

Human Epidermal Growth Factor Receptor-3 mRNA Expression as a Prognostic Marker for Invasive Duct Carcinoma not Otherwise Specified

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ABSTRACT

Introduction: Breast cancer is the most common cancer in women and the Erythroblastosis Oncogene B(ErbB) receptor family holds crucial role in its pathogenesis. Human Epidermal Growth Factor Receptor 3 (*HER-3*) gene over expression in breast tissue has been associated with aggressive clinical behaviour and bad prognosis.

Aim: To evaluate *HER-3* mRNA expression level as a prognostic marker for breast cancer and to correlate its level with other established prognostic parameters.

Materials and Methods: This study was carried out on specimens of 100 cases that were divided into 40 patients presented with fibroadenoma and 60 patients presented with Invasive Ductal Carcinoma (IDC) not otherwise specified and underwent modified radical mastectomy. All specimens were investigated for *HER-2/neu*, ER and PR expression by

Immunohistochemistry (IHC) and quantitative assay of *HER-3* mRNA expression using real time PCR technique.

Results: There was a significant high *HER3* mRNA level in carcinoma cases compared to fibroadenoma. In malignant cases, *HER3* mRNA level was significantly associated with advanced T stage, advanced N stage, number of positive lymph nodes, large tumour size and cases associated with an adjacent in situ component. Moreover, *HER-3* mRNA level was of highest values in Her-2/neu positive group followed by triple negative cases with the lowest level in luminal group ($p < 0.05$).

Conclusion: *HER-3* gene is upregulated in IDC especially those carrying poor prognostic features. *HER-3* mRNA level may identify a subset of patients with a poor prognosis, and who could undergo further evaluation for the efficacy of *HER3* targeted anticancer therapy.

Keywords: Breast cancer, Molecular subtypes, Radical mastectomy

INTRODUCTION

Breast cancer is the most common cancer diagnosed in women and the most common cause of female cancer death in both developed and developing countries [1]. In Egypt, breast cancer is still the most common cancer in women with a lower incidence rate than that of USA, however, demographic studies demonstrated that breast cancer will be a greater public health concern in Egypt in the future [2].

Type 1 Growth Factor Receptor (T1GFR) family consists of four known members: HER-1 (Human Epidermal Growth Factor Receptor -1) [also known as Epidermal Growth Factor Receptor, (EGFR), and *c-erb* B-1], HER-2 (*c-erb* B-2), HER-3 (*c-erb* B-3), and HER-4 (*c-erb* B-4) [3]. The T1GFRs are trans membrane receptors with an extracellular ligand-binding domain, an α -helical trans-membrane segment, intracellular tyrosine kinase domain, that exist as monomers in their inactivate form, and a C-terminal phosphorylation tail [4]. Upon ligand binding the T1GFRs form homodimers or heterodimers (with themselves or other family members) and initiate a signaling cascade through activation of its tyrosine kinase, leading to cellular proliferation and transformation [5].

HER-3 was first identified in 1989 by Kraus et al., and is encoded by the *ERBB3* gene [6]. The human *ERBB3* gene is located on the long arm of chromosome 12 (12q13). It is encoded by 23,651 base pairs and translates into 1342 amino acids [7]. The ErbB2-ErbB3 heterodimers are considered the most active of the possible ErbB dimers, in part because ErbB2 is the preferred dimerization partner of all the ErbB family members, and ErbB3 is the preferred partner of ErbB2 [8].

ErB receptor family consists of HER-1, *HER-2* and *HER-3*, where the latter cannot form homodimer and has a weak intra-cellular tyrosine kinase activity [9]. C-terminal region of *HER-3* can bind the SH2 domain of Phosphatidylinositol, 3 Kinase/Akt PI3K by six consensus phosphotyrosine sites, implicating its crucial role in the activation of the PI3K/Akt pathway. It seems clear that activation of PI3K/Akt signaling by *HER-3* is able to overcome HER-targeted inhibition. *HER-3* is capable of restoring its signaling activity despite inhibition of other ErbB kinases due to the redistribution of signaling functions to different ErbB family members [10]. This character of *HER-3* is not noticed in EGFR, *HER-2*, and *HER-4*. Expression and translocation of *HER-3* from the nucleus to the membrane was reported to be responsible for resistance to EGFR or *HER-2* targeted therapy [11].

Activation of *HER3* leads to *HER3* phosphorylation and subsequent activation of signaling pathways, such as phosphatidylinositol 3-kinase (PI3K)/Akt and RAF/MEK/Extracellular Signal Regulated Protein Kinase (ERK) pathways, which promote tumour cell proliferation and survival [12]. *HER-3* was reported as one of the most potent oncogenic factors in promoting breast cancer tumorigenesis [13] and is associated with resistance to *HER2* inhibitors in *HER-2+* breast cancers and anti oestrogen therapy in ER+ breast cancers [14].

The aim of this study was to evaluate *HER-3* mRNA expression level as a prognostic marker for breast cancer and to correlate its level with other established prognostic parameters such as stage, grade, lymph node status and molecular subtypes.

MATERIALS AND METHODS

This study was carried out at Medical Biochemistry and Pathology Departments, Faculty of Medicine. It included 100 female patients.

They were classified into two groups, Group I: 40 patients presented with fibroadenoma, and group II: 60 breast patients presented with IDC not otherwise specified and underwent modified radical mastectomy. These cases were received in Pathology Department, in the period between January 2014 and August 2015. Fresh part of the tumour mass was collected in centrifuge tube and kept in -80°C . Slices from the tumour mass were then immersed in formalin and was submitted to routine tissue processing ending with paraffin embedded blocks formation. Tumours were graded according to the criteria of Nottingham modification in the Bloom-Richardson system [15]. Tumour staging was performed according to TNM staging system [16].

Quantitative assay of *HER-3* mRNA expression using reverse transcriptase polymerase chain reaction technique

Tissue samples were prepared for total RNA isolation using Qiagen RN easy plus Universal Kit from, USA then RNA quality and purity were assured [17] RNA was stored in -80°C till used, then first step-PCR or cDNA synthesis (reverse transcription step) using QuantiTect Reverse Transcription Kit, Qiagen from USA, using Applied Bio systems 2720 thermal cycler (Singapore) for only one cycle as follows: 10 min at 42°C then, 5 min at 95°C to inactivate Reverse Transcriptase and finally for 5 min at 4°C . GAPDH primers were used in RT-PCR reaction as RNA loading control. Second step- PCR or cDNA amplification (real time PCR step): The cDNA was used in SYBR green based quantitative real time PCR for Relative Quantification (RQ) of *HER-3* gene expression by SensiFASTMSYBR Lo-ROX Kit, USA, using the following designed primers (Midland, Texas): 1- *HER3* Forward primer sequence: 5' GGTGCTGGGCTTGCTTTT3', Reverse primer 5' CGTGGCTGGAGTTGGTGTTA 3' and 2-GAPDH Forward primer sequence: 5' GAAGGTGAAGGTCGGAGATC3', Reverse primer: 5' GAAGATGGTGATGGGATTC 3' lastly, data analysis with the Applied Biosystems 7500 software version 2.0.1. The RQ of *HER-3* gene expression was performed using comparative $\Delta\Delta\text{Ct}$ method [18] where the amount of the target (*HER-3*) mRNA, is normalized to an endogenous reference gene (GAPDH) and relative to a control.

Immunohistochemical Procedure

Several 5 μm thick sections of paraffin embedded blocks were deparaffinized, dehydrated and then placed in Citrate Buffered Saline (CBS) (pH 6.0) and steamed for 20 min. Endogenous peroxidase activity was blocked by incubation with 6% H_2O_2 in methanol. The primary antibodies used were mouse monoclonal anti human oestrogen receptor alpha, clone 1D5 (ready to use), mouse monoclonal anti human progesterone receptor, clone PgR 636 (ready to use) and *HER2/neu*, clone 250, diluted 1/50 (Dako, Copenhagen, Denmark). The antibodies were incubated overnight on the slides. Immunoreactivity for Estrogen Receptor (ER) alpha, Progesterone Receptor (PR) and *HER2/neu* was visualized using Envision+ (DakoCytomation, Glostrup, Denmark) with DAB chromogen as substrate and Mayer's hematoxylin as counterstain. ER positive breast carcinoma positive for ER, PR and *HER2/neu* were used as positive control for ER alpha, PR and *HER2/neu*. For all reagents, negative controls were prepared by substituting the primary antibodies with cross matched isotopes.

Interpretation of Immunostaining

For ER and PR, the assessment of the immunohistochemical staining was evaluated according to the American Society of Clinical Oncology/College of American Pathologist (ASCO/CAP) guidelines [19], in addition to Histo Score (H-score) [20]. For *HER-2/neu*, immunoreactivity was evaluated according to ASCO/CAP guidelines [21].

Positive *HER-2/neu* cases were defined as 3+ positivity ($> 10\%$ intense and complete membrane staining); however, score 0 or 1+ was considered negative and 2+ cases were excluded.

Immunohistochemical Subtyping

According to the IHC results of ER, PR, *HER-2/neu*, cases were classified into groups equivalent to molecular subtypes: luminal subtype: ER+ and or PR+, *HER-2/neu*-; *HER-2/neu* positive subtype: ER-, PR-, and *HER-2/neu* +; and lastly, the triple negative (TN) subtype: ER- PR- Her-2 neu [22,23].

STATISTICAL ANALYSIS

Results were collected, tabulated and statistically analyzed by IBM personal computer and statistical package SPSS version 20. Data was expressed into two phases: I – Descriptive: 1) Mean value and Standard Deviation (SD), median, range: for quantitative data; 2) Frequency and percentage for qualitative data. II- Analytic: Mann-Whitney U test for comparison of two independent quantitative variables not normally distributed. K (Kruskal Wallis) test: for comparison of more than two independent quantitative variables not normally distributed. Spearman Correlation Coefficient (r): for comparison between two dependents quantitative not normally distributed variable. The p-value < 0.05 was considered statistically significant.

RESULTS

This study was conducted on a total number of 100 female patients divided into two groups; 40 patients presented fibroadenoma and 60 patients presented with IDC not otherwise specified. The clinicopathological characteristics of malignant cases are presented in [Table/Fig-1]. The malignant cases were classified according to ER, PR and *HER2/neu* immunohistochemical expression into 35 cases of luminal type, 9 cases showed positive Her-2 neu expression [Table/Fig-2] and 16 cases were triple negative.

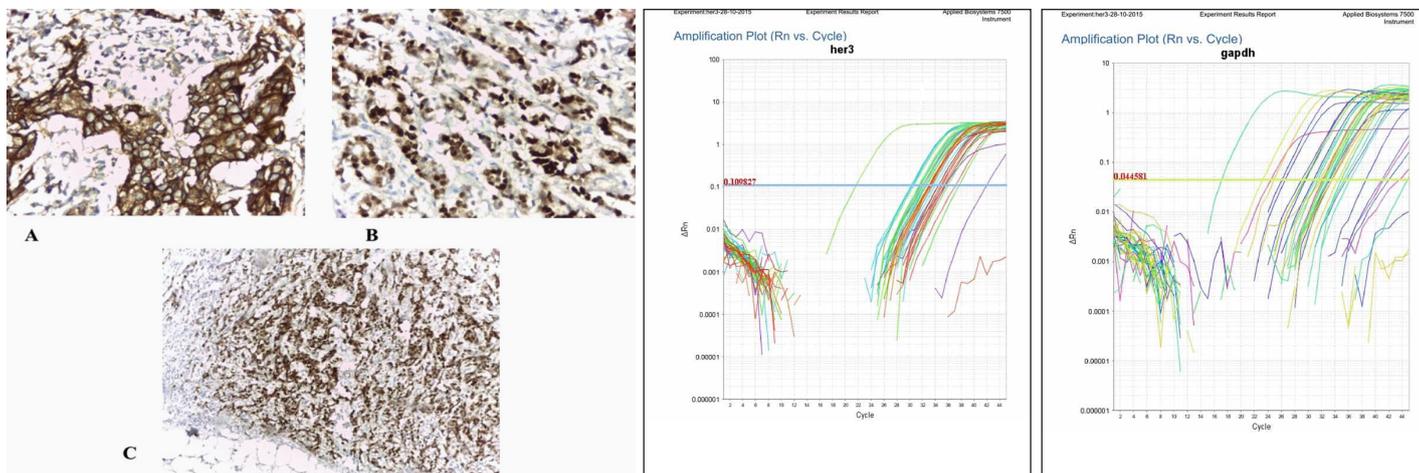
HER-3 mRNA level in the studied malignant and benign cases.

There was significant difference between benign and malignant breast lesions regarding *HER-3* mRNA level, since, its mean and median values were high in malignant cases compared to benign one ($p < 0.05$) [Table/Fig-3-6].

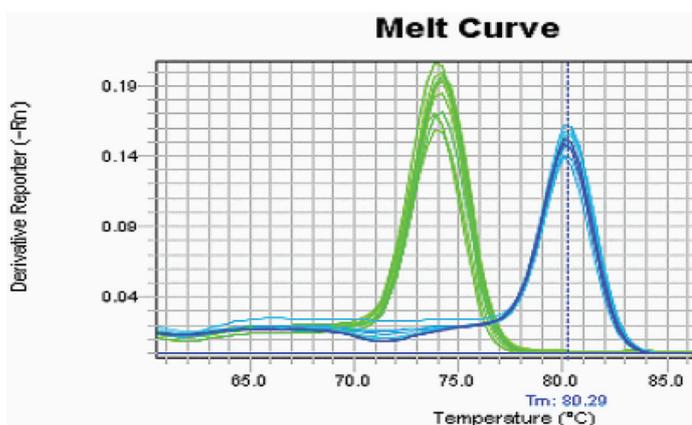
The relationship between *HER3* mRNA level and the studied

		Malignant cases		Control group
		Number	Percentage	
Age (years)	mean \pm SD	47.9 \pm 13.4		48.6 \pm 13.5
	median	48.5		46
	range	21-82		21-82
Tumour size (cm)	mean \pm SD	2.9 \pm 1.9		
	median	2		
	range	0.75-8.5		
T stage	1	26	43	
	2	24	40	
	3	10	17	
	4	0	0	
N stage	0	16	27	
	1	22	37	
	2	14	23	
	3	8	13	
Number of positive lymph node	mean \pm SD	5.7 \pm 4.5		
	median	5		
	range	1-17		
Type	Luminal	35	58	
	Her-2/neu positive	9	15	
	triple negative	16	27	
Nearby insitu component	negative	46	77	
	positive	14	23	

[Table/Fig-1]: Clinicopathological data of studied cases.



[Table/Fig-2]: A:Her-2/ neu+strong membranous staining (+++) (immunohistochemical staining x200), B: Strong diffuse nuclear expression for oestrogen receptor (immunohistochemical staining x 400), and C: Strong diffuse nuclear expression for progesterone receptor (immunohistochemical staining x 100). **[Table/Fig-3]:** Amplification plot showing the expression of Her3 mRNA in all examined samples. **[Table/Fig-4]:** Amplification plot showing the expression of reference gene (GAPDH) in all examined samples.



[Table/Fig-5]: Melting curve analysis identifying specificity of chosen primers.

		Control group	Malignant cases	Test p value
RQ of HER3mRNA	Mean±SD	0.7±0.6	108.5±98.1	5.8** <0.05
	Median	0.5	80	
	Range	0.02-2.8	7-411	

[Table/Fig-6]: Comparison between control and malignant groups regarding RQ of *HER-3* mRNA expression. *HER-3*: human epidermal growth factor receptor 3, **: Mann Whitney test, RQ: relative quantification.

clinicopathological parameters in breast carcinoma

The mean and median values of *HER3* mRNA level were significantly associated with breast carcinoma of advanced T stage ($p<0.001$) and advanced N stage ($p<0.001$). Furthermore, its level was significantly of highest values in Her-2/neu positive group followed by triple negative cases with the lowest level in luminal group ($p<0.05$). There was a statistical positive correlation between *HER-3* mRNA level and large tumour size ($p<0.001$) and number of positive lymph nodes ($p<0.001$). Moreover, breast carcinoma with an adjacent carcinoma in situ showed higher values of *HER-3* mRNA level compared to pure cases without in situ component ($p<0.05$) [Table/Fig-7]. On the other hand no significant association was identified with other parameters such as tumour grade and patient's age.

DISCUSSION

The development of breast cancer involves multiple steps of progression, which started with ductal hyper proliferation, followed by carcinoma in situ, invasive carcinoma, and eventually metastatic disease [24]. In human breast cancers both *HER-3* mRNA and protein are up regulated. Compared to normal breast tissue, *HER3* protein over expression has been reported in 50–70% of human breast cancers and seems to be associated with metastasis,

		No. of cases	RQ of <i>HER3</i> mRNA			Kruskal wallis	p-value
			Mean±SD	Median	Range		
Age		60				-0.03*	0.8
Tumour size		60				0.92*	<0.001
T stage	early (T1 and T2)	50	77.9±57.4	67.5	7-228	7.5	<0.001
	advanced (T3 and T4)	10	261.4±118.4	290.5	39-411		
N stage	early (N0 & N1)	38	56.5±43.5	44	7-184	7.5	<0.001
	advanced (N2 and N3)	22	198.2±101.9	170	47-411		
Number of positive lymph nodes		60				0.93*	<0.001
Type	Luminal	35	44.3±26.3	39	7-92	107.1	<0.05
	Her2 positive	9	284±78.8	267	178-411		
	Triple negative	16	149.9±55.5	127	89-314		
Nearby insitu component	Negative	46	74.5±72.9	57.5	7-378	6.2	<0.001
	Positive	14	219.9±88.3	217	89-411		

[Table/Fig-7]: Correlation between RQ of *HER3* mRNA levels and clinicopathological data of studied malignant cases. *: r (Spearman correlation), *HER2*: human epidermal growth factor receptor 2, *HER3*: human epidermal growth factor receptor 3, RQ: relative quantification.

tumour size, and risk of local recurrence [10].

In the present study, there was a significant statistical increase of *HER-3* mRNA expression fold change (RQ) in IDC group when compared to benign cases, Kalemi et al., demonstrated similar findings and *HER3* was found to exhibit the strongest mRNA expression among other T1GFR family members but instead of benign group, healthy subjects were investigated [25]. Studies utilizing IHC on paraffin embedded tissue have reported *HER-3* over expression ranging from 13.8% to 75% of breast cancers [26,27]. Upregulation and reactivation of *HER-3* function as escape mechanisms from EGFR inhibition and may play a role in tumour resistance to EGFR targeting tyrosine kinase inhibitors [28].

The current study demonstrated *HER-2/neu* over expression by IHC in 15% of breast cancer patients (9/60) agreeing with others [29] but it was much lower than other studies [30] who reported 79.5% (39/49). This could be attributed to the increasing aggressiveness of their tumours due to the younger age of their patients, also the staining intensity required to diagnose *HER-2/neu* over expression and the used antibodies vary among studies.

The current study demonstrated the highest level of *HER-3* mRNA expression in *HER-2*/neu positive cases (aggressive and fast growing) compared to other molecular subtypes such as triple negative and luminal types. The least level of expression was in luminal group (The highest survival rates and fairly low recurrence rates) while higher levels of *HER-3* was found in triple negative group (poorer prognosis than luminal one).

Significant coexpression of *HER-2* and *HER-3* in breast cancer has been inconsistently reported in the literature [31,32]. Formation of dimers between *HER-3* and *HER-2* seems to be crucial for the *HER-2*-driven signals in those tumours with *HER-2* over expression [4]. Ocana et al., stated that, the expression of *HER-3* is associated with worse survival in solid tumours [33]. The influence of *HER-3* may be greater in those tumours where *HER-2* is commonly over expressed.

HER-3 over expression by immunoprecipitate and immunoblotting protein quantification, RT-PCR, and genomic transfection have been demonstrated in both preclinical invitro and invivo studies to promote breast cancer oncogenesis and tumour growth [30]. The present study demonstrated the association of *HER-3* mRNA level with several bad prognostic parameters in breast carcinoma such as advanced T stage, large tumour size and number of positive lymph nodes agreeing with others [27].

In 1992, Lemoine et al., reported the association between *HER-3* expression with a worse prognosis [34] and Travis et al., found that tumours with *HER-3* expression appeared to develop locally recurrent disease [26]. A Malaysian study by Naidu et al., suggested that it could be involved in the progression of tumours from preinvasive to invasive stage [35]. Furthermore it was associated with established poor prognostic factors. Miller et al., stated that *HER-3* is associated with targeted therapeutic resistance to *HER-2* inhibitors in *HER-2*+ breast cancers and anti oestrogen therapy in ER+ breast cancers [14].

LIMITATION

The limitations of the current study are lacking follow up and survival data together with absence of normal breast tissue to serve as true control.

CONCLUSION

HER3 gene was upregulated in IDC especially those carrying poor prognostic features. *HER-3* mRNA level may identify a subset of patients with a poor prognosis, and who could undergo further evaluation for the efficacy of *HER-3* targeted anticancer therapy.

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