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Tumor necrosis factor: An inflammatory microenvironment marker in primary breast cancer patients

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ABSTRACT

Aim: The present study was planned to analyze plasma levels of tumor necrosis factor (TNF) in invasive ductal primary breast cancer (BC) patients in a South Indian population. TNF alpha (TNF α) and TNF beta (TNF β) are produced during inflammation as proinflammatory cytokine markers. The plasma levels of TNF α and TNF β (lymphotoxin α) were correlated with clinicopathological features of BC. **Materials and Methods:** Blood samples were collected from patients before treatment. We analyzed plasma levels of TNF α, and TNF β in 70 female BC cases and 35 age-matched healthy controls using Millipore magnetic bead kits. **Results:** Plasma TNF α levels in BC cases were significantly elevated (median 10.1 pg/ml) when compared to the control groups. Plasma values of TNF α and TNF β both were significantly elevated in BC patients with hormone receptor negative cases. Plasma TNF α level was elevated in lymph node metastasis and triple negative BC. Plasma values of TNF α inversely correlated with estrogen receptor and progesterone receptor positivity. **Conclusion:** The plasma levels of TNF α were more significantly overexpressed than TNF β in BC patients. Further, the patients with aggressive cancer had higher levels of inflammation markers. The present study shows that TNF levels were elevated in hormone receptor negative and triple-negative cases.

Key words: Breast cancer, Inflammation, Tumor necrosis factor

Introduction

Breast cancer (BC) was the second most commonly diagnosed cancer but has now overtaken cancer of the cervix uteri.[1] The death due to cancer is mainly because of drug resistance, disease recurrence and metastasis. The molecules present in the tumor microenvironment are responsible for metastasis.[2] Non-cancerous cells such as fibroblasts, immune cells, and blood vessels form the tumor microenvironment of cancerous cells. The proteins produced by these cells nourish the growth of cancer cells.

The proinflammatory cytokine, tumor necrosis factor (TNF), is a multifunctional cytokine involved in cellular events such as induction of other cytokines, apoptosis, necrosis, inflammation, and immunity.[3] The earliest cytokine produced in inflammatory processes is TNF, which generates a cytokine cascade and helps in the production of interleukin (IL)-1, IL-6 including other mediators, along with TNF itself.[4] The presence of TNF in the microenvironment affects tumor growth and progression.[5] TNF exists in two forms, namely; a soluble free form and a membrane-bound form. These are identified as cytokine TNF alpha (TNF α) produced by macrophages and TNF beta (TNF β) (lymphotoxin [LT]-α/LTα) released from lymphocytes.[6] TNF α binds to receptors TNF-responsive (TNFR)1 (p55) and TNFR2 (p75) and trigger the signal transduction cascades leading to inflammation and tumor cell survival.[7] In rodent models, administration of TNF can cause tumor regression. Thus, TNF has a contextual role, either promoting tumor progression *in vivo* or tumor regression when administered in experimental animals.[8]

The TNF α gene is located on the short arm of chromosome 6 at position 21.3 (6p21.3). The TNF β gene maps to chromosome 6p23–6q12. TNF β binds to the same receptor as TNF α. The biological function of TNF α and TNF β is similar, as there is 35% identity and 55% homology at amino acid sequences and structural similarity in tertiary, quaternary domains of these proteins.[9] The aim of the study was to elucidate the association between plasma levels of TNF with BC prognostic parameters such as age, menopausal status, clinical stage, lymph node status, tumor grade and estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor (HER)-2/neu status.

Materials and Methods

This study was approved by the Institute Ethical Committee, and informed consent was obtained from every individual who participated in the study. Patients with histopathologically confirmed invasive ductal carcinoma of breast registered

between August 2014 and December 2014 were selected. Clinicopathological parameters including age, menopausal status, lymph node status, tumor node and metastasis stage, tumor grade, hormone receptor status, and HER-2/neu status were recorded from case files. The study population comprised 105 subjects with a mean age of 49 years. 70 consecutive BC patients with ages ranged from 26 to 78 years were included and evaluated. The control group included 35 healthy, age-matched (±2 years) female subjects. Blood samples (5 ml) were collected from all subjects in heparin Vacutainers before treatment. The samples were centrifuged immediately at 3000 rpm for 3 min, and separated plasma was stored at −80°C until analysis. Samples were analyzed for TNF α and TNF β levels by a standard procedure using Millipore kits. The MILLIPLEX® MAP human cytokine/chemokine panel magnetic bead kits were used to measure the levels. The stock standard concentration was 10000 pg/ml which was serially diluted to plot a standard curve. Luminex reader recorded the analyte concentration based on the standard curve.

Statistical analysis was carried out using R-statistics software version 3.3.1. Mann–Whitney U-test calculated the median value and test of significance. Spearman's rank correlation compared TNF values with hormone receptor status.

Results

Clinicopathological characteristics of BC patients are shown in Table 1. In this study population, 73% patients were postmenopausal, 74% patients were in higher stages (Stage 3 and 4), 69% patients had lymph node metastases, and 63% patients had higher grades. Triple negative cases accounted for 23% of all cases. The circulating levels of TNF α were significantly elevated in BC patients as compared to controls as shown in Table 2. The median values for TNF α in control and cases were 6.2 (3.2, 10.9) pg/ml and 10.1 (3.6, 32.8) pg/ml, respectively. ER-negative and PR-negative patients showed the highest significance value for TNF α ($P < 0.001$). Patients with lymph node metastasis and those whose tumors were triple negative also showed significantly higher values for TNF α. Triple negative BC cases showed the median value of 33.5 (12.1, 46.9) ($P < 0.005$) for plasma TNF α levels [Table 2].

As shown in Table 3, a significant positive correlation was found between plasma TNF α and TNF β levels ($r = 0.75$, $P < 0.001$). Further, a significant negative correlation was observed between TNF α and ER positivity ($r = -0.58$, $P < 0.001$). A significant inverse correlation was also observed between TNF α and PR positivity ($r = -0.47$, $P < 0.001$). However, TNF β levels were not correlated with hormone receptor status.

Discussion

TNF has physiological and pathological functions in normal and diseased individuals. In the present study, plasma levels of TNF α and TNF β were significantly increased in patients when compared to the control group. Pfeilschifter *et al*.[10] showed that blood levels of proinflammatory cytokines were increased significantly in the postmenopausal normal population. In contrast, neither the normal nor the postmenopausal cancer cases showed increased levels of TNF α and TNF β in the present study. However, plasma TNF α level was significantly elevated in patients with lymph node metastases. Berberoglu *et al*. demonstrated that serum TNF-α level was found to be elevated in patients diagnosed with advanced stage and increased number and size of metastatic sites.[11] Researchers observed that along with TNF α, cytokines IL-6, and IL-8 were correlated with late-stage and lymph node metastasis.[12]

In the tumor microenvironment, cytokines, chemokines, growth factors, and DNA-damaging agents are secreted by infiltrating immune cells and activated fibroblasts.[13] Chronic inflammation in BC may be linked to cancer recurrence, and increased levels of inflammation markers are associated with reduced survival rates.[14,15] The major role of TNF α in cell transformation, proliferation, angiogenesis, invasion, and metastasis of many cancers has been studied.[16] TNF α production is regulated by a positive feedback mechanism with autocrine function, promoting tumor cell growth, invasion, metastasis, neoangiogenesis, and tumor cell survival.[17] Synthesis of estrogen in breast tumor tissue is regulated by

Table 1: Clinicopathological features of BC patients

Clinicopathological parameters	Number of BCs (%)
Age (years) (mean 49 range 26–78)	
<50	39 (56)
≥50	31 (44)
Menopause	
No	19 (27)
Yes	51 (73)
TNM stage	
I–II	18 (26)
III–IV	52 (74)
Lymph node metastasis	
No	22 (31)
Yes	48 (69)
Tumor grade	
I–II	26 (37)
III	44 (63)
ER status	
Positive	39 (56)
Negative	31 (44)
PR status	
Positive	35 (50)
Negative	35 (50)
HER status	
Positive	20 (30)
Negative	50 (70)
Triple negative	16 (23)
Others	54 (77)

BC: Breast cancer, TNM: Tumor node and metastasis, ER: Estrogen receptor, PR: Progesterone receptor, HER: Human epidermal growth factor

Table 2: Plasma levels of study parameters in controls and BCs with clinicopathological factors

Characteristics	TNF α in pg/ml (median Q1, Q2)*	*P* value	TNF β in pg/ml (median Q1, Q2)	*P* value
Control (35)	6.2 (3.2, 10.9)	<0.05#	3.2 (3.2, 3.2)	0.43
Overall case (70)	10.1 (3.6, 32.8)		3.2 (3.2, 4.3)	
Menopause (case)				
No	8.8 (3.9, 25.2)	0.62	3.2 (3.2, 3.7)	0.30
Yes	11.3 (3.3, 37.7)		3.2 (3.2, 4.6)	
TNM stage				
I–II	6.7 (3.4, 12.6)	0.28	3.2 (3.2, 3.2)	0.32
III–IV	12.4 (3.6, 39.4)		3.2 (3.2, 4.6)	
Lymph node metastases				
No	3.9 (3.2, 8.0)	<0.01#	3.2 (3.2, 3.2)	0.32
Yes	12.8 (4.0, 40.3)		3.2 (3.2, 4.5)	
Tumor grade				
I–II	4.7 (3.2, 12.7)	0.12	3.2 (2.4, 3.2)	<0.05#
III	12.6 (3.9, 37.8)		3.2 (3.2, 4.6)	
ER status				
Positive	4.03 (3.2, 9.3)	<0.001#	3.2 (3.2, 3.2)	<0.01#
Negative	34.2 (15.2, 77.2)		3.2 (3.2, 12.3)	
PR status				
Positive	4.0 (3.2, 12.4)	<0.001#	3.2 (3.2, 3.2)	<0.01#
Negative	25.7 (7.5, 69.2)		3.2 (3.2, 11.8)	
HER status				
Positive	18.3 (3.9, 58.7)	0.32	3.2 (3.2, 4.2)	0.24
Negative	8.7 (3.4, 28.8)		3.2 (3.2, 3.9)	
Triple negative	33.5 (12.1, 46.9)	0.005#	3.2 (3.2, 11.9)	0.15
Others	5.7 (3.2, 20.9)		3.2 (3.2, 4.8)	

*Q1=Quartile 25%, Q2=Quartile 75%. #*P*<0.05 is significant. BC: Breast cancer, TNM: Tumor node and metastasis, ER: Estrogen receptor, PR: Progesterone receptor, HER: Human epidermal growth factor

Table 3: Spearman's rank correlation between study parameters and ER, PR, and HER-2/neu status in BCs

Study parameters	*r* value	*P* value
TNF α and TNF β	0.75	<0.001#
TNF α and ER positivity	−0.58	<0.001#
TNF α and PR positivity	−0.47	<0.001#
TNF α and HER-2/neu positivity	0.14	0.26
TNF β and ER positivity	−0.24	0.068
TNF β and PR positivity	−0.22	0.072
TNF β HER-2/neu positivity	0.14	0.22

#*P* < 0.05 is significant. ER: Estrogen receptor, PR: Progesterone receptor, HER: Human epidermal growth factor, BC: Breast cancer, TNF α: Tumor necrosis factor alpha, TNF β: Tumor necrosis factor beta

TNF α. ER may also inhibit TNF-activation by repressing the TNFR element and TNF promoter.[18] Studies by Hwang *et al*.[19] have suggested that TNF α plays an important role in the molecular events that link inflammation with development and evolution toward cancer in the presence of CD9. In our study, triple negative BC cases showed significantly elevated plasma TNF α level. In triple negative BC with the non-availability of estrogen and PRs, elevated levels of TNF α activate multiple cell signaling cascades leading to poor prognosis and aggressive behavior of tumors.[20]

In this study, TNF α and TNF β showed a positive correlation with each other. There was a negative correlation between TNF α with ER and PR positivity [Table 3]. Thus, plasma levels of cytokines may reflect the status of the tumor microenvironment. Goldberg and Schwertfeger[21] showed that inflammation within the tumor microenvironment of BC correlated with increased invasiveness and poor prognosis. Increased cytokine overexpression in cancer tissue versus normal tissue confirmed that various cytokines expressions correlated with negative ER and PR status in BC.[22] It is still unknown fact that whether TNF-α present in the microenvironment is secreted by cancer cells as an evasion mechanism to counteract the host immunity or whether it is produced by the host immune system to abolish the tumors.[8,23] When low doses of TNF-α derivative was supplemented with chemotherapy and radiotherapy, a synergistic activity was observed by sensitizing chemotherapy drugs in *in vitro* and *in vivo* experiments.[24]

In conclusion, the present study showed that pre-operative plasma concentration of TNF α and TNF β increased significantly with ER and PR negativity. Plasma TNF α levels

were significantly elevated in lymph node metastasis and triple negative BC patients, indicating that the plasma TNF α level could be one of the important prognostic markers which also mimics the tumor microenvironment status. Knowledge of the tumor microenvironment is essential in designing new targeted therapies that will add to the therapeutic armamentarium.

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