Research Article

Immunohistochemical profiling of TNF-α in invasive ductal carcinoma of the breast - A cohort study from Rawalpindi, Pakistan

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Abstract

Breast cancer remains the most prevalent malignancy among women globally, and its incidence increasing, especially in developing countries. In Pakistan, breast cancer poses a significant public health challenge, with a notably high incidence rate among women. Tumor necrosis factor alpha (TNF-α) is a multifunctional cytokine that is commonly up-regulated in various tumors and is considered a tumor promoter element. Reports suggest the involvement of varying levels of TNF-α affecting Breast Cancer susceptibility. Given the diverse genetic, environmental, and lifestyle factors influencing cancer susceptibility, the expression and role of TNF- α in breast cancer may vary across regions. Therefore, it is crucial to explore its expression within specific to understand local disease biology and prognosis. We investigated the expression of TNF-α in 50 different female patients of invasive ductal carcinoma (IDC) of breast using immunohistochemical analysis. Fifty breast tumor biopsy samples and 17 lymph node biopsy specimens were collected. Our findings reveal an elevated expression of TNF-α in most cases analyzed within the local population. Elevated levels of TNF-α were particularly pronounced in metastatic tumors. Additionally, we observed a progressive increase in TNF-α expression in the endothelium as the tumor grade advanced (p-value= 0.044*). This study emphasizes the significant pro-tumor role of TNF in breast cancer within the local population, with elevated expression particularly evident in metastatic and higher-grade tumors.

Keywords: Breast cancer, Inflammation, TNF-α, Lymph node metastasis, Necrosis.

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Introduction

Tumor necrosis factor (TNF), a pleiotropic cytokine is one of the chief contributors of inflammatory responses [1]. During chronic inflammation, the cytokine supports tumor survival and growth by preventing apoptosis and cytotoxicity while stimulating angiogenesis [2]. Clinically, TNF-α is known to be elevated in serum and an over-expression of TNF-α has been reported in a variety of pre-neoplastic and malignant diseases. On the contrary, recently established pre-clinical cancer models provide significant evidence to favor the link between constant, low level

TNF-α exposure and the increased ability of cells to become malignant. There is some contradiction regarding the levels of TNF-α favoring tumorigenesis and a conclusive set of studies is required. However, the constant presence of such inflammatory cytokines in the tumor microenvironment may promote cancer development [3]. Normally, in the mammary gland, the expression of TNF-α occurs in circumstances such as development, proliferation, and branching morphogenesis [4]. However, in some cases of Breast cancer (BC), TNF-α is shown to be mitogenic, as in the case of rat model in which mammary tumor formation is induced by 1-methyl-1- nitrosourea [5]. In a series of experiments, mice models deficient for TNF-α showed a reduction in tumor progression accompanying decreased cell proliferation of ductal and lobular cells of breast tissue. Moreover, in mice treated with the anti-TNF antibody, breast tumor growth was inhibited to a greater extent compared with the control antibody [6]. Suppression of the TNF-α mediated signaling pathway also inhibits NF-κB-mediated gene transcription, cell migration, and invasion of BC cells [7]. In a recent study, TNF- α has been shown to synergize with TGF-b to upregulate MMP-9 expression at both transcriptional and translational levels, promoting breast cancer invasion and metastasis through chromatin remodeling mechanisms [8] . Our study aimed at analyzing TNF-α expression in different cell populations at the sites of breast tumor. The study's focus is on a cancer sample cohort from the Rawalpindi region. This is significant because cancer characteristics can vary among different populations due to genetic, environmental, and lifestyle factors. Examining TNF-α expression in this specific region can uncover unique patterns that may influence treatment strategies tailored to this population. Furthermore, to assess the role of TNF-α in BC, we correlated the expression with the clinicopathological features of the tumor such as lymph node metastasis and grade. We also observed the effect of aging on the levels of TNF-α present in the tumor.

Materials and Methods

Sample collection

A total of 67 specimens (50 tumor biopsies and 17 lymph node biopsies) were collected from 50 female patients diagnosed with invasive ductal carcinoma (IDC) who underwent surgery at Benazir Bhutto Hospital, Rawalpindi. Patient demographic data and tumor characteristics were retrieved from hospital records. Table 1 summarizes the features that exhibited a strong correlation with TNF-α. The study complied with ethical standards and regulatory guidelines. Formalin-fixed, paraffin-embedded tissue samples were used for immunohistochemical analysis.

Immunohistochemical staining

Immunohistochemistry was performed in accordance with the previously described methodology to ascertain the protein expression of TNF-α in the paraffin embedded tissue samples [9]. Tissue sections (4 µm thick) were incubated with Abcam UK's mouse monoclonal anti-human TNF-α antibody (ab1793). The samples were exposed to the HRP-labeled goat anti-mouse secondary antibody (ab47827, abcam UK) for one hour at room temperature. The staining detection system was the DAB staining Kit (Abcam Cat: ab64238, UK). Sections were dehydrated, mounted in organic medium, and counterstained with haematoxylin. Phosphate-buffered saline (PBS) without the primary antibody served as the negative control. As a positive control, tissue samples from human tonsils were employed.

Evaluation of staining

A pathologist independently evaluated each

slide. Positive staining was defined as brown color staining in epithelial, endothelial, or stromal cells. It was measured by counting the positive cells at 200X in three representative high-power fields for each section using a Labomed TCM400 inverted microscope (Labo America Inc., USA), and the stained sections were photographed using ProgRes Capture Pro 2.6 (JENOPTIK Laser, Optik, Systeme GmbH, Germany). Finally, it was calculated as a percentage of the average number of positive cells each section. The percentage of TNF-α positive cells was evaluated on a scale of 1-4 (1: < 25%, 2: 25-50%, 3: 50-75%, and 4: >75% positive). Additionally, TNF-α expression levels were categorized into two groups based on scores: low (1, 2) and high (3, 4) expression.

Statistical analysis

Data on participants' age, gender, histology results, and tumor grades from their individual medical histories were gathered. The association between TNF-α expression in the tumor, endothelium, and lymphocytic population and several patient and tumor characteristics, including age, disease grade, lymph node status, and metastasis, was ascertained using a Spearman's Rank Order correlation. A p-value of <0.05 was considered statistically significant. The Statistical Package for Social Sciences, version 16.0 (SPSS Inc., Chicago, IL, USA), was used to conduct statistical analyses.

Results

Expression of TNF-α in invasive ductal carcinoma of breast

Previous studies support the role of TNF-α in promoting cancer [10 - 13] with its expression verified in various human malignancies [3, 14]. In the present study, the overall expression of TNF-α was strong in 90% of tissue biopsies of IDC.

Association of TNF--α with patient's age

To investigate the correlation between TNF-α expression and patient's age, expression levels were analyzed across different age groups. TNF-α expression was well pronounced in patients with age 40 or below, with all cases in this group showing strong expression. In contrast, among patients older than 40 years 84.8% (28 out of 33) exhibited elevated TNF levels. A statistically significant association was identified between TNF-α expression and age (P=0.014*, r_s = -0.345), indicating that younger patients tend to have higher TNF-a expression in tumors (Table 1, Figure. 1a and Figure. 2a).

Table 1: Association of TNF-α expression in tumor cells with clinicopathological features of invasive ductal carcinoma of Breast.

Clinicopathological factors	TNF-α in tumor cells				
	very low (n=1)	Low (n=4)	Moderate (n=15)	High (n=30)	P-value
Age					
Equal to or below 40	0	0	3	14	**0.014***
above 40	1	4	12	16	
Grade					
I	0	0	1	5	0.067
II	1	1	7	17	
III	0	2	7	7	
Lymph node status					
No metastasis	1	4	11	20	0.218
metastasis present	0	0	4	10	

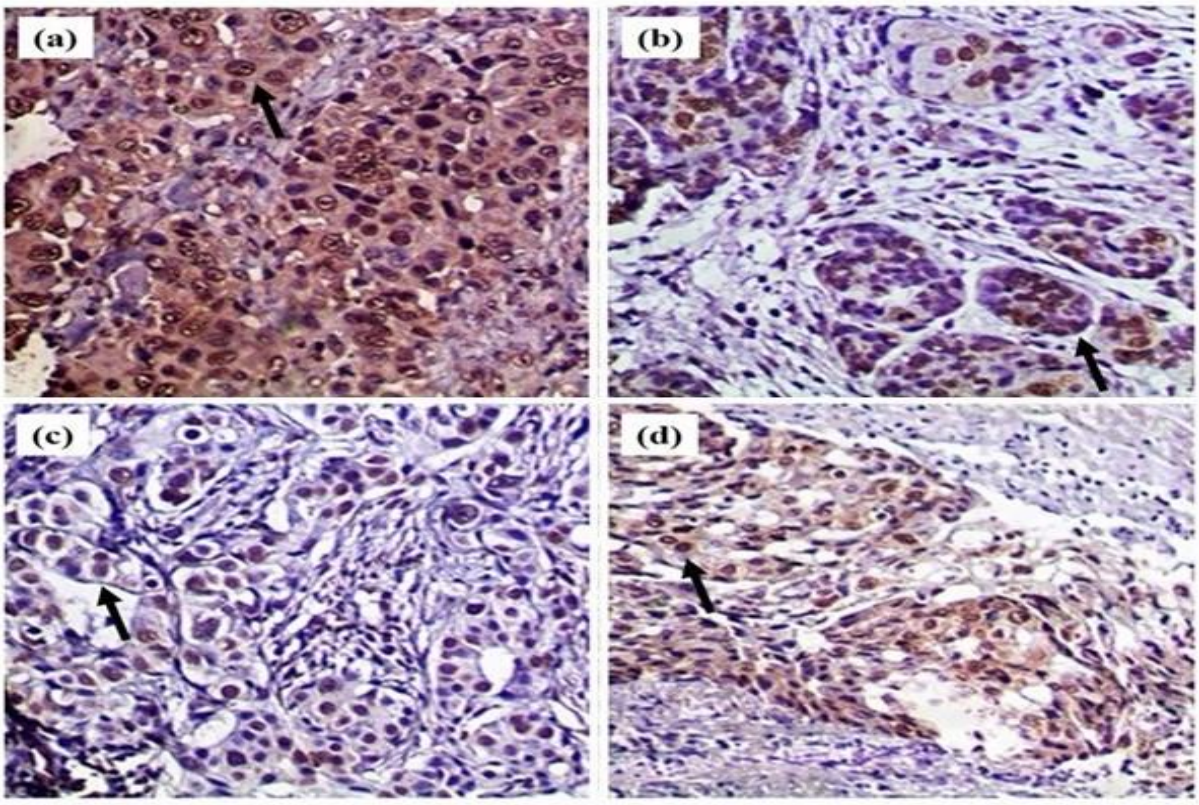

Figure 1: TNF-α immunohistochemical staining in tissue slices from invasive ductal carcinoma (IDC). TNF-α staining of invasive ductal carcinoma (IDC) tissue section (a)in patients with age below 40 (b) with age above 40; (c) non-metastatic; (d) Metastatic (The initial magnifying power was 200X.).

Association of TNF--α with tumor grade

While previous studies have reported an association between increased TNF-α production and higher tumor grades [15]. our findings revealed a gradual decrease in TNF-α expression with increasing tumor grade. In grade I, all cases expressed TNF-α strongly. While 24/26 i.e. 92.3%, among grade II and 14/16 i.e. 87.5% of grade III, showed high expression. We observed a potential association of TNF-α expression in tumors with grade of tumor (P=0.067). However, we did observe a moderately strong correlation of TNF-α expression in endothelium with the grade of tumor (P= 0.044*, r_s =0.493). In total of 17 cases of grade II and III endothelium was observed to express TNF-α. A total of 6/10 (60%) in grade II expressed TNF-α highly in endothelium while all 7/7 (100%) patients of grade III expressed TNF-α strongly (Table 2 and Figure. 2c).

Table 2: Association of TNF-α expression in tumor endothelium and lymphocytes with clinicopathological features of invasive ductal carcinoma of Breast.

Clinicopathological factors	TNF-α in tumor endothelium (P-value)	TNF-α in lymphocytes (P-value)
Age	0.385	0.469
Grade	0.044*	0.172
Lymph node status	0.943	0.062

Association of TNF-α with metastasis

TNF-α is known to be a supporter of cancer metastasis [11, 16]. We found that all (100%) of the metastatic samples showed high expression of TNF-α while 31/36 i.e. 86% of non-metastatic exhibited high expression of TNF-α. However, the

difference in TNF-α expression between metastatic and non-metastatic samples was not statistically significant. There was a slight trend observed for increasing TNF-α expression in metastatic tumor (Table 1, Figure. 1d and Figure. 2b).

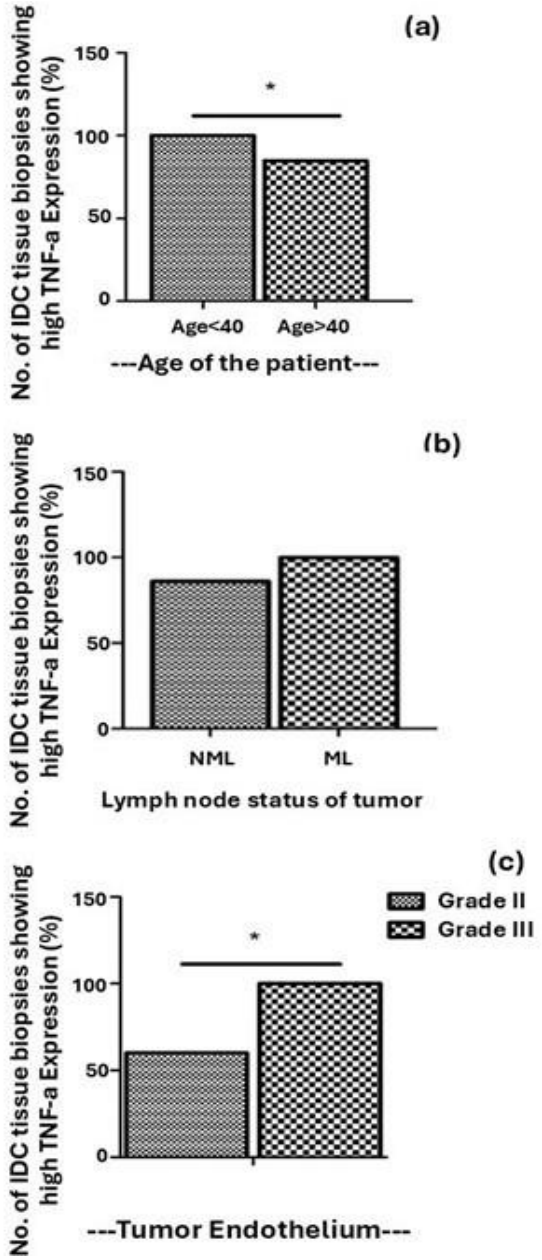

Figure 2: TNF-α expression compared statistically to different clinicopathological characteristics of invasive ductal carcinoma (IDC) tumors; (a) with age of patient; (b) in non-metastatic lymph node (NML) and metastatic lymph node (ML); (c) Graph showing association of endothelial TNF-α expression with grade of tumor. (* indicates significant correlation at 0.05).

Discussion

Tumor necrosis factor alpha (TNF-α) is expressed intracellularly and secreted from a variety of cells such as macrophages, mast cells, some T and natural killer (NK) cells connective tissue cells like fibroblasts, endothelial cells and cancer cells [17, 18]. Under normal circumstances mammary gland up regulate the expression of TNF-α during development, proliferation and branching morphogenesis [4]. Different studies describe the role of TNF-α to be tumor promoting in Breast Cancer (BC) [5, 6, 19]. TNF-α expression analyzed through Immunohistochemistry was found to a varying extent in all the tissue sections of invasive ductal carcinoma (IDC) from 50 patients. With the help of a pathologist different cell populations expressing TNF-α were analyzed and statistical comparison was done among various parameters like age less than or more than 40, metastasis and no metastasis and varying grades of tumor.

We analyzed the correlation of various factors such as expression of TNF-α with clinicopathological features of the tumor and patient's age. However, not all tested parameters showed significant associations. TNF-α expression was shown to be not associated with lymph node metastasis in the tumor. The reasons may lie in the fact that we tested a small population of IDC patients. Still in our small sample sized population we observed some significant findings which are as such.

The downstream proinflammatory cytokines of TLR4 signaling create an inflammatory milieu which favors tumorigenesis if it persists in the microenvironment. TNF-α is an important multifunctional cytokine that has been shown to act as a double-edged sword in tumors. Different studies indicate the role of TNF-α as a communicator between tumor and its microenvironment and cause

of tumor promotion [6, 20]. As the immune status of the body alters with age, so does the inflammatory responses that may arise against any threat in the body. The expression of mediators of these responses and their role in the body differs gradually with age. Our study identified an inverse correlation between patient age and TNF-α expression, with higher expression observed in younger patients. This may be due to the fact that the immune system is more active at an early age, and it declines as the body ages.

It has been established that low grade cancers have less potential of metastasis as compared to high grade, poorly differentiated cancers that spread rapidly (cancer.gov). TNF-α may be one of the many factors that help in tumor growth and quick spread. As reported earlier the higher incidence of TNF-α expression may be caused by the increased cell density in high grade tumors and the relative increase in infiltrating cells found in these cases [15]. As a supporter of metastasis, TNF-α influences the endothelium; promoting chemotaxis, stimulating growth, and activating angiogenesis [21, 22]. In our study we observed high TNF-α expression especially in the endothelium of the poorly differentiated tissue sections. In a research TNF-α was shown to induce undifferentiation in MCF-7 BC cells [23]. Moreover TNF-α can activate the expression of TFG-β (transforming growth factor beta) and subsequent epithelial to mesenchymal stem cell transition in cancer which corresponds to high grade of tumor [24 - 27]. This accumulating evidence supports our observation that high TNF-α level is associated with higher tumor grades.

Statements and declarations

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