Differential Expression of EGFR in Primary Tumor and Lymph Node Deposits of Breast Carcinoma

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Abstract— Epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptors. Its stimulation by endogenous ligands, EGF or transforming growth factor-alpha (TGF-α) results in activation of intracellular tyrosine kinase, therefore, cell cycle progression. High levels of EGFR expression are correlated with poor prognosis and resistance to radiation therapy in a variety of cancers. This study was conducted on Tissue samples include 6 excision biopsies from benign cases and 60 modified radical mastectomy biopsies from malignant cases. Benign lesions include fibrocystic disease of breast (4 cases) and fibroadenoma (2 cases), while malignant tumors include invasive duct carcinoma (50 cases), invasive lobular carcinoma (4 cases) and duct carcinoma in situ (6 cases). High significant differences in EGFR expression percentage and intensity between benign (that were all negative for EGFR), and malignant breast lesions. Also, parameters of EGFR expression were significantly lower in lymph node metastatic deposits in relation to primary breast tumors. There was no significant difference in EGFR positivity between all stages of primary breast cancer and between EGFR positivity in primary breast cancer and metastatic lymph node deposits, Percentage of EGFR cellular positivity and intensity of staining were higher in primary breast cancers of all stages compared to metastatic deposits in lymph nodes, however, the differences between both groups were non-significant. so Evaluation of EGFR expression in the primary breast cancer tissue and the metastatic deposits of breast cancer in axillary lymph nodes, it will reflected on diagnosis and therapy.

Index Terms— Benign, Breast Cancer, EGFR, Immunohistochemistry, Lymph node, Malignant and Metastasis.

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1 INTRODUCTION

Breast cancer is the most commonly occurring female cancer and the leading cause of cancer deaths worldwide. Breast cancer occurs in approximately 1 in 8 women and 1 in 37 women with breast cancer succumbed to the disease. Over the past decades, new diagnostic tools and treatments have substantially improved the prognosis of women with local diseases. However, women with metastatic disease still have a dismal prognosis without effective treatments [1]. Normal breast cells become cancerous because of mutations in the DNA, and although some of these are inherited, most DNA changes related to breast cells are acquired during one's life. Proto-oncogenes help cells to grow. If these cells mutate, they can increase growth without any control. Such mutations are referred to as oncogenesis and such uncontrolled cell growth can lead to cancer[2].

There are many types of breast cancer such as ductal carcinoma in situ, invasive ductal carcinoma, mucinous carcinoma, adenoid cystic carcinoma and Lobular carcinoma. Breast cancer is a heterogeneous disease and it encompasses a variety of entities with distinct morphological appearances and clinical behaviours [3]. In recent years, it has become evident that this diversity is the result of genetic alterations. The analysis of gene expression data has suggested that breast cancers can be divided into molecular subtypes which have distinct clinical features, with markedly differing prognoses and

clinical outcomes. These subtypes consist of two ER positive types (Luminal A and Luminal B) [3] - [6].

Triple-negative breast cancers are a group of primary breast cancers which lack the expressions of the oestrogen receptor (ER), the progesterone receptor (PR) and HER-2. Although the triple-negative phenotype has been considered as sufficient to identify the 'basal-like' tumours, increasing evidence has shown that the terms 'basal-like' and 'triple-negative' are not synonymous [7]. Axillary lymph node (ALN) status is an important prognostic factor and determinant of treatment for patients with breast carcinoma. Clinical trials have proven that Sentinel lymph node (SLN) is equivalent to axillary lymph node for staging of the axilla in patients with clinically node-negative disease and is associated with significantly less morbidity[8].

The protein encoded by EGFR gene is a transmembrane glycoprotein that is a member of the protein kinase superfamily. This protein is a receptor for members of the epidermal growth factor family. EGFR is a cell surface protein that binds to epidermal growth factor. Binding of the protein to a ligand induces receptor dimerization and tyrosine autophosphorylation and leads to cell proliferation. Mutations in this gene are associated with lung and breast cancer [9] – [12].

The aim of this project to evaluate the expression of EGFR in the primary breast cancer tissue and the metastatic deposits of breast cancer in axillary lymph nodes, in order to check if there is a difference in tumor behaviour between both locations, that will be reflected on diagnosis and therapy

2 MATERIAL AND METHODS

Paraffin sections from 60 breast cancer cases and there corresponding axillary lymph node metastases as well as 6 benign breast lesions were included in the present study. They were taken from the archival material of the pathology department of Theodor Bilharz Research Institute, Cairo, Egypt. Sections were cut on positively charged slides and were subjected to the following procedures:

1) Routine histopathological examination using paraffin sections stained by hematoxylin and eosin stain, with special reference to:

- Diagnosis benign and malignant lesions
- Diagnosis of grade, stage and type of breast carcinoma
- Diagnosis of metastatic deposits in regional lymph nodes

2) Immunohistochemical study of tissue sections using monoclonal antibody against EGFR antigens.

Immunohistochemical Method

Anti-EGFR antibody (Santa Cruz Biotechnology) was used for immunohistochemical (IHC) detection of the expression of EGFR protein in tissue. Tissue sections were processed for IHC analysis of EGFR protein as follows. IHC examinations were carried out on 4-5 μm thick sections. For anti-EGFR IHC, antigen retrieval was performed with 10 mM sodium citrate buffer, pH 6.0, at 90°C for 30 min. Sections were incubated in 0.03% hydrogen peroxide for 10 min at room temperature, to block endogenous peroxidase activity, and then in blocking serum (0.04% bovine serum albumin, A2153, Sigma-Aldrich, Shanghai, China, and 0.5% normal goat serum X0907, Dako Corporation, Carpinteria, CA, USA, in PBS) for 30 min at room temperature. Anti-EGFR antibody (A11): sc- 80652 EGFR Antibody (A11) is a mouse monoclonal IgG2a provided at 200 μg/ml rose against a truncated extracellular domain of EGFR of human origin (Santa Cruz Biotechnology, USA). The antibody was used at a dilution of 1:100. The antibody was incubated overnight at 4°C. Sections were then washed three times for 5 min in PBS. Non-specific staining was blocked 5% normal serum for 30 min at room temperature. Finally, staining was developed with diaminobenzidine substrate and sections were counterstained with hematoxylin. PBS replaced EGFR antibody in negative controls.

2.1Quantification of protein expression

The expression of EGFR was semi quantitatively estimated as the total membrane-cytoplasmic immunostaining scores, which were calculated as the product of a proportion score and an intensity score. The proportion and intensity of staining was evaluated independently.

All immunostained slides were analyzed and scored, the EGFR positive staining was indicated by brown cytoplasmic, membranous, or both cytoplasmic and membranous staining of the hepatocytes.

The score used for EGFR interpretation according to Morinaga et al., 2006 [13], Buckley et al., 2008[14] and Harder et al., 2009[15] is the number of positive cells evaluated under x400 magnification (Extent of expression) and was assessed as:

0 = no positive cells
1+ = 1-10% positive cells,
2+ = 11-50% positive cells,
3+ > 51% of cells with positive staining.

2.2Statistical analysis

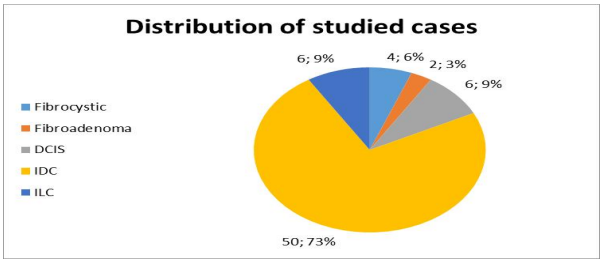
Pearson's Chi square test was used to compare the differences in percentages of positive results between groups. ANOVA and student t-tests were used to compare groups' means. Fisher's exact chi square test was used to compare between percentages. SPSS 20.0 for Windows was used for all statistical analyses. Significant differences between groups were achieved if ($p<0.05$).

3 Results

Tissue samples include 6 excision biopsies from benign cases and 60 modified radical mastectomy biopsies from malignant cases. Benign lesions include fibrocystic disease of breast (4 cases) and fibroadenoma (2 cases), while malignant tumors include invasive duct carcinoma (50 cases), invasive lobular carcinoma (4 cases) and duct carcinoma in

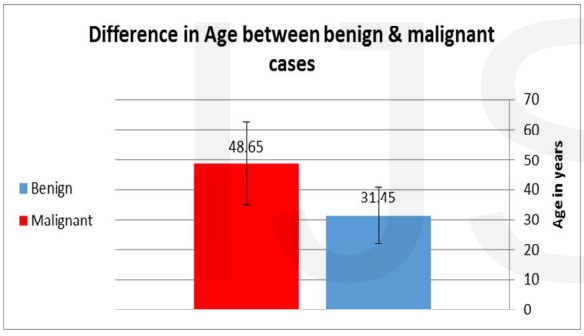
situ (6 cases).

Histogram (1): Distribution of studied cases



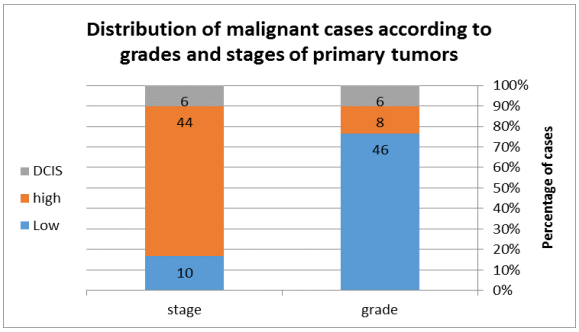
Our study was conducted on breast and lymph node biopsies from 60 female patients ranging in age from 18 to 55 years. The mean age for benign cases was 31.45±9.22 years and for malignant cases was 48.65±13.81 (p <0.01).

Histogram (2): Age difference between benign and malignant studied cases:



Most of the studied cases were of low grades (G1 and G2) but high stages of malignancy (T2 and T3).

Histogram (3): Distribution of studied malignant cases according to grades and stages of breast canc



There were high significant differences in means of EGFR expression percentage and intensity between benign (that were all negative for EGFR), and malignant breast lesions. Also, parameters of EGFR expression were significantly lower in lymph node metastatic deposits in relation to primary breast tumors. (Table 1).

Table (1): Difference in EGFR expression between benign and malignant breast lesions, compared to lymph node (LN) metastasis

Significant difference with the malignant breast EGFR percentage ($p<0.01$)

*High significant difference with the malignant breast EGFR intensity ($p<0.0001$)

-Similar letters (a) denote non-significant difference between groups.

No significant difference in percentage of EGFR positive cases was achieved between low and high grade primary breast cancer cases using Ficher's exact test ($p>0.05$). Also, no significant difference in percentage of EGFR positive cases was achieved between high grade primary breast cancer and lymph node metastasis ($p>0.05$).

Table (2): Difference in EGFR expression in relation to different grades of malignancy and lymph node metastatic deposits:

P: Positive cases within group T: Total number of cases within group

There was no significant difference in EGFR positivity between all stages of primary breast cancer ($p>0.05$). No significant difference was achieved between EGFR positivity in primary breast cancer and metastatic lymph node deposits ($p>0.05$).

Table (3): Difference in EGFR expression in relation to different stages of malignancy and lymph node metastatic deposits:

P: Positive cases within group T: Total number of cases within group

Percentage of EGFR cellular positivity and intensity of staining were higher in primary breast cancers of all stages compared to metastatic deposits in lymph nodes, however, the differences between both groups were non-significant using t-test ($p>0.05$).

TABLE 1

Diagnosis		Breast EGFR %	Breast EGFR in-tensity	Breast EGFR positivity	LN EGFR %	LN EGFR Intensi-ty	LN EGFR Positivity
Benign (6)	Mean	0	0	0/6 (0%)a	-	-	-
	N	0	0		-	-	
	S. D.	0	0		-	-	
Malignant (60)	Mean	84.54	2.33	7/60 (11.67%)a	63.38*	1.91**	5/48 (10.42%)a
	N	60	60		48	48	
	S. D.	36.03	0.46		30.47	0.42	
		p<0.0001	p<0.0001		-	-	-

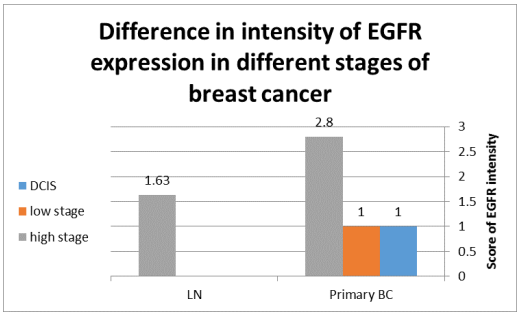
TABLE 2

GRADE	Breast Ca EGFR Positivity P/T (%)	LN EGFR Positivity P/T (%)
Low grade (46)	5/46 (10.87%)	4/40 (10%)
High grade (8)	1/8 (12.5%)	1/8 (12.5%)
Total	7/54 (12.96%)	5/48 (10.42%)

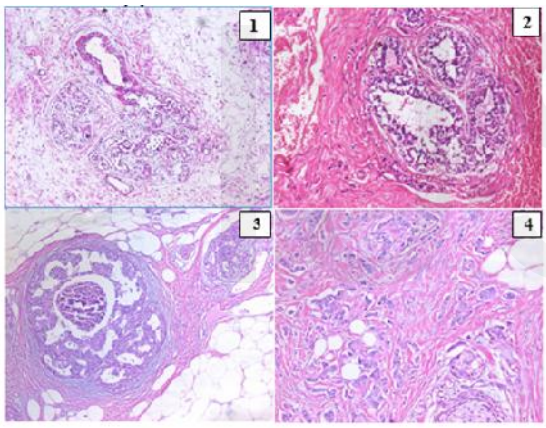
TABLE 3

STAGE	Breast Ca EGFR Positivity P/T (%)	LN EGFR Positivity P/T (%)
DCIS (6)	1/6 (16.67%)	-
Low stage (10)	1/10 (10%)	0/4 (0%)
High stage (44)	5/44 (11.36%)	5/44 (11.36%)
Total	7/60 (11.67%)	5/48 (10.42%)

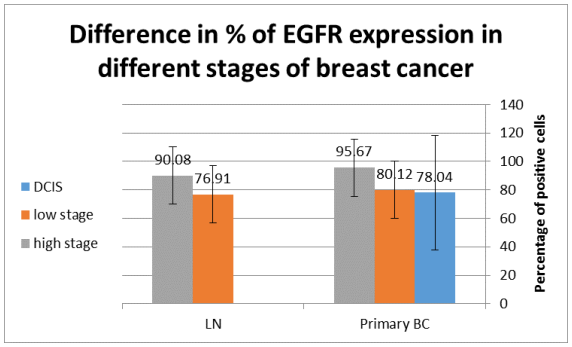
Histogram (4): Difference in EGFR intensity in different stages of breast cancer.



Gaph: show Difference in percentage of cellular expression of EGFR between different grades of Breast cancer and variants of lymph node



Histogram (5): Difference in EGFR percentage in different stages of breast cancer.



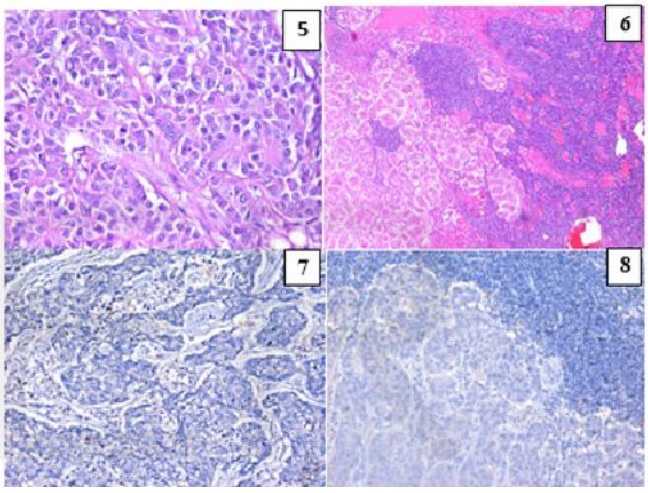


Fig. (1): Section in normal breast tissue showing breast lobules and a ductule (Hematoxylin and eosin stain, X100)

Fig. (2): Section in breast tissue showing benign fibrocystic changes (Hematoxylin and eosin stain, X100)

Fig. (3): Section in breast tissue showing Duct Carcinoma In Situ (DCIS), with cribriform pattern (Hematoxylin and eosin stain, X100)

Fig. (4): Section in breast tissue showing Invasive Duct Carcinoma (IDC) of moderate differentiation (G2) (Hematoxylin and eosin stain, X200)

Fig. (5): Section in breast tissue showing Invasive Duct Carcinoma (IDC) of high grade (G3), with visible mitotic figures (arrows) (Hematoxylin and eosin stain, X200)

Fig. (6): Section in axillary lymph node showing metastatic deposits of Invasive
Duct Carcinoma (IDC) of moderate differentiation (G2) (Hematoxylin and eosin stain, X200)

Fig. (7): Section in breast tissue showing Invasive Duct Carcinoma (IDC) of low grade (G2), showing negative EGFR expression ((IHC for EGFR-DAB, X200)

Fig, (8): Section in axillary lymph node showing metastatic deposits of Invasive Duct Carcinoma (IDC) with negative expression for EGFR (IHC for EGFR-DAB, X200)

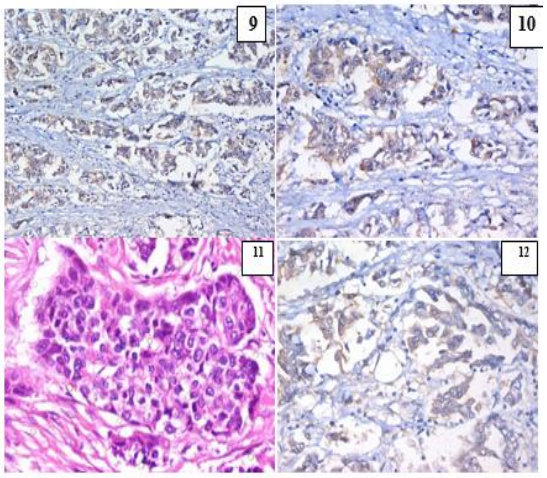


Fig. (9): Section in breast tissue showing Invasive Duct Carcinoma (IDC) of low grade (G2), showing positive EGFR expression ((IHC for EGFR-DAB, X200)

Fig. (10): Higher magnification of previous section showing high EGFR expression ((IHC for EGFR-DAB, X400)

Fig. (11): Section in lymph node showing metastatic deposit of Invasive Duct Carcinoma (IDC) of low grade (G2) (Hematoxylin and eosin stain, X200)

Fig. (12): Section in lymph node showing high expression of EGFR in metastatic deposit of IDC ((IHC for EGFR-DAB, X400)

4 Discussion

High significant differences in EGFR expression percentage and intensity between benign and malignant breast lesions. Also, parameters of EGFR expression were significantly lower in lymph node metastatic deposits in relation to primary breast tumors. There was no significant difference in EGFR positivity between all stages of primary breast cancer and between EGFR positivity in primary breast cancer and metastatic lymph node deposits, Percentage of EGFR cellular positivity and intensity of staining were higher in primary breast cancers of all stages compared to metastatic deposits in lymph nodes. so EGFR is promising marker which can use differential diagnosis of primary and metastatic carcinomas of the breast as well as a marker for future target therapy of breast cancer.

Breast cancer is a complex disease that results from the inheritance of a number of susceptible genes. [16] Although exhaustive investigations from single-locus to genome-wide association studies have been conducted, to unravel the ultimate genetic underpinnings of breast cancer still remains a challengeable task. [17] – [19] Metastasis is a major cause of mortality in Breast Cancer (BC) patients. [20] Among the different types of BC, triple negative BC (TNBC) (ER-, PR-, and HER2-) has been associated the most with poor prognosis and survival due to early metastasis to other organs and a lack of clinically established targeted therapies. [21] Hence, elucidating novel mechanisms that regulate metastasis would lead to the development of targeted therapies and new treatments for TNBC and metastatic breast cancers. [22]

The epidermal growth factor receptor (EGFR) is one of the first identified important targets of these novel antitumor agents. [23] Approximately half of cases of triple-negative breast cancer (TNBC) and inflammatory breast cancer (IBC) overexpress EGFR. Thus, EGFR inhibitors for treatment of breast cancer have been evaluated in several studies. Recent studies have shown that EGFR and its downstream pathway regulate epithelial-mesenchymal transition, migration, and tumor invasion and that high EGFR expression is an independent predictor of poor prognosis in IBC. [24] – [26] Our examined tissue samples include 6 excision biopsies from benign cases and 60 modified radical mastectomy biopsies from malignant cases. Benign lesions include fibrocystic disease of breast (4cases) and fibroadenoma (2 cases), while malignant tumors include invasive duct carcinoma (50 cases), invasive lobular carcinoma (2 cases), medullary canoma (1 case), mucoid carcinoma (1 case) and intraduct carcinoma (6 cases). Invasive duct carcinoma cases include 3 cases of tubular carcinoma, and 47 cases of non-otherwise specified (NOS) carcinoma. Most of the studied cases were of low grade but high stage of malignancy (GI&II, T2&3). [17]

There were high significant differences in means of EGFR expression percentage and intensity between benign (that were all negative for EGFR), and malignant breast lesions. [27] Also, parameters of EGFR expression were significantly lower in lymph node metastatic deposits in relation to primary breast tumors. This was in agreement with [28] who stated that EGFR expressions were significantly lower in lymph node metastases compared to primary breast tumors. No significant difference in percentage of EGFR positive cases was achieved between low and high grade primary breast cancer cases using Fisher's exact test

(p>0.05). [29] Breast cancer activity in peri-malignant tissue, was moderate in lower grade (grade 1), while marked expression was seen in higher grades (grades 2,3). [30] This can be related to severe injury of the breast cancer However, these differences were statistically nonsignificant. (p>0.05) This result was in relation with previous study of [31] – [34] which state that the histological grade between low and high grade primary breast cancer (p>0.05)

In examined EGFR expression in metastatic lymph node and EGFR expression in different grades of metastatic breast cancer activity in tissue, was moderate in lower grade (grade 1), while marked expression was seen in higher grades (grades 2,3) No significant difference was achieved between EGFR positivity in primary breast cancer and metastatic lymph node deposits (p>0.05). [35] Regarding intensity of EGFR immunoexpression, malignant breast tissue percentage showed significant difference (p<0.001), the least incidence of marked intensity of EGFR, compared to malignant breast EGFR which showed the highest value, with significant difference (p<0.0001). This is in agreement with [36] who found marked positivity in breast cancer intensity of EGFR expression was positively correlated with nuclear grade. However, [37] – [38] reported that most of their studied benign breast cancer tissue showed marked intensity followed by moderate intensity. [39] Percentage of EGFR cellular positivity and intensity of staining were higher in primary breast cancers of all stages compared to metastatic deposits in lymph nodes, however, the differences between both groups were non-significant using t-test (p>0.05). Similar results were obtained by Foulkes et al (2010) [40].

5 Conclusions

EGFR expression percentage and intensity between benign and malignant breast lesions record high significant differences. Also, parameters of EGFR expression were significantly lower in lymph node metastatic deposits in relation to primary breast tumors . There was no significant difference in EGFR positivity between all stages of primary breast cancer and between EGFR positivity in primary breast cancer and metastatic lymph node deposits, Percentage of EGFR cellular positivity and intensity of staining were higher in primary breast cancers of all stages compared to metastatic deposits in lymph nodes. So EGFR is promising marker which can use differential diagnosis of primary and metastatic carcinomas of the breast as well as a marker for future target therapy of breast cancer.

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