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Research Article

Esoteric of Tumor Microenvironment: An Association of Cytokines and the Breast Cancer. A Perspective from Pakistan's Population

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Abstract

Background: Cytokines are key signalling molecules that mechanically influence immune responses and tumour dynamics. The tumour microenvironment is pivotal in immune suppression and cancer progression.

Objective: We aim to explore the interplay between oxidative and anti-oxidative markers modulating the cytokines and eventually become the cause of breast cancer.

Materials and Methods: Two groups of clinically diagnosed 288 breast cancer women in stage II or III were included. The control group had 100 disease-free individuals. Along with cytokines, other variable measures were Malondialdehyde, 8-Hydroxy-2'-deoxyguanosine and Glutathione using a standard ELISA kit. The raw data were analyzed with standard SPSS software.

Results: The levels of cytokines, IL-6, IL-10, tumour necrosis factor, and transforming growth factor-β expressions in the BC patients were increased compared to the control group. Oxidative and anti-oxidative stress markers in BC patient' vs control were Malondialdehyde, (0.96 ± 0.05 nmol/mL vs 4.35 ± 1.15nmol/mL), 8-Hydroxy-2'-deoxyguanosine, (1.03 ± 0.09ng/mL vs 6.59 ± 1.44ng/mL), and Glutathione peroxidase (9.77 ± 1.16μg/dL vs 5.88 ± 1.29μg/dL). The *p*-value of this analysis was significant. IL-6 showed a strong association with MDA, 8-OHdG, and GSH in the Pearson correlation test.

Conclusion: Irrespective of the cause of breast cancer, the involvement of the cytokines is inevitable. Henceforth, an extensive study of cytokines associated with breast cancer holds promise for improving therapeutic interventions. Eventually, this will foster the development of more effective and personalized breast cancer treatments.

Keywords: Breast Cancer; Growth Factors; Cytokines; Tumor Necrosis Factors; Oxidants; Antioxidants

Introduction

The immune system plays a crucial role in cancer prevention. However, its imbalance favours its initiation and progression. The primary tumour escape strategies are reduced immune recognition, increased resistance, and the development of an immunosuppressive cancer environment [1]. During the multistep development of tumours, cells must be able to evade immune destruction by promoting an immunosuppressive microenvironment [2,3].

Cancer cells can adopt other immunosuppressive mechanisms to create a tolerant microenvironment. The interaction between

cancer cells and the tumour microenvironment depends on metabolic enzymes, specific genetic events, which induce cytokine and chemokine expression, and other factors, such as the state of the patient's immune cells influenced by environmental features [4].

Cytokines play a critical role in regulating the host's immune response toward cancer and determining the overall fate of tumorigenesis. The tumour microenvironment is dominated mainly by immune-suppressive cytokines that control antitumor immunity and promote survival and cancer cell proliferation, ultimately leading to enhanced tumour growth. These cytokines are classified into a broad range; however, in most tumour types, the interleukin-10, Transforming growth factor-β (TGF-β), IL-6, and IL-17 are consistently reported as immune-suppressive cytokines that help tumour growth and metastasis [5].

With the development of tumour immunity, some studies confirm the possibility of serum cytokine levels for tumour diagnosis. It has been reported that the serum level of some interleukins could serve as potential biomarkers in cancer diagnosis [6]. Thus, interleukins are critical in cancer development, progression and control. Interleukins can nurture an environment that enables and favours cancer growth while simultaneously being essential for a productive tumour-directed immune response. These properties of interleukins can be exploited to improve immune therapies to promote effectiveness and limit side effects. However, the results of different studies are contradictory [6,7].

The regulation of tumorigenesis by TGF-β signalling might depend upon its ability to influence the biology of tumour cells or the functions of immune cells. The activity of TGF-β on tumour cells has been attributed to growth inhibition, maintenance of genomic stability and stimulation of apoptosis. At the same time, effects on the immune system may be mediated by changing the function of cells, such as T cells, NK cells, neutrophils, monocytes and macrophages [8].

Epidemiological studies reveal the strong association of breast cancer (BC) with genetic and environmental factors underlying disease etiology and progression. Over the years, multiple lines of evidence indicated a significant relationship between BC at the molecular level; however, unidentified and different hypotheses have been proposed [9].

Understanding the interactions between tumours and the immune system is instrumental to identifying new molecular targets, novel immunotherapeutic approaches, and new efficacy biomarkers that may improve immunotherapy for breast malignancy.

This study aims to elucidate the risk of developing BC in response to an imbalance of cytokines produced by oxidative stress so that understanding cytokine modulation can be involved in fundamental biochemical and clinical applications for cancer immunotherapy.

Materials and Methods

Study setup

A cross-sectional comparative study was carried out at the Institute of Molecular Biology and Biotechnology, The Lahore University. The Research Ethical Committee approved experimental protocols under ethical approval No. IRB: UOL/1336/03/19. Subjects (BC patients and control) were rigorously screened. The duration of the study was two years. 288 clinically diagnosed BC women with stage II to III disease, aged 40 to 50 years, were included in the study. One hundred age-matched women with no history of any disease were taken as control. None of the controls included in the study were on any medications or had a history of chronic illness, malabsorption, malnutrition, depression, or metabolic dysfunction, which could hinder their oxidative metabolites. The demographic data were recorded in a questionnaire designed for this study.

Blood sample collection

A five mL venous blood sample was taken in a gel tube from each participant's antecubital vein for biochemical parameters. Sample tubes were centrifuged at 4000 rpm within three hours of blood collection. The supernatant was collected and stored at -80˚C until it was assayed.

Analysis of variables

After calibrating the microplate spectrophotometers "SpectraMax Plus 384," all samples underwent analysis in a single batch. The analytical study of different variables was performed using commercially available standard ELISA kits. Essentially, the protocols mentioned in the respective leaflets were observed. Abcam kits were used for the measurements of Glutathione

peroxidase (GSH) (Catalog No.: DIGT-250), Malondialdehyde (MDA) (Catalog No.: DTAC-100), and for the measurement of oxidative DNA damage marker; 8-Hydroxy-2′-deoxyguanosine (8-OHdG), (Catalog No.: SKT-120-96S). IL-6, (Cat #, ab6672), Il-10; (Cat #, ab133575), TGF-β; (Cat #, ab92486), TNF-α; (Cat #, ab6671). To reduce the fluctuations in test outcomes, the intra-assay coefficients of variation were employed, and three to four repetitions of each test were conducted on the same plate to assess the variability among data points for all parameters.

Statistical analysis

A sample size of 288 consented BC women was determined using 95% CI or confidence level, 95.0% power. The sample size was estimated using precision 3.0 software. The collected data was analyzed by SPSS v20. Variables of data were articulated as mean ± standard deviation. An Independent student's test was used to determine the significant changes in different variables in both groups. $P < 0.05$ was taken as significant. The Pearson Correlation Coefficient was used to find the correlation between cytokines and oxidants or antioxidants. The r-value of >0.5 was taken as a positive significant association between the two parameters.

Results

The data in Table 1 reveals the demographic profile of the individuals suffering from BC. A large number of the participant were married, had breastfed their children and were near the menopausal age, which was identified as 54 ± 4.15 years (Mean ± SD). Furthermore, the majority of them had a positive ER status, and histologically, 227 individuals had a category of invasive carcinoma. Most were identified as housewives in the middle class of socio-economic Status, with a mean age of 46.8 years. Family history of BC was positive for 53 participants, and a large number of them had gone through radio and chemotherapy.

The biomarkers of oxidative stress portrayed in Table 2 have a role in disease progression, as reflected by their profile in BC patients. The levels of anti-oxidant GSH were decreased in BC patients vs control (5.88 ± 1.29µg/dL vs 9.77 ± 1.16µg/dL) in combating the oxidative stress in the micro milieu of the tumour

Parameters	Values with number of individuals (n)	
Age (years) Mean ± SD	46.8 ± 13.5	
Socio-economic status (n)	Lower-class n = 54 Middle-class n = 215 Upper-class n = 19	
Occupation (n)	Housewife n = 247 Working women n = 41	
Family history (n)	Positive family history of BRCA in mother/siblings n=53	
Marital Status (n)	Married n = 265 Unmarried n = 23	
Age at first pregnancy (n)	Nulliparous n=17 Less than 25 years n = 152 More than 25 years n = 119	
Breastfeeding history (n)	Breastfed one child n = 116 Breastfed 2 child n = 88 Breastfed more than 2 child n = 84	
Age at menarche Mean ± SD	14 ± 2.13 years	
Age at menopause Mean ± SD	54 ± 4.15 years	
Menopausal status (n)	Premenopausal n = 119 Postmenopausal n = 169	
Site of cancer (n)	Right n = 141 Left n = 147	
Duration of cancer (n)	Less than 2 years n = 98 2 to 4 years n = 141 More than 4 years n = 49	
Histologically diagnosed (n)	In situ carcinoma; Ductal n = 26 Lobular n = 35	Invasive carcinoma; Ductal n=198 Lobular n=29
ER status (n)	Positive n = 201 Negative n = 87	
Mode of treatment (n)	Radiotherapy n = 89 Radiotherapy and Chemotherapy n=199	

Table 1: Demographic data in BC patients included in the study.

VARIABLES	CONTROL (n = 100) Mean ± SD	BC PATIENT (n = 288) Mean ± SD	*P*-VALUE
MDA (nmol/mL)	0.96 ± 0.05	4.35 ± 1.15	0.003 ¶
8-OHdG (ng/mL)	1.03 ± 0.09	6.59 ± 1.44	0.001 ¶
GSH (**µg**/dL)	9.77 ± 1.16	5.88 ± 1.29	0.018 ¶

Table 2: Oxidative and anti-oxidative profile of all individuals in the study.

¶ *p*-value < 0.05. A significant difference as compared to the control vs. BC Patient

The collected data was analyzed by SPSS v20 for the Student's t-test.

environment. The increased lipid peroxidation and MDA levels were higher in BC patients (4.35 ± 1.15nmol/mL) compared to controls (0.96 ± 0.05nmol/mL). The DNA damage profile markers, 8-OHdG in the BC patient group, portrayed significantly increased levels compared to the control group (6.59 ± 1.44ng/mL vs. 1.03 ± 0.09ng/mL) with p-values of 0.001.

VARIABLES	CONTROL (n = 100) Mean ± SD	BC PATIENT (n = 288) Mean ± SD	*P*-VALUE
TNF-α (pg/mL)	20.25 ± 6.35	31.22 ± 3.66	0.002 ¶
TGF-β (pg/mL)	172.59 ± 16.35	256.35 ± 16.54	0.001 ¶
IL-6 (pg/mL)	6.35 ± 1.59	12.99 ± 3.29	0.001 ¶
IL-10 (pg/mL)	5.58 ± 1.77	7.88 ± 2.49	0.000 ¶

Table 3: Cytokine profile in control subjects and BC patients.

¶ *p*-value < 0.05. A significant difference as compared to the control vs. BC Patient.

The collected data was analyzed for student's t-test.

According to Table 03, the levels of TNF-α activated in inflammatory situations were significantly increased in BC patients (31.22 ± 3.66pg/mL) compared to the healthy group (20.25 ± 6.35pg/mL). Highly significant increase of TGF-β levels (256.35 ± 16.54pg/mL vs. 172.59 ± 16.35pg/mL) were observed compared to control individuals. The mean values of IL-6 and IL-10 were also evidently high in BC patients.

Variables	R - value
IL-6 vs Oxidant and Anti-Oxidant	
IL-6 vs. MDA	0.889
IL-6 vs. 8-OHdG	0.723
IL-6 vs. GSH	0.784
IL-10 vs Oxidant and Anti-Oxidant	
IL-10 vs. MDA	0.878
IL-10 vs. 8-OHdG	0.752
IL-10 vs. GSH	0.771
TNF-α vs Oxidant and Anti-Oxidant	
TNF-α vs. MDA	0.989
TNF-α vs. 8-OHdG	0.841
TNF-α vs. GSH	0.734
TGF-β vs Oxidant and Anti-Oxidant	
TGF-β vs. MDA	0.843
TGF-β vs. 8-OHdG	0.751
TGF-β vs. GSH	0.791

Table 4: Correlation of serum cytokine; IL-6, IL-10, TNF-α and TGF-β (measured by ELISA) with oxidant and anti-oxidant.

Table 4 Correlation of BC patient's cytokines with oxidative and anti-oxidative markers. An r-value of more than 0.500 indicates a strong correlation between the two variables.

IL-6's physiological and pathological function is widely documented and seems to play an essential role in modulating BC. It shows a significant positive correlation with MDA, 8-OHdG, and GSH markers in Table 4. Similarly, the expression of other cytokines (IL-10, TNF-**α** and TGF-β) were significantly in positive correlation. The practical observation in this table was that all cytokines showed a high level of r value with MDA. This imbalance related to MDA is suggestive of the dominance of oxidative stress, which is also evident when observing the cytokines with GSH. Needless to mention an imbalance in these variables will eventually propagate inflammation and modulate immune responses, thus creating a microenvironment favoring oncogenesis.

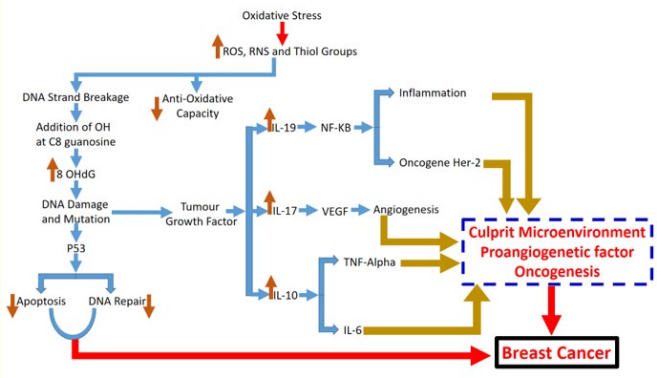

Figure 1: Illustrates the western blotting of cytokine profiles in the control and BC patients.

Figure 1 showcases the flow chart of the pathways leading to BC by the involvement of different cytokines.

Figure 1 illustrates the flow chart when, under oxidative stress, increased amounts of ROS, which plays a vital role in BC, are produced. To begin with, it causes depletion of antioxidants and DNA strand breakage, leading to the addition of the OH group at the C8 position of guanosine and hence increasing the formation of 8-OHdG. This renders the mechanism of p53, DNA damage continues, and mutation will decrease the level of p53, leading to a decrease in BAX-1, cytochrome-c, and caspase 3 and, evidently, a decrease in apoptosis, ultimately contributing to BC. Increasing amounts of ROS continue to orchestrate different other pathways; changes in the neutrophil-lymphocytes ratio and C - reactive protein increase the level of IL-17 and activate TGF-β, which in turn increases the vascular endothelial growth factor contributing to angiogenesis of in-situ tumour and facilitating its spread. IL-17 thus increased and modulated IL-19, NF kappa B, and HER2. An increase in the level of IL-10 due to an increase in ROS and tumour growth will activate TNF-α. IL-10 is dependent upon IL-6 through IL-23. An increase in IL-6 will act upon estrogen receptor alpha (ER-α) and cause tumour growth. This tumour microenvironment will release cytokines and pathogens-associated molecular patterns, which will attach to toll-like receptors and will cause the release of NF-KB. An increase in the level of NF-KB and inflammation TIMP will start acting as a carcinogenic factor with NF-KB, leading to metastasis and hence worsening the prognosis.

Discussion

Demographic variables can be used for the categorization and analysis of BC. Several demographic variables are considered to understand its prevalence, incidence, and impact on different groups. In our study, demographic variables of patients showed that middle-aged women, positive marital Status, and professional housewives of the middle class are at risk of developing BC.

A review and previous meta-analyses have documented various predisposing factors of BC. The ailment in thyroid dysfunction has been documented as a critical factor potentiating the proliferative capacity of the breast tissue [10-12]. Although the exact mechanism of association between thyroid dysfunction and BC is not well known, some hypotheses have been suggested, such as T3 and T4, which can be anti-apoptotic and thus have a proliferative effect. Modulation of gene expression of TGF-α in B-cell [13,14], causing phosphorylation by MAPK pathways, binding in the integrin [15,16], stimulates estrogen-like effects [17]. Moreover, excessive or insufficient iodine intake can be a crucial player in thyroid hormone production and could also be a risk factor for BC [18]. Thus, different hormonal and molecular players should be considered in every patient when analyzing the association of BC [9].

The population-based and other study results suggested that factors other than oncological may play a role in the initiation and progression of BC. Although specific genetic mutations, such as BRCA1 and BRCA2, are associated with an increased risk of BC [19], their association with the male breast has also been reported [20]. According to our study, the levels of TNF-α and TGF-β were recorded as significantly increased in BC subjects compared to the healthy group. Similar observations have been reported by other groups [21-23].

The most emerging concern in cancer treatment is hijacking and restraining the activity of antitumor immune cells in the tumour niche due to a highly immune-suppressive environment [5]. Among cytokines, some play an antitumor role, such as interferons (IFNs) and IL-12 [24]. Other cytokines exert pro-tumorigenic and immunosuppressive effects like IL-4, IL-10, and TGF-β. Cytokine IL-10 expression positively correlated with invasion and larger tumour size, suggesting a role in carcinoma progression and aggressiveness [25]. Our findings are similar, where the mean values of IL-6, 10, 17, and 19 were significantly raised in the BC patients contrary to control groups. Moreover, IL-4 and IL-10 have been found to increase the resistance of cancer cells to chemotherapy [26] through the up-regulation of anti-apoptotic proteins like Bcl-2 and Bcl-xL [27-29].

IL-6 has garnered significant attention among the interleukins investigated in BC research. Its effects on tumour growth, metastasis, and the surrounding environment have been thoroughly investigated. In several studies, higher levels of IL-6 have been linked to more aggressive types of BC and worse prognosis [30-32]. However, because BC is a complex disease, scientists are closely monitoring the mechanisms of interleukins, such as TNF-α, IL-8, and IL-10, because of their roles in tumour growth, inflammation, and the immune system. Even though IL-6 has a variety of impacts

on cancer cells and the tumour microenvironment [31], research is still being done to determine the precise roles that different interleukins and cytokines play in BC.

Similarly, in our study, IL-6 seems vital in its association with other study variables. Henceforth, it is suggested that cytokines can be predictive indicators of the therapeutic response and the prognosis of patients with BC [33]. Furthermore, their interplay during cellular proliferation can be utilized to develop vaccination or chemo-drugs to combat tumour growth.

Conclusion

The present data call attention to the usefulness of screening for BC after a particular age and advise on ebbing stress factors from their life. Similarly, screening potential genes and serum measurement of oxidative parameters should alert the individuals.

Additionally, the article draws ideas for further research to develop targeted treatment for a more successful outcome in patients with BC. Assessment of the autoimmune profile should be considered, especially for those with thyroid disorders.

These findings highlight the importance of TGF-β in generating and maintaining a highly immune-suppressive tumour environment that restricts the activity of effector immune cells and promotes tumour growth. Therefore, targeting TGF-β alone or with other combinatorial therapies has notable clinical benefits for treating cancer patients. Furthermore, analysis of cytokine could lead to the development of new treatment approaches.

Declarations

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- **Availability of data and materials:** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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