each other.

The mean age reported in this study was 48.4 years, which is in line with the results of the Egyptian study by Ismail et al. [21] who reported a mean of 47.7 years. This age is much lower than that reported in the western countries being 57 years [22].

All the immunohistochemically positive tumors for both HER2 and HER3 expressed high level of mRNA by RT-PCR confirming the reliability of the immunohistochemical method. A weak RT-PCR band could be seen among some of the immunohistochemically negative tumors, this could be due to the low level of mRNA expression indicating that low levels of mRNA translated into low levels of protein that were not enough for immunohistochemical detection or alternatively the protein was not expressed at all.

HER 2 expression was 79.5% (39/49) by IH vs. 81.6% (39/49) by RT-PCR. Our results are much higher than Selim et al., Kurebayashi and Tsutsui et al. [23,24,25] who reported 15%, 40% and 55%, respectively. This could be attributed to the increasing aggressiveness of our tumors due to the younger age of the patients and that 95% of them had tumor size ranging from 2-7

cm. Another explanation could be that the staining intensity required to diagnose HER2 over expression varies from study to study and from antibody to antibody [26].

No significant association was found between HER 2 expression and age (although in our cases all 5 patients under 35 years were HER2 positive), going with the results of Pierce et al. and Zeillinger et al. [27,28] but against Arisio et al. [29] who suggested that age is one of the most predictors of progression and metastasis. Also no significant association was encountered with menopausal status, tumor size, grade or presence of intraduct component; this in concordance with Huang et al. and Jeziorski et al. [30,31]. The direct association with lymph node involvement and the inverse association with ER and PR status encountered in this work is in agreement with Ciocca et al and Jeziorski et al. [31,31] who reported lack of expression of HER 2 in association with high levels of steroid receptors.

In this work, HER 3 expression was 46.9% (23/49) by IH vs. 67.3% (33/49) by RT-PCR. Our results are in concordance with those of Naidu et al. [33] who reported HER 3 expression of 60% but much higher than those of Lemoine et al., Travis et al. and Suo et al., who reported 22%, 35% and 32%, respectively [8,14,34].

A controversy still exists as regards the prognostic significance of HER3 expression in breast carcinoma. In 1992, Lemoine et al. [34] reported its association with a worse prognosis and Travis et al. [8] found that tumors with HER 3 expression appeared to develop locally recurrent disease. A Malaysian study by Naidu et al. [33] suggested that it could be involved in the progression of tumors from pre-invasive to invasive stage. Further more it was associated with established poor prognostic factors such as high histological grade and EGFR. On the contrary, Knowleden et al. [35] reported that it was associated with the prognostic favorable ER phenotype. Paulowski et al. [36] demonstrated that it was associated with a better prognosis being inversely related to histopathological grade and directly associated with the presence of ER and PR.

Our results go with the opinion of worse prognosis because HER 3 expression was directly associated with the presence of intraduct

component which is usually associated with high incidence of local recurrence. Sixty percent of LN positive cases showed HER 3 expression and 70% of HER 2 positive cases showed HER 3 expression too, though the association was not statistically significant. Furthermore, out of the 13 patients who developed recurrence, 10 (77%) were HER 3 positive. The prognostic impact of HER 3 needs to be verified on a larger number of cases.

In this study HER 4 expression was detected in 55.1% (27/49) of our cases, which is in concordance with Srivivasan et al. [11] who reported a 49%. These results are much lower than those of Suo et al. [14] who reported HER 4 expression in 82% of their cases. No significant association was encountered between HER 4 expression with age, menopausal status, presence of intraduct component or tumor size. Although HER 4 expression was much higher (85%) in low-grade (I and II) compared to high grade (III) tumors (14%), no significant association was detected. This is in line with a study by Kew et al. [37] who demonstrated that, in contrast to other members of HER family; higher levels of HER 4 expression were associated with more differentiated histological grade tumors.

The direct significant association with established good prognostic markers including ER and PR encountered in this work is in agreement with Knowlden et al., Powlawski et al. and Zhene et al. [14,35,36].

On the other hand, an inverse association was detected between HER 4 expression and established bad prognostic markers including lymph node involvement and HER 2 expression. These results are in concordance with those of Kew et al., Powlawski et al. and Suo et al. [14, 36]. Suo and his colleagues [14] reported that the prognosis in patients expressing both HER 2 and HER 4 was much better than those expressing HER 2 alone, which suggests that HER 4 antagonizes the HER 2 effect on the clinical course of breast carcinoma. So, for best results of HER 2 application in breast cancer management, clarifying the status of HER 4 may be of importance [14]. In this work, in spite of its inverse association with poor prognostic factors, it showed expression in 9 cases (69%) of the 13 who developed recurrence, which may indi-

cate that HER 4 may still need to be thoroughly studied with a larger number of patients and a longer period of follow up.

In conclusion, the results of this work showed that immunohistochemical staining could be used as a screening test for both HER 2 and HER 3 while RT-PCR should be only restricted to negative immunohistochemical cases. HER 3 could be considered as one of the poor prognostic markers in addition to EGFR and HER 2; whereas HER 4 could not yet be considered as one of the favorable prognostic markers. Further studies on a larger number of patients are needed to verify.

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