

Tumor-promoting roles of IL-4 and TGF- β 3, their implications in the progression of breast tumors

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Immunosuppressive cytokines are the main components of the tumor microenvironment and perform a vital function in controlling the immune response to malignant neoplasms.

The objective: to study the influence of interleukin-4 (IL-4) and transforming growth factor- β 3 (TGF- β 3) on the development of breast tumors in women.

Materials and methods. The concentration of cytokines IL-4 and TGF- β 3 in blood serum was determined in 40 women with benign breast tumors, 40 women with malignant breast tumors, and 40 healthy patients without breast pathology, who were included in the control group.

Breast cancer (BC) patients were divided into two groups; the first group included patients with the II stage of BC, who were considered to have a low level of BC, and the second group included patients with III and IV stages of BC, who were considered to have a high level. The method of solid phase immunoenzymatic analysis was used to determine the level of cytokines.

Results. The results showed that women with benign breast tumors (86.82 ± 1.67 pg/ml) had no statistically significant difference in IL-4 levels compared to the control group (88.25 ± 1.56 pg/ml). However, a significantly higher level of IL-4 ($P=0.0001$) was found in women with BC (97.12 ± 1.84 pg/ml) compared to the control group.

In addition, the results showed that the concentration of TGF- β 3 did not increase significantly in women with benign breast tumors (80.84 ± 2.88 pg/ml) compared with patients with BC and controls (80.84 ± 2.88 and 87.89 ± 2.41 pg/ml, respectively). However, the level of TGF- β 3 was significantly higher ($P=0.01$) in women with BC compared to the control group.

Conclusions. The results of the current study indicate that the concentrations of TGF- β 3 and IL-4 in the blood serum of women may be useful predictors for the early detection of breast cancer, as well as serve as a prognostic indicator of its development.

Keywords: interleukin-4, transforming growth factor- β 3, breast tumors.

Значення IL-4 і TGF- β 3 у виникненні пухлини, їхній вплив на прогресування пухлин грудної залози Б. Р. Сахар, Р. М. Х. Аль-Джумайлі

Імуносупресивні цитокіни є основними компонентами мікрооточення пухлини і виконують життєво важливу функцію у контролі імунної відповіді на злоякісні новоутворення.

Мета дослідження: вивчення впливу інтерлейкіну-4 (IL-4) і трансформівного фактора росту- β 3 (TGF- β 3) на розвиток пухлин грудної залози у жінок.

Матеріали та методи. Визначали концентрацію цитокінів IL-4 та TGF- β 3 у сироватці крові у 40 жінок із доброякісними пухлинами грудної залози, 40 жінок зі злоякісними пухлинами грудної залози та 40 здорових пацієнток без патології грудної залози, які увійшли до контрольної групи.

Хворих на рак грудної залози (РГЗ) розподілили на дві групи; до першої групи увійшли пацієнтки з II стадією РГЗ, що вважали низьким рівнем РГЗ, а до другої групи – пацієнтки з III та IV стадіями РГЗ, що вважали високим рівнем. Для визначення рівня цитокінів застосовували метод твердофазного імуоферментного аналізу.

Результати. Результати засвідчили, що жінки з доброякісними пухлинами грудної залози ($86,82 \pm 1,67$ пг/мл) не мали статистично значущої відмінності у рівнях IL-4 порівняно з контрольною групою ($88,25 \pm 1,56$ пг/мл). Проте у жінок з РГЗ ($97,12 \pm 1,84$ пг/мл) виявлено достовірно вищий рівень IL-4 ($P=0,0001$) порівняно з контрольною групою.

Крім того, результати продемонстрували, що концентрація TGF- β 3 не збільшилася значно у жінок з доброякісними пухлинами грудної залози ($80,84 \pm 2,88$ пг/мл) порівняно з пацієнтками з РГЗ та контрольної групи ($80,84 \pm 2,88$ і $87,89 \pm 2,41$ пг/мл відповідно). Проте рівень TGF- β 3 був значно вищий ($P=0,01$) у жінок з РГЗ порівняно з показником контрольної групи.

Висновки. Результати поточного дослідження свідчать, що концентрації TGF- β 3 та IL-4 у сироватці крові жінок можуть бути корисними предикторами для раннього виявлення раку грудної залози, а також слугувати прогностичним індикатором його розвитку.

Ключові слова: інтерлейкін-4, трансформівний фактор росту- β 3, пухлини грудної залози.

The breast tumor is a neoplastic accumulation of abnormal tissue in the breast include fibrocystic and fibroadenoma or malignant breast cancer. Femoradenomas account for approximately 7% of breast tumors, while breast cancer comprises 10%; the remainder is attributed to benign shapes [1].

However, the presence of immune-regulatory cytokines within a tumor microenvironment induces the re-

cruitment of supplementary immune suppressive cells. Specifically, it alters the phenotype and function of effector immune cells in order to stimulate the development of tumors. Immune cells with changed phenotypes make more cytokines that weaken the immune system. This makes the environment around the tumor less susceptible to the effects of immune cells that fight tumors [2, 3].

These immune regulatory cytokines exhibit properties that promote tumor growth through cytokines that control the functioning of the immune system. These cytokines can take control of the cell by changing signals within cancer cells and immune cells surrounding the tumor. This occurs through an autocratic or positive feedback loop [4].

Macrophages and T cells produce many cytokines, one of which is called interleukin 4 (IL-4). It has been linked to increased susceptibility to asthma and is responsible for controlling the growth of T-helper 2 (Th2) cells. Although previous studies have shown that interleukin 4 (IL-4) has anti-cancer capabilities, high levels of IL-4 have been observed in the tumor tissues of patients diagnosed with kidney, prostate, colon, and lung cancer [5].

The IL-4 plays a key role in breast cancer growth, as many previous studies have shown. It helps malignant cells become resistant to apoptosis and spread to other parts of the body [6]. Breast cancer is characterized by a high level of expression of the IL-4 receptor, which is required for the actions of IL-4 on its target cancer cells [7].

Transforming growth factor- β (TGF- β) represents an evolutionarily conserved family of secreted polypeptide factors that regulate many aspects of physiological embryogenesis and adult tissue homeostasis. In mammalian tissues, TGF- β regulates homeostasis under typical physiological circumstances by inhibiting cell proliferation and promoting apoptosis. However, the multifaceted nature of TGF- β signaling in carcinogenesis should not be overlooked. Initially, TGF- β functions as a tumor suppressor; however, as the disease advances, it converts into a tumor promoter and loses its ability to modulate cancer cells.

When cancer cells generate excessive TGF- β , they generate a local fibrotic and immunosuppressive microenvironment. This microenvironment is conducive to tumor development and is linked to the invasiveness and metastasis of cancer cells [8]. There is a protein known as TGF- β 3 that is encoded by the TGF- β 3 gene and is found in humans. The processes of embryogenesis, development, cell differentiation, and cytokines all involve the action of TGF- β 3. Like many other cytokines, it belongs to the transforming growth factor beta superfamily [9].

The objective: the main purpose of this study is to explore the impact of IL-4 and TGF- β 3 on development the breast tumors by studying the serum levels of IL-4 and TGF- β 3. Age, body mass index, and stages of cancer, have also been linked to IL-4 and TGF- β 3 to get a better understanding of the progression of breast tumors in affected women.

MATERIALS AND METHODS

A total of 120 Iraqi women were enrolled in this study. Eighty women diagnosed with breast tumors (pre-treatment) were randomly selected during their attendance at Al-Karama Hospital's Oncology Center / Wasit and Al-Hussein Teaching Hospital's Oncology Center / Karbala, Iraq. The research was conducted between October 2023 and February 2024.

Blood serum were taken from forty women who had benign breast tumors (non-malignant breast tumors) and forty women who had malignant breast tumors (Breast cancer), forty women who served as healthy controls group.

Then, the breast cancer (BC) patients were divided into 2 groups; group one consisted of patients with stage II breast cancer, which was considered to be low level of BC, while group two was consisted of patients with stage III and IV of BC levels, which considered to be high level. The age distribution of women diagnosed with breast cancer was 33 to 75 years (52.17 ± 1.48 years), whereas women diagnosed with benign breast lesions were 16 to 65 years (30.00 ± 1.89 years).

In addition, 40 healthy women aged 24-54 years (39.50 ± 2.77 years) participated in this study. Also, patients with breast cancer were categorized into two groups: Group 1, comprising individuals in stage II (low level), and Group 2, comprising individuals in stages III and IV (high level).

Breast cancer stages

Staging is an important step in the treatment of breast cancer, just as it is in other forms of cancer. As a result, standardized staging tools are required. Physicians utilize the American Joint Committee on Cancer (AJCC) staging system to determine cancer progression and make treatment decisions. The AJCC has considered anatomic information, such as tumor size (T), lymph node status (N), and the presence or absence of distant metastasis (M), to be fundamental for cancer staging. There are 4 major stages of breast cancer: stage 1 (zero), which is non-invasive ductal carcinoma in situ (DCIS), and stages I through IV (1 through 4), which are used for invasive breast cancer.

The Body Mass Index (BMI)

The Body Mass Index is a measurement of body weight that is derived by dividing the weight in kilograms by the height in meters squared Chapter Two: Literature Review 23 (kg/m^2). BMI has historically been used to categorize people's weight in relation to others, classifying them as underweight, normal weight, overweight, or obese, with a BMI between 25 and 29.9 kg/m^2 indicating overweight and a BMI higher than or equal to 30 kg/m^2 indicating obesity.

Ethical clearance

The Ethical Committee (CSEC/0623/0044 on June 8, 2023) of the Department of Biotechnology, College of Science, University of Baghdad, approved the research after receiving written informed consent from all participants.

Measurement of IL4 and TGF- β 3 levels

The Sandwich-ELISA method (Cat. No. E-EL-H0101 and E-EL-H2339), purchased from Elabscience, (USA) was used to measure interleukin-4 (IL-4) and transforming growth factor- β 3 (TGF- β 3), respectively in blood serum of samples. The ELISA microplate was coated with an antibody specific to human IL-4 and TGF- β 3.

The specific antibody was introduced into the wells of the ELISA microplate containing the samples. Human biotinylated detection antibodies for IL-4 and TGF- β 3 were added to each microplate well, along with an Avidin-Horseradish peroxidase (HRP)-conjugated antibody. Then we left the dishes to settle for a while. The preserved components were rinsed away. In each well, a substrate solution was introduced. It is only possible to see the blue color in wells containing human IL-4 and TGF- β 3, biotinylated detection antibodies, and Avidin-HRP conjugate.

Then, a stop solution terminated the reaction of the enzyme with the substrate, giving the result of yellow color. Optical density (OD) is determined using spectrophotometry at

a wavelength of 450±2 nm. Proportional to the concentration of human IL-4 and TGF-β3, the OD value is determined.

Statistical analysis

All of the statistical analysis was carried out with the help of the SPSS version 24.0 software. The data were expressed as the mean ± SE. The three parameters or more were compared using the ANOVA. T Student tests were additionally utilized to compare the two numerical or categorical parameters. If the P values were less than 0.05, then the differences were determined to be statistically significant.

RESULTS AND DISCUSSION

Assessment of IL-4 and TGF-β3 levels in breast cancer patients according to the age and BMI.

The results of table 1 showed the BC patients with age group <50 had a high level of IL-4 at low-level stages (I+II) with significant differences (p<0.025) when compare with age group >50 at the same level. In contrast, a high level in high-level stage (III+IV) was shown in the age group >50 with no significant differences (p<0.612) when compare with age group >50 at the same level. Similar results was found with TGF-β3 level with no significant differences as shown in table 1.

Regarding to BMI of the patients, the level of IL-4 showed a higher mean level at morbid weight group in low-level stage and overweight group in high-level stages (III+IV), whereas TGF-β3 results showed higher mean levels at normal weight and obesity weight groups in both stages (low and high level), respectively. However, there was statistically no significance in the levels of IL-4 and TGF-β3 among BC patients as shown in table 1.

However, the women at 50 years are more likely to get breast cancer especially after menopause and hormone levels changes such as oestrogen, progesterone and testosterone. Also, from ages 50 to 60, the risk of breast cancer increases to one in 43 [1].

Determination the levels of IL-4 in breast tumors

This study's findings revealed there was no statistically significant difference in the levels of IL-4 in benign breast tumors (86.82±1.67 pg/ml) when compared with the control group (88.25±1.56 pg/ml). On the other hand, there was a significant increase (p-value 0.0001) in the levels of IL-4 in breast cancer women (97.12±1.84 pg/ml) when compared with the control group in table 2.

Determination the level of TGF-β3 in breast tumors

The study showed that there was no statistically significant difference in the level of TGF-β3 between the group with benign breast tumors (80.84±2.88 pg/ml) and the control (75.69±2.83 pg/ml). While, there was a significant (p-value 0.01) increase in the concentration of TGF-β3 among women with breast cancer (87.89±2.41 pg/ml) compared to the control group (table 3). Furthermore, a notable disparity in TGF-β3 levels was observed between benign breast tumors and breast cancer, as illustrated in table 3.

Assessment of Serological Markers IL4 and TGF-β3 According to Low and High Levels of Breast Cancer Stages

As shown in table 4, in BC patients at high levels, we found a significant difference (p≥0.001) in IL-4 level

Table 1

Serum levels of IL-4 and TGF-β3 at low and high level stages of BC patients according to age and BMI

		Age groups	
Variable		IL-4 (pg/ml)	TGF-β3 (pg/ml)
Low levels (I+II) N=13	<50 years	98.03±3.02	80.76±3.31
	>50 years	89.83±2.72	85.67±5.03
P-value		0.025	0.431
High levels (III+IV) N=27	<50 years	97.29±3.95	96.34±4.78
	>50 years	102.40±4.71	95.30±3.91
P-value		0.612	0.902
BMI (kg/m²)			
Low levels (I+II) N=13	Underweight	-	-
	Normal weight	100.30±8.4	90.38±2.8
	Over weight	98.05±2.9	78.76±3.49
	Obesity weight	90.72±4.3	84.26±5.4
	Morbid weight	109.31±10.02	85.80±1.42
P-value		0.28	0.702
High levels (III+IV) N=27	Underweight	-	-
	Normal weight	98.41±3.82	96.81±3.9
	Over weight	105.67±5.24	94.87±12.1
	Obesity weight	98.88±7.17	98.24±6.7
	Morbid weight		
P-value		0.784	0.979

Note. BMI – the Body Mass Index.

Table 2

Assessment of IL-4 levels in the benign and breast cancer women and control

Groups	Mean ± SE
	IL-4 (pg/ml)
Control (n=40)	88.25±1.56 b
Benign (n=40)	86.82±1.67 b
Malignant (n=40)	97.12±1.84 a
LSD value	5.476 **
P-value	0.0001
Means followed by the same letter do not differ statistically among themselves while the means having the different letters differed significantly. ** (P<0.01).	

(95.509±3.21 pg/mL) compared with the control group (75.924±2.55 pg/mL). Moreover, no significance was found in low-stage BC patients at 83.319±3.04 pg/mL compared with control. Moreover, there was a significant difference between the levels of low stages and high stages.

In addition, the results of table 5 showed that BC patients at low levels have a significant difference (p≥0.001) in TGF-β3 level (101.387±2.14 pg/ml) compared with the control group (87.453±1.51 pg/ml). Furthermore, a significant differences

was found in BC patients at high-level stage 94.563 ± 1.73 pg/ml in comparison with control 87.453 ± 1.51 pg/ml; also, showed a high significant of TGF- β 3 serum concentration was recorded in stages (I and II) of BC patients compared to that in patients with stages (III and IV).

The results of this study agree with those of [10, 11] which showed that breast cancer patients had higher levels of IL-4 in their serum than people with benign tumors or a control group. Another study discovered that the level of IL-4 was high when the expression of estrogen receptors was strongly increased [6].

Multiple studies have shown a strong relationship between interleukin-4 (IL-4) and the development of tumors. Different forms of malignant tumor cells have been subjected to tests to examine this connection. It has been observed that stimulation of cancer cells by Interleukin-4 enhances their resistance to apoptosis by facilitating tumor progression [6, 12].

The secretion of the essential differentiation cytokine known as IL-4 is responsible for promoting the formation of Th2 cells. The Th2 subset of lymphocytes is accountable for the eradication of tumor cells via the activation of granulocytes and eosinophils, as well as the inhibition of angiogenesis [13].

Interleukin-4 impacts allergy reactions via interaction with various immune cells, including B lymphocytes, monocytes, dendritic cells, and fibroblasts. Additionally, IL-4 has been shown to possess antitumor and anti-inflammatory capabilities. Janus kinase transducers, signal transducers, and activators of transcription pathways (JAKs and STATs) are responsible for facilitating IL-4 signal transduction.

Furthermore, activated T lymphocytes, mast cells, and basophils are the only cells capable of expressing and synthesizing this entity simultaneously. In breast cancer, the expression of the IL-4 receptor is highly elevated, and the presence of this receptor is necessary for IL-4 to exert its effect on malignant cells [14]. A complicated interaction of chemokines, sex hormones (including prostaglandins and estrogens), and cytokines controls inflammation during carcinogenesis [15, 16].

It has been shown that the cytokine IL-4 is connected with a variety of different forms of cancer. Plasma levels of IL-4 have been linked to several cancers, including melanoma, squamous cell carcinoma, renal cancer, lung cancer, leukemia, and breast cancer [17, 18]. The results of Gaggianesi, M. *et al.*, indicate that IL-4 affects the control of estrogen-synthesizing enzymes as well as the induction of apoptosis in breast cancer cells that have been grown in vitro.

An investigation into the effects of inhibiting IL-4 by the use of the IL-4 receptor (IL-4R) antagonist IL-4DM on malignancies in the mammary gland was carried out. As a result of their research, they discovered that inhibiting the activity of the mitogen-activated protein kinase (MAPK) pathway prevented the proliferation of cancer cells, the invasion of healthy cells, and the formation of tumors [19].

The growth factor beta-3 signaling pathway is important in the tumor microenvironment. Many cell types interact with TGF- β . This cytokine controls many cellular and molecular systems throughout development and disease. TGF- β has a dual function in carcinogenesis. Primary

Table 3

Assessment of TGF- β 3 levels in the benign and breast cancer women and control

Groups	Mean \pm SE
	TGF- β 3 (pg/ml)
Control (n=40)	75.69 \pm 2.83 b
Benign (n=40)	80.84 \pm 2.88 ab
Malignant (n=40)	87.89 \pm 2.41 a
LSD value	8.147 **
P-value	0.01

Means followed by the same letter do not differ statistically among themselves while the means having the different letters differed significantly. ** (P \leq 0.01).

Table 4

Serum levels of IL-4 according to low and high level stages of BC and control

Groups	Mean \pm SE
	IL-4 pg/ml
Control (n=40)	75.924 \pm 2.55a
Low levels (n=22)	83.319 \pm 3.04a
High levels (n=18)	95.509 \pm 3.21b
LSD value	8.638
P-value	\leq 0.001

Means followed by the same letter do not differ statistically among themselves while the means having the different letters differed significantly. ** (P \leq 0.05).

Table 5

Serum levels of TGF- β 3 according to low and high level stages of BC and control

Groups	Mean \pm SE
	TGF- β 3 pg/ml
Control (n=40)	87.453 \pm 1.51 a
Low levels (n=22)	101.387 \pm 2.14 b
High levels (n=10)	94.563 \pm 1.732 c
LSD value	6.492
P-value	\leq 0.001

Means having with the different letters in same column differed significantly. ** (P \leq 0.05).

TGF- β acts as a tumor suppressor, promoting growth inhibition and apoptosis. In advanced tumor development, cancer cells may exploit the regulatory activities of TGF- β to promote tumor growth, bypassing its suppressive effect.

The TGF- β signaling system regulates tumor genesis and progression. It helps cancer cells invade, EMT (epithelial-to-mesenchymal transition), the immune system functions less well, angiogenesis improves, stromal cells become activated, and metastases grow [20–23].

However, the current findings are consistent with those of Sudheer, K. *et al.*, who discovered that patients

with positive lymph nodes in BC had significantly elevated levels of TGF- β 3 [24]. Furthermore, the findings of this research align with those of Ghellal, A. *et al.*, who observed elevated levels of TGF- β 3 in the plasma of early stage BC patients, which were linked to lymph node metastases. Another study also found an association of TGF- β 3 with breast cancer regardless of menopausal status, tumor stage, grade, and size [25–27].

The current study is consistent with Hachim, M. *et al.*, finding higher levels of TGF- β 1 and TGF- β 3 in breast cancer patients compare with control. Transforming growth factor-beta (TGF- β) is synthesized inside the tumor microenvironment by several cellular components, such as tumor cells, stromal cells, immune cells, and vascular cells [28]. Depending on the stage of tumor progression, TGF- β demonstrates dualistic functionality in tumorigenesis, functioning as a suppressor of tumorigenesis in premalignant cells while promoting tumorigenesis in malignant cells [29].

Although extensive research has been devoted to this contradictory function, the mechanism underlying this transition remains unknown. A failure in the T β R regulation system may lead to tumor cell resistance to TGF β 's suppressive effects. A lot of the time, solid tumors have problems with the TGF- β signaling pathway because T β R or SMAD effectors are lost or not working [30, 31]. Cancer cells that possess an inactive T β R or a damaged SMAD pathway exhibit a loss of the tumor-suppressing actions mediated by TGF- β , leading to a persistent state of cellular proliferation.

In addition, research has demonstrated that TGF- β can be converted by cancer cells into a cancer-promoting agent, thereby facilitating a variety of processes, including invasion, metastasis, chemoresistance, and immune evasion.

The primary roles of TGF- β include restricting cellular proliferation and promoting complete epithelial cell differentiation, impeding cancer onset and advancement [32]. In cancerous cells, transforming growth factor beta works as a suppressor of carcinogenesis by obstructing the progression of the cell cycle. Additionally, it induces programmed cell death, also known as apoptosis, in cells that are in a pre-malignant condition. Through gene ablation research, it has been shown that taking away parts of the TGF- β signaling pathway could lead to the development of cancer.

This is in addition to its main job of keeping cells in balance [33]. The cycle of cell division is controlled by the activity of cyclin-dependent kinase-dependent enzymes. One of the mechanisms by which TGF- β inhibits cell proliferation is by lowering cyclin-dependent kinase (CDK) activity. Because it lowers CDK activity, TGF- β stops the cell cycle from progressing through the G1/S phase.

This is because CDK activity is essential for the transition from the G1 phase to the S phase of the cell cycle.

Changing growth factor (TGF) also causes the production of several CDK inhibitors, such as p15, p21, and p57. Cell growth may be inhibited, and cellular [34, 35].

The physiological role of IL-4 is modulating the immune system to promote the generation of Th2 cells and inhibit the formation of Th1 cells that secrete IFN-g, a crucial factor for successful anti-tumor immune responses. The phenomenon observed, whereby the immune system exhibits a bias towards a Th2 response rather than a Th1 response, resembles a frequently seen pattern in individuals diagnosed with cancer.

In this context, the immune system's diversion is a further subversive consequence resulting from the tumor cells' release of IL-4. This particular cytokine facilitates the survival of tumor cells by impeding the development of an unfavorable Th1 environment [18, 36].

According to Soufla, G. *et al.*, there was correlation between tumor stage and the elevated serum levels of TGF- β 3, the levels of TGF- β 3 transcripts were significantly higher in cancer samples than in normal tissues [37]. TGF- β is vital in breast cancer development in vivo because it promotes attack on receptor-positive BC cells through a urokinase-plasminogen activator (uPA)-dependent process [38].

The change of TGF- β from an anti-proliferative to a pro-invasive and metastatic role is facilitated by HER2/EGFR via the modification of Smad3 nuclear localization via AKT. This process ultimately contributes to the development of breast cancer [12]. The development of breast cancer was exacerbated by a synergistic impact resulting from the elevated expression of TGF- β and human epidermal growth factor receptor 2 (HER2) [39].

CONCLUSIONS

During this study, it was shown that individuals who have breast cancer have elevated levels of serum TGF- β 3 and IL-4. These particular cytokines are of significant importance as they actively contribute to the modulation of the tumor immune milieu, hence exerting influence on the progression of carcinogenesis. Numerous prior studies have also shown that the profoundly immunosuppressive tumor microenvironment is primarily accountable for the proliferation of tumors and their resistance to various chemotherapy and immunotherapy agents.

Consequently, the strategic approach of specifically addressing and diminishing the prevalence of immunosuppressive cytokines, namely TGF- β 3 and IL-4, inside the localized environment of the tumor has significant potential for counteracting immunosuppression. This intervention amplifies the effectiveness of therapeutic interventions for breast cancer and stimulates the activation of immune responses against tumor cells.

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