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Predictive Role of EGFR, IGF-1R, VEGFR2 and HIF-1α Expression on Survival of Patients with Triple-negative Breast Cancer

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ABSTRACT

Background: Triple-negative breast cancer (TNBC) carries a poor prognosis and therapeutic options are limited to date. The aim of this study was to investigate to what extent the epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor 2 (VEGFR2), hypoxia inducible factor-1 alpha (HIF-1α) and insulin-like growth factor-1 receptor (IGF-1R) are expressed in TNBC and to determine if these expressions have a predictive role.

Methods: Of 923 breast cancer patients who were treated and followed in Academic Center for Education, Culture and Research (ACECR) from 2006-2010, 104 of them had TNBC. Immunohistochemistry analyses were performed on tissue microarray blocks with antibodies for EGFR, VEGFR2, HIF-1α and IGF-1R.

Results: We analyzed tumor samples from 104 patients with classic primary invasive ductal carcinoma (IDC). Fifteen patients (14%) were in stage I, 46.6% in stage II, 30.1% in stage III and 5.8% in a metastatic stage (stage IV). The median overall survival (OS) was 48 months. EGFR was expressed in 15 (14%), VEGFR2 in 63 (61%), IGF-1R in 81 (78%) and HIF-1α in 57 (55%) samples. EGFR expression was significantly associated with poor outcome in terms of OS (P = 0.021, OR = 3.9).

Conclusions: Among the four investigated tumor markers, only EGFR was significantly associated with survival of patients with TNBC.

Introduction

Triple-negative breast cancer (TNBC) is an important subtype of breast cancer which does not express estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor (HER-2/neu). Therefore, targeted therapies such as hormone therapy or trastuzumab are not very effective modalities for treatment of this type of breast cancer.[1] TNBCs account for 10-20% of all breast cancer cases diagnosed in Asian and Western populations, but it is much more common in patients of African descent.[2-4] Standard treatment regimen for TNBC has not yet been established, and insufficient data are available.[5-7]

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Researchers have conducted several trials considering molecular mechanisms involved in development of TNBC which will hopefully lead to development of targeted therapies.[5,8] In this regard, receptor tyrosine kinases (RTKs) have been suggested as potential selective anti-cancer targets.[9] RTKs are the main mediators of the signaling network that transmit extracellular signals into the cell, mediate cell-to-cell communication, and regulate several cellular processes, such as proliferation, differentiation, and cell survival.[10] Several cell markers that belong to the family of RTKs and their role in development of human cancers have been studied by investigators and these markers include epidermal growth factor receptor (EGFR), insulin-like growth factor-1 receptor (IGF-1R), hypoxia inducible factor-1 alpha (HIF-1α) and vascular endothelial growth factor receptor (VEGFR).[11-14]

The purpose of this study was to determine the association of various pathological and biological factors including over-expression of HIF-1α, VEGFR2, EGFR and IGF-1R with overall survival (OS) of patients with TNBC.

Methods

Study population

This study investigated a consecutive series of 923 cases of primary invasive breast carcinoma recruited from Iranian Center for Breast Cancer (ICBC) between 2005 and 2010. Of these patients, 104 cases were diagnosed with TNBC and were selected for further study. Patients' clinical history and tumor characteristics were assessed. Information on therapy and local, regional, and distant recurrence was recorded on a prospective basis, as well as survival.

Laboratory assessment

Formalin-fixed paraffin-embedded blocks from the primary tumors were retrieved for these 104 patients. Three biopsies, 0.6 mm in diameter, were obtained from each donor block corresponding to a previously marked area on a slide of invasive tumor and three tumor tissue microarray (TMA) blocks were constructed in triplicate using a tissue array machine according to the manufacturer's instructions (Beecher Instruments, ALPHELYS, France). Two to three µm sections were cut from TMA blocks and placed on silanized glass slides. The sections were deparaffinized in xylol for 2-5 min and rehydrated in graded dilutions of ethanol in water.

Antigens were retrieved using 10 mM Tris/EDTA buffer, pH 9.0 (Dako) for 20 minutes (min) at 90°C in microwave. Antigen retrieval for EGFR was performed using Proteinase K (Dako) for 4 min. Nonspecific binding was blocked by incubation with 2% bovine serum albumin in Tris-buffered saline (TBS) (15 min at Room Temperature (RT)) and subsequently, tumor sections were incubated with 100µl of each 1:100 dilution of mouse monoclonal anti-EGFR antibody (Dako, clone H11), 1:500 dilution of mouse monoclonal anti-IGF-1R antibody (Abeam, clone 3C8B1, 3G5C1), 1:250 dilution of polyclonal anti-VERGFR2 antibody (Abeam, ab39256) and 1:750 dilution of mouse monoclonal anti-HIF-1α antibody (Abeam, clone ESEE122) (1hour at RT). Endogenous peroxidase activity was blocked with 3% H2O2 (5 min at RT), followed by incubation with EnVision + Dual Link System-HRP (Dako) for 30 min at RT and finally, Liquid DAB+ Substrate Chromogen System (Dako) was applied for 10 min at RT. Before the slides were mounted, all sections were counterstained for 1 minute with hematoxylin and dehydrated in alcohol and xylene. TBS with 0.1% Tween-20 was used as a washing buffer. Staining with hematoxylin and eosin was carried out for morphological overview and evaluation of the quality of the biopsies (Table1).

When more than 1% of cells were stained, EGFR cytoplasmic membrane positivity was considered positive EGFR staining. For IGF-1R and VEGFR2, only invasive tumor cells were considered and the membrane and cytoplasmic staining intensity was scored using a semi-quantitative scale (0-3). In statistical analysis, moderate to strong immunoreactivity (2-3) was compared to weak or absent staining (0-1). HIF-1α positivity was considered when 1% or a higher percentage of positive cells was present (Nucleic and cytoplasmic).

For all staining procedures, the highest score out of three scores from the same tumor was used for the analysis. If one or two staining cores out of three failed to stain, the highest value of the remaining core(s) was included in the analysis. Only the invasive tumor component was considered when judging the staining. The tumor array was examined by two investigators to whom the clinical data were blinded and all divergent results were re-examined in order to reach a conclusive decision.

Statistical Analysis

Statistical analysis was performed using statistical package for the social sciences (SPSS) 20 (SPSS Inc., Chicago). We examined the association between triple-negative phenotype and other clinicopathological variables. Cut-off values for different biomarkers included in this study were chosen before statistical analysis. The association of variables with survival was analyzed initially by Kaplan-Meier and log-rank tests. P value of <0.05 was considered significant.

Table 1. Clone, dilution and source of antibodies used to detect expression of investigated markers

Antibody	clone	dilution	source
EGFR	H11	1:100	Dako, Denmark
VEGFR2	Polyclonal ab39256*	1:250	Abeam, UK
IGF-1R	3C8Bl, 3G5C1	1:500	Abeam, UK
HIF-1α	ESEE122	1:750	Abeam, UK

** Product number*

Results

One hundred and four patients with TNBC were included in this study. The patients had a median age of 50 years (range, 29-87 years). Pathologically, all tumors were ductal carcinoma of no special type (NST). All patients received Anthracycline and Taxane-based chemotherapy (AC4-T4). The median follow-up time was 53 months (range, 15 to 80 months). During the follow-up period, 24 (23%) patients died. The median OS was 48 months. Fifteen patients (14%) were in pathologic stage I, 57 (56%) in stage II, 25 (24%) in stage III, and 7 (6%) in stage IV at the time of presentation. A total of 61 (58%) patients had a tumor size between 2 to 5 cm. Fifty eight (56%) patients had axillary node metastases at the time of diagnosis and others were node negative (Table 2) .

We analyzed the association between clinicopathological variables and OS. The results of univariate analyses are summarized in table 3. Tumor size larger than T2 tumors, lymph node involvement greater than N1 tumors, presence of lymphovascular invasion, and higher tumor grade were associated with shorter OS, but there were no significant survival differences between patients with or without perineural invasion (Table 3).

EGFR expression was observed in 15 (14%) patients (Table 2). In survival analysis, EGFR expression was significantly associated with lower survival (P = 0.021, OR = 3.99). EGFR expression was significantly more common in advanced stages (P = 0.042, OR = 3.095), but no significant association was observed between EGFR expression and other clinicopathological variables. Co-expression of EGFR with the other three markers was assessed, but no significant results were found.

Univariate survival analysis showed no association between HIF-1α (P = 0.835, OR = 1.09), VEGFR2 (P = 0.156, OR = 0.51) or IGF-1R expression (P = 0.235, OR = 2.18) and OS (Table 3)

Table 2. Clinicopathological features and expression of biological markers among study population

	N (%)
Tumor size	
< 2cm	27 (26%)
2-5cm	61 (59%)
> 5cm	16 (15%)
Lymph node	
0	46 (44%)
1-3	25 (24%)
4-9	21 (2.0%)
> 9	12 (12%)
Grade	
I	1 (1%)
II	51 (49%)
III	52 (50%)
Age	
< 50	54 (51.9%)
≥ 50	50 (48.1%)
Perineural invasion	
Positive	46 (44%)
Negative	58 (56%)
Lymphovascular invasion	
Positive	52 (50%)
Negative	52 (50%)
Types of surgery	
BCS	33 (32%)
MRM	71 (68%)
VEGFR2	
Negative	41 (39%)
Positive	63 (61%)
EGFR	
Negative	89 (86%)
Positive	15 (14%)
HIF-1α	
Negative	47 (45%)
Positive	57 (55%)
IGF-1R	
Negative	23 (22%)
Positive	81 (78%)

Table 3. Univariate analysis of tumor features predicting patients' survival

	Odds ratio	95% confidence interval	P-value
IGF-1R positive	2.18	0.58 - 8.13	0.235
HIF-1α positive	1.09	0.43 - 2.77	0.835
VEGFR2 positive	0.51	0.20 - 1.30	0.156
EGFR positive	3.99	1.26 - 12.6	0.021
Vascular invasion	2.86	1.06 - 7.69	0.033
Perineural invasion	1.89	0.74 - 4.81	0.179
Grade 3	3.72	1.33 - 10.42	0.009
N 2-3	34.87	9.00 - 134.6	< 0.001
T 3-4	6.79	2.17 - 21.2	< 0.001
Stage IIIA-IV	3.095	1.01 - 9.46	0.041

Discussion

In the current study, we have investigated the prognostic value of different tumor markers in a large series of TNBC patients. The most common histological type was ductal carcinoma of NST and the majority of tumors with this pathology were grade 3. There were positive associations of larger tumor size (T3, T4), greater nodal involvement (N2, N3), higher tumor grades, vascular invasion, EGFR expression, and higher stage at presentation with poor outcome in terms of OS and disease free survival (DFS) in univariate analysis.

The frequency of TNBC is reported to be 10 to 17% of all breast cancers. However, clinical data on TNBC in Middle Eastern populations are limited.[2-4] Among the 923 breast cancer cases in this study, we identified 104 (11.2%) patients with the triple-negative phenotype. Currently, routine clinical management of breast cancer relies on traditional prognostic factors including nodal status, histological tumor grade and primary tumor size. A single therapeutic strategy is not effective in all patients due to heterogeneity of breast cancer and tumors with similar pathological features still vary in response to the same treatment protocol.[15]

The clinical display of breast cancer is seldom easy to predict. Hence, novel tumor markers have been recently suggested for assessment in order to delineate the course of the disease more efficiently, especially in cases where routine tumor characteristics fail to fully elucidate the outcome of patients with TNBC.[5]

Some studies revealed a positive association between increased proliferation, higher histological grade and poorer survival and higher expression levels of HIF-1α.[16] In contrast, no association between above mentioned prognostic indexes and HIF-1α expression was found in this study.

TNBC frequently overexpresses EGFR, and this feature is correlated with poor prognosis in many studies and used as a promising potential therapeutic target.[17,18] In this study, overexpression of EGFR was an indicator of poor prognosis in TNBC. In another study, EGFR mutations were found in 8 of 70 samples (11.4%). Mutations were predominantly exon 19 deletions (4 of 8), which clustered in the region within the kinase domain of EGFR but these mutations were independent of EGFR expression.[19]

Evidences regarding the role of functional IGF signal transduction pathway in estrogen-unresponsive breast cells are not conclusive. Although most studies suggest that estrogen-unresponsive cells show nono mitogenic response to IGFs, one report has demonstrated that these cells show proliferative response to these growth factors.[20,21] The relatively high expression of the IGF receptors in TNBC cells witnessed in other studies suggests that the IGF signaling pathway may have a significant role in controlling cell survival and proliferation and in this category of patients.[22] But in our study, there was no correlation between IGF-1R overexpression and DFS or OS. Additionally, there was no association between overexpression of VEGFR2 and DFS or OS in our series of patients.

The major limitations in our clinical analysis include the cumbersome nature of procedures and limited sample size.

Our observation suggests that expression of EGFR in patients with TNBC is associated with poor survival, while such an association was not observed for HIF-1α, IGF-1R, and VEGFR2. Thus effectiveness of EGFR targeted therapies in this subtype of breast cancer should be the concern of further investigations.

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