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IMMUNOHISTOCHEMICAL STUDY IN COMPARISON OF ONCOGENE HER1 AND HER2 WITH HORMONAL EXPRESSION IN PRIMARY IRAQI BREAST CANCER

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ABSTRACT

Breast cancer is a leading cause of cancer-related deaths in women worldwide.The objective of this current project was to expression the HER1 (Human epidermal growth factor receptor 1), HER2 (Human epidermal growth factor receptor 2), ER (Estrogen receptor), PR (Progesterone receptor), Ki67 and CK18 (Cytokeratin 18) among primary breast cancers. The expression of HER1, HER2, ER, PR, Ki-67, and CK18 was studied by immunohisto chemical (IHC) analysis in 50 patients breast cancer. The molecular classification was used in commonly breastcancer by IHC analysis. Based on this analysis, there were characterized five molecularly different subclasses: luminal type A and B, HER-2, basal-like and unclassified. In multivariate analysis, weexamined the significance of HER1 with other biomarkers which was showed HER1 significant difference with HER2, ER and Ki67.In addition, Luminal A subtype presented a higher percentage (38%) than other molecular subtypes but Luminal B and unclassified subtypes recorded a lower percentage (12%).HER1 a significant difference ($p \leq 0.05$) association with TNM(Tumor size, Lymph Node status, Metastases) stage (I+II) of the patients was showed. Also, we found a significant difference($p \leq 0.05$) association between HER2 with age ≥50 and TNM stage (I+II). Moreover,significant difference ($p \leq 0.05$) association was noticedbetween both ER and CK18 with invasive ductal carcinoma.Our findings suggest that HER1 plays an important role in the pathogenesis of primary breast cancer and associated with clinicopathological parameters and molecular subtypes.

KEY WORDS: Breast cancer,HER1,immunohistochemistry,molecular classification.

INTRODUCTION

Breast cancer is a collection of cells that proliferated abnormally and formed a lump in the breast area, and is characterized by its ability of cancer cells to spread from one place to another and this means that if they left and not treated as soon as may lead to the spread of the disease in the body and then death [1].Itis no longer seen as a single disease but rather a multifaceted disease comprised of distinct biological subtypes with diverse natural history, presenting a varied spectrum of clinical, pathologic and molecular features with different prognostic and therapeutic implications [2]. The most common way to classify breast tumors is according to the status of three specific cell surface receptors;these are the estrogen receptor (ER), the progesterone receptor (PR) and the Human Epidermal Growth Factor Receptor (HER2) 2/neu receptor[3][4].

The HER (human epidermal growth factor receptor) family of receptor tyrosine kinases [5](HER1/EGFR (epidermal growth factorreceptor)/c-erbB1, HER2/c-erbB2, HER3/c-erbB3 and HER4/c-erbB4) shares a high degree of structural and functional homology [6][7][8].HER2 (human epidermal growth factor receptor 2) is one of the first oncogenes to be Identified [9] [10] and is one such gene that can play a role in the development a breast cancer [11].Normally, HER2 receptors help control how a healthy breast cell grows, divides, and repairs itself. But in about 25% of breast cancers, the HER2 gene doesn't work correctly and makes too many copies of itself (known as HER2 gene amplification)[12]. All these extra HER2 genes tell breast cells to make too many HER2 receptors (HER2 protein overexpression)[13].Studies carried out on humans to be hyper- tumor gene expression cerbB-2 is associated with a bad warning in cases of breast cancer. The studies indicated that hyper- expression of this gene is also associated with a high risk of recurrence in early cases of ER positive or passivity. This gene, called a number of other labels, including:HER-2/new, generally considered the presence of this gene tumor useful indicator warning in cancers of the breast, ovary, uterus and digestive system. This makes breast cells grow and divide in an uncontrolled way[14][15].HER-1 is another member of ErbB receptor family and it has similar, although not identical, downstream signals as HER-2[16].HER1 is a 170 kD transmembrane receptor encoded by the human HER1 gene [17] and it protein has an extracellular ligand binding domain, a transmembrane region, and an intracellular domain with intrinsic protein tyrosine kinase activity and multiple autophosphorylation sites clustered at the C-terminal tail [18].HER1 expression has predictive or prognostic value in a number of malignancies, but the predictive and prognostic significance of HER1 in patients with breast carcinoma was

uncommonly studied, leading to insufficient data concerning its biologic significance in particular in patients treated with preoperative chemotherapy[19][20].

ER and PR are the standard biological factors that most accurately predict response to types of hormonal therapy[21].Also, Ki-67 antigen is a nuclear protein, are detected and determined by its interaction with the monoclonal antibody clone of k67[22]. On the other hand, the tumors with high proliferative possess considerable potential for a complete remission after the application of the additional new treatment[23].In normal breast, both luminal epithelial, and the myoepithelial cells exhibit different and distinctive keratin phenotypes. CK 7, 8, 18 and 19 are expressed in the luminal cells, while smooth muscle actin (α) and cytokeratins (CKs) 5, 14 and 17 are found in the myoepithelial/basal cells [24].

The present study,evaluate the expression ofHER1,HER2, ER, PR, Ki67 and CK18 markers among primary breast cancer.Also, we correlated HER1with other markers and correlation of these markers with clinicopathological parameters of patients. In addition, we definite molecular subtypes dependent of these markers.

MATERIALS AND METHODS

Patients and Breast tumor samples

Paraffin-embedded tissue blocks were collected from 50cases which diagnosis as primary breast cancers and submitted for HER1, HER2, ER, PR, Ki67 and CK18 analysis by Immunohistochemical(IHC) from the archives of the Histopathology Unit/Central public health lab/ Ministry of health, Baghdad/ Iraq, in a period extended through November 2013 to January 2014. The mean age of patients was (47.72±8.9 year).

Immunohistochemistry and Scoring

Immunohistochemical analysis was performed as specified by the manufacturer using the labeled streptavidin-biotin (LSAB). Briefly, 4µm paraffin sections were placed in an oven overnight at 50°C then 70°C for 1 hour. The slides were dewaxed in xylene, rehydrated in graded alcohol, incubated in proprietary retrieval at 95°C (in a water bath) for 20 minutes, and cooling the slid at room temperature for 15 minutes. The slides then were placed on an Autostainer plus (DAKO) using the primary antibody, polyclonal rabbit anti-human c-erbB-2 Oncoprotein (Dako Denmark A/S), monoclonal mouse anti-humanERα (Clone 1D5, Dako Denmark A/S), monoclonal mouse anti-human PR(Clone 636, DakoDenmark A/S), monoclonal mouse anti-humanKi-67 (Clone MIB-1, DakoDenmark A/S)and monoclonal

mouse anti-human CK18 (Clone DC 10, DakoDenmark A/S).The slides were placed in water, mounting in aqueous media.

Hercep Test Kit(monoclonal mouse anti-human, code K1497, Dako) was used for detection of HER1. Briefly, the specimens are first incubated with Peroxidase Block for five minutes to quench endogenous Peroxidase activity. The specimens are then incubated for five minutes with a protein block to suppress nonspecific bindingof subsequent reagents, followed by 15-minute incubation with an appropriately characterized and dilutedmouse primary antibody or negative control reagent (user provided). This is followed by sequential 15-minute incubations with anti-mouse immunoglobulins-HRP, fluorescyl-tyramide hydrogen peroxide (amplification reagent) and anti-fluorescein-HRP. Staining is completed by a five-minute incubation with 3,3'diaminobenzidine tetrahydrochloride (DAB)/hydrogen peroxide, which results in a brown precipitate at the antigen site.

HER1 was scored based on the proportion of positively staining cells, were as follows: 0 = none; 1= < 1%, 2 = 1–10%, 3 = 10–33%, 4 = 33–66% and 5= >67% [21]. HER2 was scored as (0) - no staining is observed or cell membrane staining is observed in less than 10% of the tumor cells, (negative). (+1) - A faint perceptible membrane staining can be detected in more than 10% of the tumor cells. The cells are only stained in part of their membrane, (negative). (+2) - A weak to moderate complete membrane staining is observed in more than 10% of the tumor cells, weakly positive (equivocal). (3+) -A strong complete membrane staining is observed in more than 10% of the tumor cells (strong positive) [25].

For ER and PR, using (Allred Method Staining) examined each slide independently, the proportion of cells staining was quantified, (0) - no stained cells, (1)- stained cells <1/100, (2) - 1/100 ≤ stained cells < 1/10, (3) - 1/10 stained cells < 1/3, (4) - stained cells = 1/3 < 2/3 ,(5) - stained cells > 2/3 and the intensity of staining was assessed on a 0 = none ,1 = weak, 2 = intermediate, 3 = strong, to obtain the total score was added staining score and intensity score [26]. Ki67 is a nuclear protein. It was scored as percentage of positively stained cells among the total number of malignant cells scored [27]. The staining intensity of Ck18 was assessed as weak staining, moderate staining or strong staining in each tumor samples of breast cancer [28].The definition for each molecular subtype was based on the expression of ER, PR, HER2 and EGFR as previously described in (Table 1).

Table (1):Definition of each subtype was based on molecular classification.

Molecular subtype	ER and/or PR	HER2 over-expression	EGFR
Luminal A	+	-	**- or +**
Luminal B	+	+	**- or +**
HER2	-	+	**- or +**
Basal like	-	-	+
Unclassified	-	-	-

Statistical analysis

The statistical analyses were performed using Statistical Package for the Social Sciences software system SPSS-22 statistical software(SPSS Incorporation, Chicago, IL, USA).For analyzing differences between HER1 and other parameters, and also the categorical two-tailed Pearson's Chi-squared test was used. Probability values of $P \leq 0.05$ were considered significant in all analyses. The Minitab version 15 statistical programs were used for analysis the association between the clinical parameters and the staining results of all parameters using Chi-square test, $P \leq 0.05$ was considered significant.

RESULTS

In this study, 50 cases of invasive carcinoma of the breast were studied by IHC.The whole series 50 cases had complete data on age, TNM(Tumor size, Lymph Node status, Metastases)stage, grade and histological type. These 50 cases formed the basis of this study. Of these cases, 1 case (2%) was age (20-29 year),7cases(14%) were age (30-39 year),22 cases (44%) were age (40-49 year), 17 cases (34%) were age (50-59 year) and 3 cases (6%) were age (60-69 year).

While TNM stage showed 3 cases (6%) were IA, 13 cases (26%) were IIA, 10 cases (20%) were IIB, 17 cases (34%) were IIIA, 3 cases (6%) were IIIB and 4 cases (8%) were IIIC.In addition, grade was detected in all cases, 5 cases (10%) were grade I, 38 cases (76%) were grade II and 7 cases (14%) were grade III. Furthermore, most of the histological type was ductal carcinoma 39 cases (78%)and 11 cases (22%) were lobular carcinoma(Table 2).

Table (2): Clinicopathologicalparameters in 50 breast cancer patients.

Items	NO. of cases	NO. of cases (%)
1) Age group		
(20-29)	1	2%
(30-39)	7	14%
(40-49)	22	44%
(50-59)	17	34%

(60-69)	3	6%
2) TNM Stage		
IA	3	6%
IIA	13	26%
IIB	10	20%
IIIA	17	34%
IIIB	3	6%
IIIC	4	8%
3) Grade		
I	5	10%
II	38	76%
III	7	14%
4) Histological type		
Ductal carcinoma	39	78%
Lobular carcinoma	11	22%

In the whole 50 breast cancer patients, the 31% HER1 positive of breast cancer cases (Fig. 1A), 10 (32.3 %) were of score 1+ to 2+, 9 cases (29%) were of score 3+, 2 cases (6.6%) were of score 4+ and 10 cases (32.3%) were of score 5+.On the other hand, of the 15%HER2 positive cases (Fig. 1B), 3 (20%) had score 1+, 4 cases (26.6%) had score 2+ and 8 (53.3%) score 3+ . While, there were 21 cases (42%) ER positive(Fig. 1C) and 22 cases (44%) PR positive(Fig. 1D) than 41 cases (82%) Ki67 positive (Fig. 1E) and 38 cases (76%) CK18 positive (Fig. 1F, Table 3).

From our results, there were highly significant ($p \leq 0.05$) associations between HER1and HER2 ($p=0.001$). Statistically a significant ($p \leq 0.05$) difference was found between HER1 as compared with ER and Ki67 ($p=0.045$, 0.026) respectively, while there was no statistically significant ($p \leq 0.05$) differences between HER1 as compared with PR and CK18.

Table (3): Expression of HER1, HER2, ER, PR, Ki67 and CK18 parameters inbreast cancer patients.

	All cases	**Positive n (%)**	**Negative n (%)**
HER1	50	31(62%)	19(38%)
HER2	50	15(30%)	35(70%)
ER	50	21(42%)	29(58%)
PR	50	22(44%)	28(56%)
Ki67	50	41(82%)	9(18%)
Ck18	50	38(76%)	12(24%)

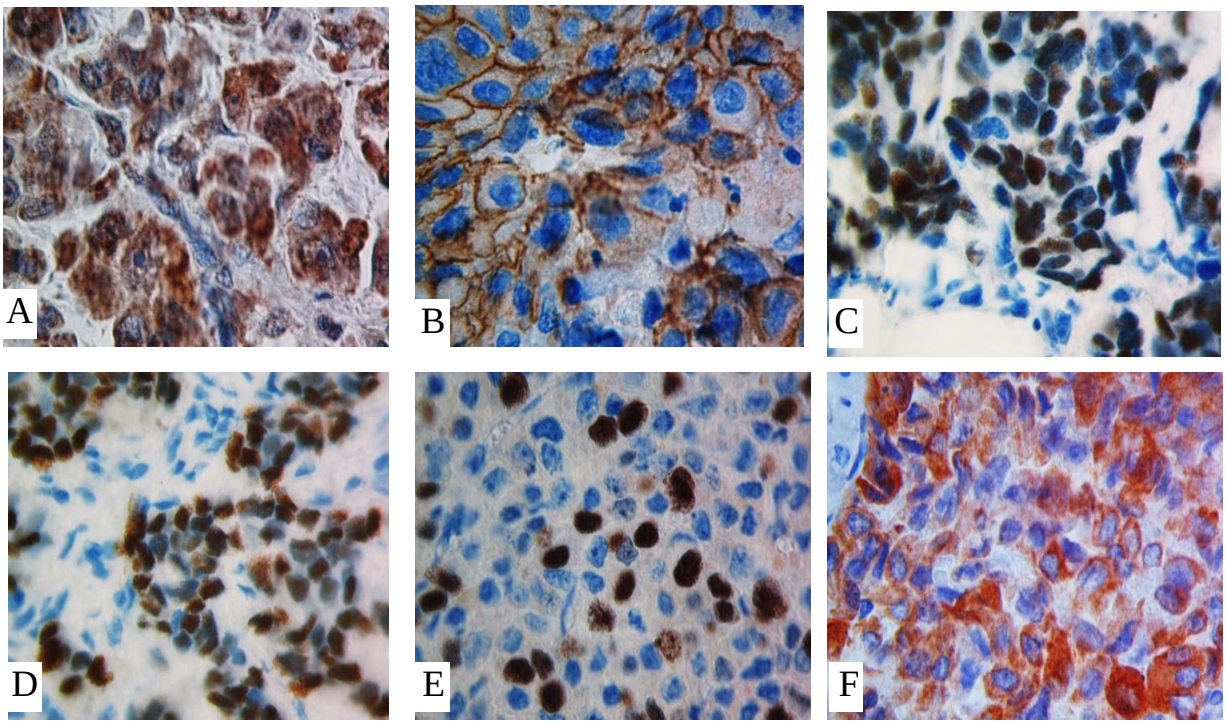

Figure 1: Immunohistochemical profile of breast cancer, (A) HER1 positive staining; (B) HER2 positive staining; (C) ER positive staining; (D) PR positive staining; (E) Ki67 positive staining and (F) CK18 positive staining, Magnification A-F, ×400.

In this study, we performed IHC evaluation of 50 cases with invasive breast carcinoma,testing the reactivity for HER1,HER2,ER,PR and CK18, which are necessary to obtain amolecular classification. As a result we found luminal A type in 19 (38%)cases, luminal B in 6 (12%) cases, HER2 type in 9 (18%) cases, basal like carcinoma in 10 (20%) cases, and unclassified in 6 (12%) cases (Fig.2,Table 4).

Table (4): Correlation between molecular classification and pathological response in (50)breast cancer patients.

Molecular subtype	N. of cases	N. of cases (%)
Luminal A	19	38
Luminal B	6	12
HER2	9	18
Basal like	10	20
Unclassified	6	12

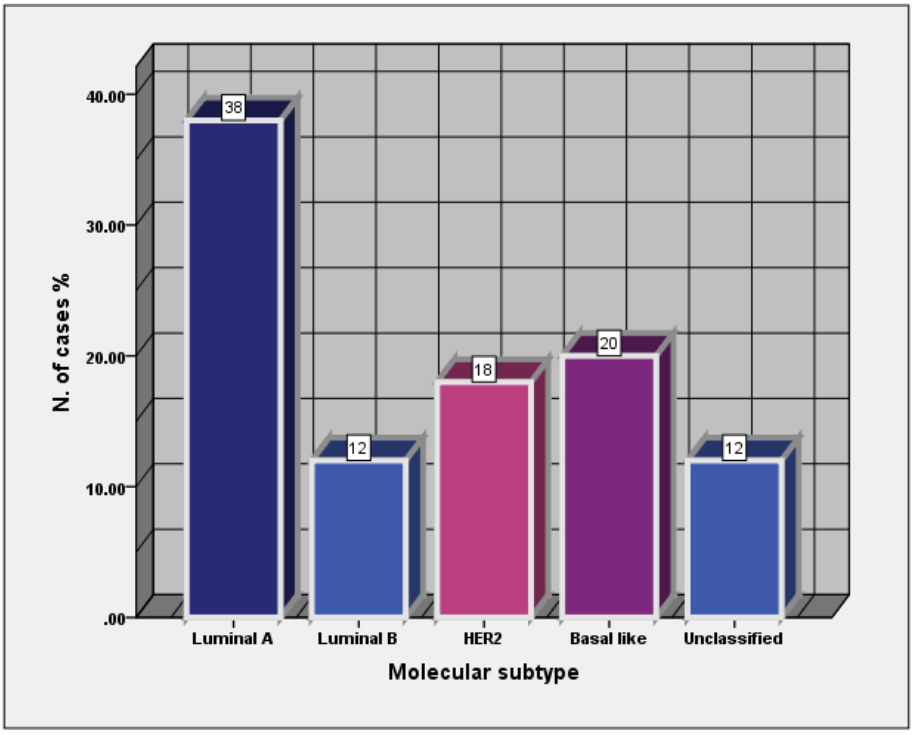

Figure 2:Percentage of different molecular subtypes of 50 breast cancer patients

In the present study, we examined the clinical pathologic variables that were associated with HER1, HER2, ER, Ki67 and CK18.There was no statically significant p≤0.05 association between HER1 with prognostic factors, but we found to be a significant in grade I + II (p= 0.016). There was a significant p≤0.05 association between HER2 and both age ≥ 50 and stage I+II (p = 0.048 andp = 0.022, respectively). While, there was a significant p≤0.05 association between ductal carcinoma and both ER and CK18(p=0.001 and p=0.59 respectively) but there was no significant with other clinical pathologic variables. In addition, we found no statically significant p≤0.05 between Ki67 and clinical pathologic variables (Table 5).

Table (5):Association of HER1, HER2, ER, Ki67and CK18 with Clinicopathological parameters in 50 breast cancer patients.

Items	HER1			HER2			ER			Ki67			CK18		
	+	-	p-value	+	-	p-value	+	-	p-value	+	-	p-value	+	-	p-value
1) Age															
≥ 50	17	11	**NS**	4	20	**0.048**	8	12	**NS**	19	3	**NS**	17	5	**NS**
< 50	14	8		11	15		13	17		22	6		21	7	
2) TNM Stage															
I+II	12	14	**0.016**	5	21	**0.022**	10	16	**NS**	24	2	**NS**	21	5	**NS**

III	19 5		12 12		7 17		18 6		22 2	
3) Grade										
I+II	27 16	**NS**	11 32	**NS**	20 23	**NS**	35 8	**NS**	31 12	**NS**
III	4 3		2 5		1 6		7 0		6 1	
Histological type										
Ductal	26 14	**NS**	13 27	**NS**	14 25	**0.001**	33 6	**NS**	32 7	**0.059**
Lobular	5 6		2 8		7 4		8 3		6 5	

Note: NS, not significant.*P* ≤0.05 is significant

DISCUSSION

Breast cancer is a form of cancer that affects the breast tissue and milk glands and is the second leading cause of cancer death in women (after lung cancer), which is the most common cancer among women.In our study, we describe the status of the prognostic biomarkers, HER1, HER2, ER, PR, Ki67 and CK18, used in breast cancer diagnosis in 50 primary breast cancers.

In fact, limited number of studies has been focused on HER1in primary breast cancer in Iraq. In the current study there was significant difference (p≤0.05) between HER1 with HER2, ER and Ki67.Ansquer et al., 2005 was founded a significant correlation between HER1 and HER2. Some of earlier reports have revealed that the presence of HER2 is associated with increased worse prognosis of breast cancer also, inhibition of HER1 activity has been led to blocking HER2 [29][30][31].

Molecular subtypes were confirmed by IHC analysis. The finding of this study shows an increase in Luminal A subtype (38%) as compared with other subtypes. Numerous studies have revealed that Luminal A subtype has best differentiated tumors and has the better prognosis compared to other subtype. Conversely, Luminal B (12%) and Unclassified (12%) subtypes a worse prognosis than Luminal A. In addition, Basal like subtype (20%) is occurs and more frequently associated with visceral organ and HER2 subtype (18%) is often associated with nodal metastasis[32][33][34].

From the above results, a significant difference association between HER1and TNM stage (I+II) of the patients was showed. Several studies have shown that the level of HER1 correlates with stage, survival, disease progression and response to therapy [35][36][37].Also, we found a significant difference association between HER2 with age ≥50 andTNM stage (I+II).This compares with the findings of Goud et al., 2012 showed positive correlation younger breast cancer patients with HER2. Cheng et al., 2013 have demonstrated the

Coactivator-associated arginine methyltransferase 1 (CARM1) overexpression in younger patients was more common compared with older patients, and might contribute to the clinical characteristics and positively correlated with HER2 expression of younger patients. Previous studies have reported the same relationships with age, TNM stage, grade, tumor size and lymph node [40][41]. Other study hasn't found an association age and TNM stage with HER2 [42]. Our finding regarding was greatly associated between the development of breast cancer disease and HER2 expression.

In addition, our study showed that significant difference in ER and CK18 between invasive ductal and lobular carcinoma ($P \leq 0.05$). However, it is believed that invasive lobular carcinoma they may be associated with both morphologically and biologically different from invasive ductal carcinoma[42].

CONCLUSION

HER1 may be considered susceptibility genetic markers for the risk of breast carcinogenesis but not suitable indicators of disease aggressiveness. In addition, a better understanding of molecular biology and more clinical data are necessary before HER1 and HER2 measurements should be used to select endocrine therapy in routine practice.

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