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Pathophysiology

Folate receptor α is associated with poor clinicopathological perspectives in breast carcinoma

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ABSTRACT

Background: Breast carcinoma is the commonest malignancy in females. Folate is required for the biosynthesis of nucleotide bases, amino acids, and other cellular methylation reactions in proteins and phospholipids. The high affinity folate receptor alpha (FRα) has been shown to be expressed in several kinds of human cancers.

Methods: In this descriptive-analytic study, sections from formalin-fixed paraffin-embedded tissue blocks of 50 cases of invasive ductal breast carcinoma (IDC) as well as 15 cases of non-neoplastic breast specimens were immunohistochemically stained with FRα antibody. Histopathological evaluation for various clinicopathological parameters was done and was correlated with FRα expression.

Results: Positive FRα expression was more frequently detected in IDC (64%) compared to non-neoplastic breast specimens (20%). In IDC, Positive FRα expression was significantly associated with high tumor grade (p = 0.007), large tumor size (p < 0.001), high lymph node stage (p = 0.004), presence of angiolymphatic emboli (p = 0.001), presence of perineural invasion (p = 0.001). Significant association between FRα positivity and negative hormone receptors (estrogen and progesterone) (p < 0.001) and triple negative cases (p = 0.0021).

Conclusion: Our work demonstrates that FRα is over expressed in IDC compared to non-neoplastic breast tissue. Folate receptor α expression was associated with poor clinicopathological perspective. This work suggests that FRα may be an independent prognostic factor and supports the possibility of using FRα-targeted therapies of breast carcinoma. However, our work requires validation on larger cohort with correlation with survival data of patients.

1. Background

Breast cancer is considered as the most common cancer and the leading cause of carcinoma death in females worldwide [1]. Despite the great advance in early detection and treatment of breast cancer; recurrence, metastasis and mortality remain high. The molecular targeted therapy of breast cancer is offering hope to selected groups of breast cancer patients [2].

Folate, or vitamin B9, is a basic component of cell metabolism and DNA synthesis and repair. It is required for the biosynthesis of nucleotide bases, amino acids, and other cellular methylation reactions in proteins and phospholipids. Accordingly, folate is needed in large quantities in rapidly proliferating cells [3].

Cellular uptake of folate may be mediated by a reduced folate carrier, a proton-coupled folate transporter, or high affinity folate receptor (FR). Several high-affinity FR isoforms have been identified (α, β, γ, and δ), but most studies have focused on folate receptor alpha (FRα) [4].

Normally, the expression of FRα is restricted to some types of epithelial cells (low expression levels), such as the kidney, retina, choroid plexus and placenta. Folate receptor α binds with a low affinity to reduced folate and a much higher affinity to oxidized folate and it then transports the folate into the cytoplasm by endocytosis [4].

Folate receptor α is a high affinity glycosyl phosphatidyl inositol anchored membrane protein. It is upregulated in some cancers of epithelial origin such as ovarian, endometrial and lung cancers; perhaps reflecting their increased need for folate to support rapid cell division. Moreover, the expression levels of FRα have been associated with disease stage, aggressiveness and survival in some of these tumors [3–5].

Therapeutic strategies targeting FRα have shown great promise in preclinical studies and early clinical trials. Folate has been recently utilized to deliver attached drugs selectively into pathologic cells that overexpress FRα. In such a way, folate conjugation allows selective delivery of non-specific drugs into pathologic cells. As a consequence, normal tissues lacking FRα are spared the toxicity that commonly limits non-targeted therapies [6].

Because of its potential as a tumor-specific target, we sought to examine the expression of FRα in breast cancer in greater detail. To address this issue, and to assess the possibility of growth advantage of FRα-positive tumors, we studied its expression in a set of good and poor-outcome breast cancers according to various histopathological indicators.

2. Patients and methods

2.1. Patients and materials

The study was conducted retrospectively on 65 formalin-fixed paraffin embedded blocks of a spectrum of breast specimens. These specimens were divided into two groups; neoplastic and non-neoplastic groups. The neoplastic group was in the form of 50 specimens of invasive duct carcinoma of the breast (IDC), not otherwise specified (NOS) with a range of pathological grades. Only IDC cases were studied and no other types of special breast carcinomas were included in the study. The non-neoplastic group included 10 specimens of benign proliferative breast lesions (fibrocystic disease of the breast) and 5 specimens of morphologically normal breast tissue obtained from reduction mammoplasty specimens. Tissue blocks were obtained from the archive of the Surgical Pathology Laboratory, Assiut University Hospital, Faculty of Medicine, Assiut University, Egypt at the period from 2012–2015. The study was approved by Research and Ethical Committee at Faculty of Medicine, Assiut University. The available clinicopathological features were retrieved from the hospital medical records, including patient age, tumor site, laterality, tumor size, lymph nodes, surgical margins state and hormone receptors state. Tumor stage was assessed according to AJCC Cancer Staging Handbook of the American Joint Committee on Cancer [7]. Measurement of tumor size was made on received mastectomy specimens before sampling of the specimens. The greatest diameter was assessed and used to classify tumors into T1, T2, T3 and T4 groups.

2.2. Pathological review

Hematoxylin and eosin stained sections were examined for detailed histopathologic features. Invasive duct carcinoma (NOS) specimens were histologically graded according to the Elston's modification of Bloom-Richardson system [8]. A total of three representative sections for each case were examined and the high power field area was 0.152 mm^2 according to our microscope. Scores are assigned for the proportion of tubule formation (score of 1–3), the degree of nuclear pleomorphism (scores of 1–3) and the mitotic count (scores 1–3). The scores were combined and accordingly, cases were classified into grade I (total scores of 3–5), grade II (scores 6 or 7) or grade III (score 8 or 9). By examining the three most representative sections of each tumor, assessment for the presence or absence of angiolymphatic tumor emboli [9], perineural invasion [10], tumor immune response [11], estrogen receptor (ER) [12], progesterone receptor (PR) [12] and human epidermal growth factor receptor-2 (Her-2 neu) status [13] was done.

2.3. Immunohistochemical methodology

Immunohistochemical staining for the sections was performed using the avidin–biotin complex method. Tissue sections of 4 μm

Table 1
Clinicopathologicalparameters of studied breast carcinoma cases (n = 50).

Factor	Number	Percentage
Total	50	100%
Age range	25–75 years (mean, 53.2)	
Age category		
<50 year	19	38%
≥50 year	31	62%
Histological grade		
Grade I	5	10%
Grade II	30	60%
Grade III	15	30%
Tumor size		
T1	6	12%
T2	18	36%
T3	24	48%
T4	2	4%
Lymph node stage		
N0	9	18%
N1	13	26%
N2	14	28%
N3	14	28%
Ductal carcinoma in situ		
Present	20	40%
Absent	30	60%
Lymphovascular emboli		
Present	17	34%
Absent	33	66%
Perineural invasion		
Present	17	34%
Absent	33	66%
Peritumoral immune response		
Weak	31	62%
Moderate	8	16%
Strong	11	22%
ER		
Positive	28	56%
Negative	22	44%
PR		
Positive	17	34%
Negative	33	66%
Her-2 neu		
Positive	11	22%
Negative	39	78%

thickness were taken from tissue array blocks. Paraffin sections were de-waxed and then rehydrated through descending graded ethanol series down to distilled water. To block the endogenous peroxidase, the rehydrated sections were treated with 6% hydrogen peroxide for 10 min. For epitope retrieval, sections were microwaved in citrate buffer, pH 6 for a total 20 min. Non-specific staining had been blocked by superblock (UV block) for 10 min.

Sections were incubated with antihuman FRα (FOLR1, cat # NBPI-6936, United States biological life sciences).

Secondary staining kits were used according to the manufacturer's instructions (Ultra Vision Detection System, Thermo scientific corporation Fremont, CA, USA). Counter staining was done with Mayer's hematoxylin and sections then are examined by light microscopy. In each staining run, sections from kidney was used as positive control for FRα [14]. Negative control slides were stained in parallel, by the omission of the primary antibodies.

2.4. Immunohistochemical evaluation of FRα expression

Manual evaluation and scoring of immunohistochemical results for FRα were carried out by 2 pathologists independently. The

reactivity for FRα was consistent with a membranous localization; although diffuse intracellular staining was also observed. The staining intensity and the proportion of FRα-positive cells were recorded. We defined 10% membranous and/or cytoplasmic staining with any intensity as the cut-off level for positive FRα results [15].

2.5. Statistical analysis

Chi squared test was used for assessment of association between expression of FRα and different clinicopathological criteria of tumors, using P < 0.05 as the cutoff.

3. Results

3.1. Clinicopathological characteristics

Of the 50 cases of IDC, 62% were older than 50 years. Thirty per cent of patients had tumors with a high histologic grade. Half of patients had T3 tumors, while 40% had T2 tumors. Tumors of T1 and T4 categories represent 6% and 4% of cases, respectively. Lymph node metastasis occurred in 82% of cases. Forty per cent of cases showed DCIS associated with IDC. Lymphovascular emboli as well as perineural invasion were found in 66% of cases. The immune response was weak in 62% and strong in 22% of cases. Only 56% of the tumors were ER positive and 34% were PR positive. Most of the cases (78%) were Her-2/neu negative. The patients' clinicopathological characteristics of neoplastic group (IDC) are summarized in (Table 1).

3.2. Immunohistochemical expression of FRα in breast specimens

Folate receptor α expression was assessed in neoplastic and non-neoplastic breast specimens. Using the criteria of ≥10% of cells exhibiting membranous and/or cytoplasmic staining, FRα was positive in the malignant cells of 32 (64%) of IDC cases, whereas 18 (36%) of cases were negative. Although most FRα staining was in the membrane, mixed cytoplasmic and membrane staining patterns were also observed. Specimens obtained from benign proliferative breast disease and normal breast showed only membranous staining in the secretory cells toward the luminal side. The myoepithelial cells were negative. Two out of the 10 (20%) non-neoplastic group were positive for FRα (Fig. 1).

Folate receptor α expression was strongly associated with high histologic grade, larger tumor size, higher nodal stage, presence of lymphovascular emboli and positive perineural invasion.

Most of grade III tumors (93.3%) expressed FRα. We also observed a significant difference in FRα expression regarding tumor size with 87.5% of T3 cases showed positive FRα expression compared to 16.7% of T1 tumors. Most of IDC cases that have high nodal stage (N2 and N3) were positive for FRα (85.7% and 78.6% of N2 and N3 cases, respectively). The majority of tumors with lymphovascular emboli (16 out of 17; 94.1%) showed positive expression for FRα and the same finding was observed in cases with tumors positive for perineural invasion in which FRα was expressed in 16 out of 17 cases (94.1%). No significant association was noted between FRα expression and patient's age, presence of associated DCIS and the degree of immune response around the tumor. Table 2 shows the details of these findings.

Regarding the relationship between FRα expression and the expression of ER, PR and HER2/neu, FRα expression was significantly associated with ER negativity (100%), PR negativity (93.3%), and Her-2, neu negativity (76.9%). The details of FRα expression in relation to ER, PR and Her-2 neu is shown in Table 3.

4. Discussion

Breast cancer is the most commonly diagnosed life-threatening malignancy in women [16]. With the improved treatment strategies and the rapid development of molecular-based therapeutic modalities, morbidity and mortality resulted from breast cancer had greatly reduced [17].

Folate is essential for rapidly proliferating cells for one-carbon metabolism as well as DNA biosynthesis, repair and methylation. Folate is taken up in cells via three mechanisms that include: the reduced folate carrier, the proton coupled folate transporter and through four glycopolypeptide folate receptors (FRα, β, γ and δ) which mediate folate uptake by endocytosis [4].

In this study, Immunohistochemical evaluation of FRα expression revealed that FRα was expressed in 64% of the breast carcinomas compared to 20% in non-neoplastic breast specimens.

In the non-neoplastic breast tissue, FRα protein expression was limited to the luminal membranes of secretory ductal cells. Expression of FRα in normal breast epithelial cells is expected and consistent with folate secretion into milk and as being as a source of bound folates for the developing embryo. As a result for this apical location, FRα is not in direct contact with folate in the circulation in contrast to FRα in cancer which can access folate in the circulation [18].

Expression of FRα in normal breast cells concurs with other studies [19–21]. Zhang et al. [21] found weak membrane staining of FRα in 30% of non-neoplastic breast tissue from patients with breast cancer. Parker et al. [20] measured the level of FRα as 3.99 pmol/mg protein in normal breast epithelial cells, compared to 7.44 pmol/mg in breast cancer cells, by quantitative radioligand binding assay.

The high expression of FRα in neoplastic tissues of breast indicates a critical role of FRα in the process of carcinogenesis. Several lines of evidence suggest that FRα may contribute to the malignant phenotype of breast tissue. Firstly, the known biologic function of FRα is to supply folates for the biosynthesis of nucleotide bases and the methylation of isoprenylated proteins such as ras. These processes are critical for maintenance of a proliferative state of malignant cells [22]. Moreover, FRα overexpression has been biochemically linked to down regulation of caveolin-1, a process shown to be directly involved in induction of the transformed phenotype. Also, decreasing FRα expression reduces the proliferation rate of breast cancer cell lines [23].

The association between FRα expression and various clinicopathological perspectives in different tumor types was a matter of contradiction In one study, FRα expression was correlated significantly with higher grade and stage in non-mucinous ovarian cancer [24]. On the contrary, in another study on lung carcinoma, FRα expression was observed in well differentiated and early stages tumors that are associated with good prognosis [25].

In breast carcinoma, the same controversy was observed. In this study, there were positive significant associations between FRα expression and poor indicators of breast carcinoma as higher tumor grade (p-value = 0.007), larger tumor size (p-value < 0.001), higher nodal stage (p-value = 0.004), presence of angiolymphatic emboli (p-value = 0.001), and perineural invasion (p-value = 0.001). No significant association between FRα expression and patient's age, presence of DCIS, and immune response.

On comparing these findings to other studies, some difference was noted. Similar to our findings, Zhang et al. found no association between FRα expression and patient's age. They also noticed significant positive association between FRα and tumor grade and lymph node stage [21]. In another study, Ginter et al. found no significant association between FRα expression and any of the clinicopathological factors in breast carcinoma [15]. These variations may be related to the difference in sample size among studies or the different method of evaluation of FRα expression.
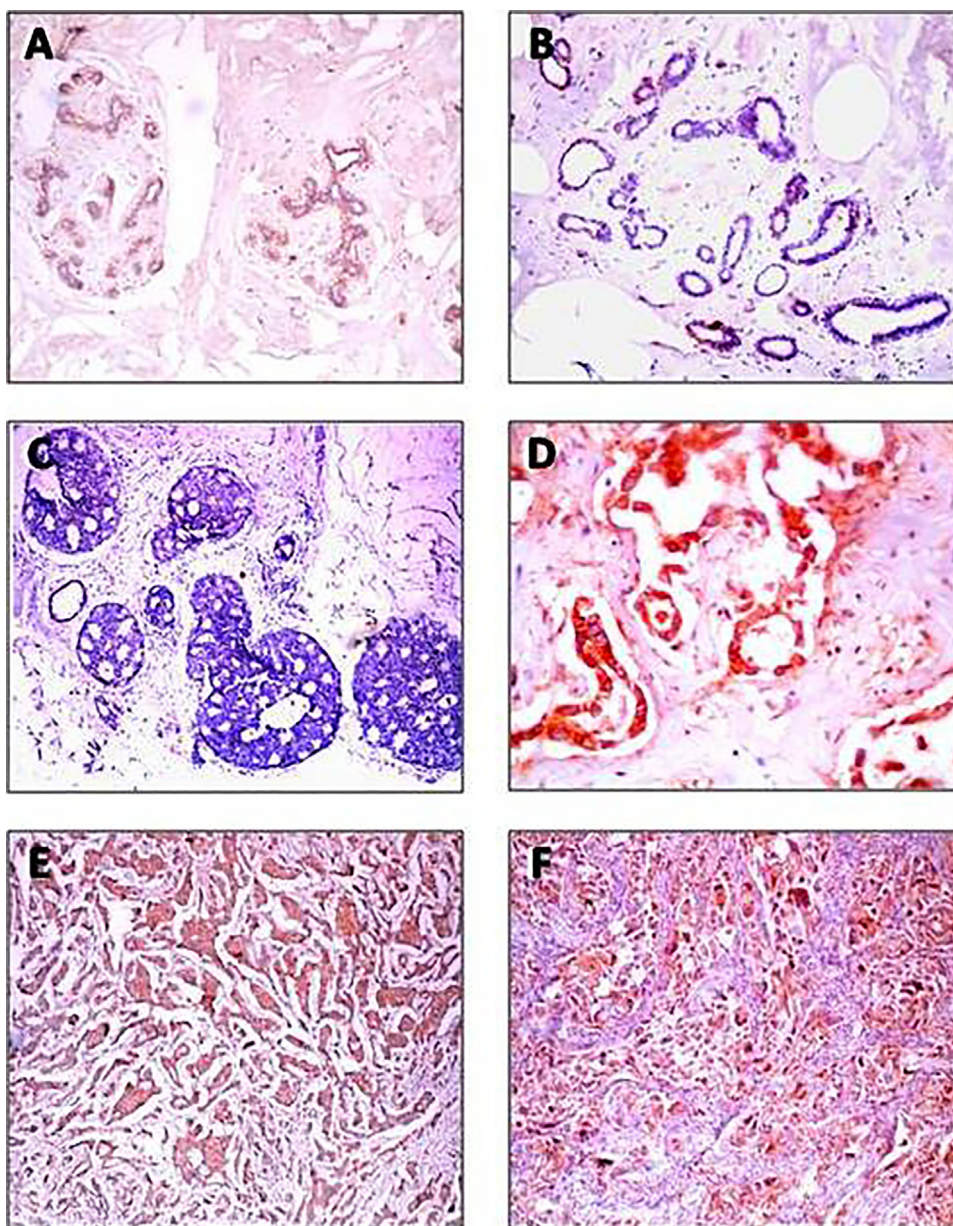
Fig. 1. Folate receptor α expression in breast tissue.
A: Folate receptor α showing membranous staining in luminal cells in normal breast tissue (X100).
B: Few luminal cells showing membranous staining for FRα in fibrocystic breast disease (X100).
C: Ductal carcinoma in situ showing negative immunohistochemical expression for FRα (X100).
D: Positive membranous and cytoplasmic immunohistochemical expression for FRα in invasive duct carcinoma (NOS), grade I (X200).
E: Positive membranous and cytoplasmic immunohistochemical expression for FRα in invasive duct carcinoma (NOS), grade II (X100).
F: Positive membranous and cytoplasmic immunohistochemical expression for FRα in invasive duct carcinoma (NOS), grade III (X100).

Although most studies have used the same clone for FRα antibody, the cut-off level for defining FRα positivity was different. We arbitrarily chose 10% as the cut-off level for FRα positivity in the current study. Based on previous studies Bremer et al. [26] and Hartmann et al. [14] found the cut-off level of 10% as the optimal cutoff value that could predict the overall survival and disease-free survival of patients. In this regard, they used this cut-off value to categorize patients into those with high and low FRα expression with decreased and increased overall survival, respectively.

Regarding the association between FRα expression and hormone receptor profile of the patients, we noticed significant association between FRα positivity and negative hormone receptors (ER negative/PR negative/Her-2neu positive/negative) and triple negative cases (ER negative/PR negative/Her-2neu negative).

Similar to our results, some studies found significantly increased FRα expression in ER/PR negative and in triple negative breast tumors, compared to ER/PR positive cases [6,27]. This finding is in keeping with data showing that ER negatively regulates FRα expression and that downregulating ER with anti-estrogen treatments promotes FRα expression in estrogen receptor-dependent manner [6]. However, the limited number of our cases makes this finding in need for further confirmation on larger sample size.

Few studies have examined tumor levels of FRα and patient survival. Zhang et al. found a trend toward worse overall and disease-free survival with FRα expression. However, the investigators declared that the significance was not achieved [21]. Unfortunately, no survival data were available for our study.

Table 2
Clinicopathological characteristics of breast carcinoma cases based on FRα immunohistochemical expression.

Clinicopathological Parameters	Folate Receptor α Expression				Total	P value
	Positive		Negative			
	Number	Percentage	Number	Percentage		
Age						
<50 years(median)	9	47.4%	10	52.6%	19	0.055
≥50 years(median)	23	74.2%	8	25.8%	31	
Histological grade						
Grade I	4	80%	1	20%	5	0.007
Grade II	14	46.7%	16	53.3%	30	
Grade III	14	93.3%	1	6.7%	15	
Tumor size						
T1	1	16.7%	5	83.3%	6	<0.001
T2	8	44.4%	10	55.6%	18	
T3	21	87.5%	3	12.5%	24	
T4	2	100%	0	0%	2	
Lymph Node Stage						
N0	4	44.4%	5	55.6%	9	0.004
N1	4	30.8%	9	69.2%	13	
N2	12	85.7%	2	14.3%	14	
N3	12	85.7%	2	14.3%	14	
Ductal Carcinoma In Situ						
Present	12	60%	8	40%	20	0.63
Absent	20	66.7%	10	33.3%	30	
Lymphovascular Emboli						
Present	16	94.1%	1	5.9%	17	0.001
Absent	16	48.5%	17	51.5%	33	
Perineural Invasion						
Present	16	94.1%	1	5.9%	17	0.001
Absent	16	48.5%	17	51.1%	33	
Peritumoral Immune Response						
Strong	5	45.5%	6	54.5%	11	0.325
Moderate	6	75%	2	25%	8	
Weak	21	67.7%	10	32.3%	31	

Table 3
Association between FRα immunohistochemical expression and ER, PR and Her-2 neu (N = 50).

Hormone Receptor	Folate Receptor α Expression				Total	P value
	Positive		Negative			
	Number	Percentage	Number	Percentage		
Estrogen Receptor						
Positive	10	35.7%	18	64.3%	28	<0.001
Negative	22	100%	0	0	22	
Progesterone Receptors						
Positive	1	5.9%	16	94.1%	17	<0.001
Negative	31	93.9%	2	6.1%	33	
Her-2 neu Receptor						
Positive	4	36.4%	7	63.6%	11	0.032
Negative	30	76.9%	9	23.1%	39	
ER-/PR-/Her-2 neu+/−	18	81.8%	4	18.2%	22	<0.001
ER-/PR-/Her-2 neu-	13	86.7%	2	13.3%	15	0.0021

Studying FRα as a target in cancer therapy has led to encouraging results. A range of FRα-targeting approaches, including folic acid derivatives, folate drug-conjugates, vaccines and monoclonal antibodies, have been developed for application for therapeutic purposes. Currently, farletuzumab, vintafolide and IMGN853 are the three FRα-targeting agents that show the most effective potential in clinical cancer trials [4]. According to our findings and in agreement with other studies, FRα expression in non-malignant breast tissue was restricted to the apical surface of epithelial cells lining breast ducts [6]. This position makes FRα in normal cells out of direct contact with folates in the circulation compared to FRα expression in malignant cells that is located in blood-accessible lateral and basal membranes. Importantly, because of this, FRα targeted therapies cannot access this compartment. Moreover, there are many folate transporters other than FRα that can provide folate to normal cells ensuring that folate heomostasis in normal cells will not be affected. Taken together, this indicates that folate conjugated therapy will not affect folate heomostasis for normal cells and predict low propability of significant toxicity by FRα-conjugated drugs in these cells [28]. These data suggest that FRα expression in breast cancer may serve as both predictive marker to guide patient

selection for new treatment approaches and also as a potential prognostic indicator.

5. Conclusions

Our study demonstrates that FRα expression is present in a subset of invasive ductal breast carcinoma that are associated with poor prognostic criteria. In this regard, FRα may play a key role in their tumorigenesis. In this regard, FRα may be used as a target molecule for the currently ongoing clinical trials on new cancer therapies.

Conflict of interest

The authors declare that they have no conflict of interests.

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