RESEARCH ARTICLE

Expression of Serum IL-22, IL-23, and TLR9 as Tumor Markers in Untreated Breast Cancer Patients

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ABSTRACT

Breast cancer (BC) is the most common cancer among women and is the most important cause of death among them. Breast cancer is a common malignancy, accounting for one-third of women cancers in Iraq. To the best of our knowledge, little literature information is available on the participation of Interleukin-22 (IL-22), interleukin-23 (IL-23), and Toll-like receptor 9 (TLR9) in BC patients. Thus, the evaluation of serum IL-22, IL-23, and TLR9 level changes can add new information on their roles in breast cancer development and progression. The present study aimed to evaluate the clinical usefulness of IL-22, IL-23, and TLR9 as tumor markers in BC.

Levels of circulating interleukin-22, IL-23, and TLR9 were estimated by the enzyme-linked immunoassay method. The serum level of IL-22 was highly significantly elevated in patients compared to the benign and healthy control groups (317.53 ± 14.33 *vs.* 68.73 ± 12.38 and 62.67 ± 19.11 pg/mL, $p \leq 0.05$), but the difference between the benign tumor and healthy control groups means was not significant. Also, these results indicated that the mean level of IL-23 was higher significantly in breast cancer patients in comparison with the benign and healthy controls (887.89 ± 199.04 *vs.* 146.86 ± 84.26 and 72.26 ± 12.76 pg/mL, $p \leq 0.05$). So, no significant difference was revealed between a benign tumor and healthy control groups (146.86 ± 84.26 and 72.26 ± 12.76 pg/mL, $p > 0.05$). Finally, a serum level of TLR9 was significantly elevated in breast cancer patients in comparison with the control groups (16.89 ± 0.75 *vs.* 9.97 ± 1.21 ng/mL, 9.06 ± 0.21 ng/mL, $p < 0.05$). On estimating IL-22, IL-23, and TLR9, the results showed the concentrations of IL-22, IL-23, and TLR9 in the sera of breast cancer were significantly elevated as compared to those in the control groups. Expression of IL-22, IL-23, and TLR9 could be used as a diagnostic tumor biomarker.

Keywords: Breast cancer, Interleukin, Toll-like receptor 9, Tumor marker.

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INTRODUCTION

Interleukin-22 is a recently-described cytokine of the IL-10 family, which is produced by T-helper-17 cells, γδ9 T-cells, NKT cells, and the newly-described innate lymphoid cells (ILCs).[1] Interleukin-22 is a cytokine having tumor-promoting characteristics, and enhances tumor-cell proliferation, protects against apoptosis, and mediates the immunosuppressive immune cell attractions, as well as, releasing the pro- and anti-inflammatory cytokines.[2] In addition, IL-22 also enhances the neo-angiogenesis and epithelial-mesenchymal transition that are the cancer hallmarks.[3] The existence of the IL-22 producing cells is correlated with a more aggressive phenotype in various cancer entities, like breast, lung, gastric, and skin cancers, which indicates a more universal IL-22 function in the progression of cancers.[4,5] The IL-22 source in those tumor entities differs, including $CD4^+$ T cells and innate immune cells.[1] IL-22 is a well known tumor-related inflammatory factor correlated with various types of cancers.[6]

IL-23 is the heterodimeric cytokine consisting of a common p40 subunits, which it shares with its relative IL-12 and the unique p19 subunits.[7] Most IL-23 is secreted by the activated M1 macrophage in the response of Toll-like receptor engagements, which acts on the induction of its expression via the STAT3 and NF-κB transcription factors.[8] From another side, the differentiation of naive $CD4^+$ T-cells into Th17-cells is induced by IL-23, representing an important therapeutic target in treating several chronic immuno-inflammatory diseases.[9] Connecting IL-23 with BC through the study of expression levels of IL-23/IL-23 receptor (R) genes reported high results in BC tissues and associated IL-23 and IL-23R expression levels with the stage, size, and metastasis of cancer.[10] It can promote tumor growth and development,[11] but conversely, it can possess anti-tumor activities in some cancers.[12]

The Toll-like receptor 9 is the intracellular DNA-receptor that recognizes both host-derived and microbial DNAs, and the activation of TLR9 initiates a fast and robust

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innate immune response, with high secretion inflammatory mediator secretions.[13,14] In breast carcinomas, the TLR9 is largely expressed,[15] as well as, TLR9 is also involved in the recognition of self CpG DNA that can lead to the development of autoimmune disorders, including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).[16,17] The expression of Toll-like receptor 9 was observed in the breast milk cells, as well as, the normal mammary gland epithelial cells.[18] The protein and mRNA of TLR9 mRNA are also largely expressed in different human cancer cell lines, as well as, in the clinical cancer samples, such as, breast, gastric, esophageal, prostatic, brain, and renal cell carcinoma.[19] In breast carcinoma, in particular, the expression of TLR9 protein was observed in both epithelial cancer cells in addition to the fibroblast-like cells related to those tumors.[20] The expression of mRNA of the TLR9 A and B isoforms were detected and studied in BC samples of the five human TLR9 isoforms (A–E).[13] The expression of tumor TLR9 is correlated with prognosis, but only among patients with triple-negative tumors to whom no therapy is targeted.[15]

To the best of our knowledge, little literature information is found on therole of serum IL-22, IL-23, and TLR9 in the development of breast cancer. Thus, the evaluation of serum IL-22, IL-23, and TLR9 level changes can add new information on their roles in breast cancer development and progression. The present study aimed to evaluate the clinical usefulness of IL-22, IL-23, and TLR9 as tumor markers in BC.

MATERIALS AND METHODS

Patients

The present study was performed on 60 untreated patients with breast cancers, who were diagnosed by oncologist consultant doctors at the Oncology Teaching Hospital, Baghdad, Iraq, with age range 40 to 60 years average age and standard error (SE) (50.73 ± 1.6) during the period from March to June 2019 for diagnosis and treatment. Diagnosis of the participant patients was confirmed based on breast ultrasound, mammography, pathological, and tissue sample features, while tumor staging was performed in accordance with the 6th edition tumor node metastasis (TNM) classification of the international union against cancer guidelines; all of them had no malignancy other than BC, recurrent breast cancer cases, and treated with chemotherapy or radiotherapy or hormone therapies were also excluded. Besides, 30 benign tumors and 30 apparently healthy women were also included in this study (control group); matched patients with mean ± SE (46.9 ± 2.58; 48.93 ± 3 years).

The clinical parameters, including tumor node metastasis (TNM), estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2) were extracted from medical recordings of patients archived in the Oncology Teaching Hospital. The highest percentage of breast cancer patients were observed in TNM stage III (46.66%), while the lowest percentage was recorded in TNM stage I (16.66%). For breast cancer, positive ER (56.66%) and PR (53.33%) were also observed to have the highest percentages, and HER-2 status (46.66%) was the highest percentage among breast cancer patients.

Blood Samples

From each volunteer, 5 mL venous blood was collected through disposable syringes. Blood samples were transferred into plain tubes, left to clot at room temperature for 10 minutes, then spun at 3,000 rpm for 10 minutes to obtain serum, which was divided into aliquots, 0.5 mL each, in tightly closed Eppendorf tubes, then stored at -20ºC until assayed for IL-22, IL23, and TLR9 detection in the sera of the studied groups.

Assay Method

Serum IL-22, IL23, and TLR9 levels were estimated by the enzyme-linked immunosorbent assay (ELISA) kit (Elabscience, USA).

Statistical Analysis

The IBM SPSS version 25 computer program was used to calculate means, standard errors, and the probability by using student t-test, ANOVA table, and Duncan test. $p < 0.05$ indicated a significant difference.

RESULTS

Estimation of Serum IL-22 Levels in the Studied Groups

The serum IL-22 level was highly significantly elevated in patients when compared with the benign and healthy control groups (317.53 ± 14.33 *vs.* 68.73 ± 12.38 and 62.67 ± 19.11 pg/mL, $p \leq 0.05$) (Table 4.6), but no significant variation was found between the benign tumor and healthy control groups means (Table 1).

Estimation of IL-23 Levels in the Studied Groups

Table 2 indicated that the mean IL-23 level was higher significantly in breast cancer patients compared to the benign and healthy control group (887.89 ± 199.04 *vs.* 146.86 ± 84.26 and 72.26 ± 12.76 pg/mL, $p \leq 0.05$). Also, no significant

Table 1: Serum IL-22 levels in breast cancer patients and the control group

		Serum IL-22 level (pg/mL)
Groups		*Mean ± SE*
Patients	Breast cancer patients	317.53 ± 14.33^{A}
Control	Benign tumor	68.73 ± 12.38^{B}
	Healthy	62.67 ± 19.11^{B}

Non-significant difference ($p > 0.05$) between means (Duncan's test); significant difference ($p \leq 0.05$) between means (Duncan's test)

Table 2: Serum IL-23 levels in breast cancer patients and controls

		Serum IL-23 level (pg/mL)
Groups		*Mean ± SE*
Patients	Breast cancer patients	887.89 ± 199.04^{A}
Controls	Benign tumor	146.86 ± 84.26^{B}
	Healthy	72.26 ± 12.76^{B}

Non-significant difference ($p > 0.05$) between means (Duncan's test); significant difference ($p \leq 0.05$) between means (Duncan's test)

Table 3: Serum levels of TLR9 in breast cancer patients and the control groups

		Serum TLR9 level (ng/mL)
Groups		*Mean ± SE*
Patients	Breast cancer patients	16.89 ± 0.75^{A}
Controls	Benign tumor	9.06 ± 0.21^{B}
	Healthy	9.97 ± 1.21^{B}

Non-significant difference ($p > 0.05$) between means (Duncan's test); significant difference ($p \leq 0.05$) between means (Duncan's test)

variation was revealed between a benign tumor and healthy control groups (146.86 ± 84.26 and 72.26 ± 12.76 pg/mL, $p > 0.05$).

Estimation of Serum TLR9 Levels among the Studied Groups

Serum TLR9 levels were significantly elevated in breast cancer patients when compared with the control groups (16.89 ± 0.75 *vs.* 9.97 ± 1.21 ng/mL, 9.06 ± 0.21 ng/mL, $p < 0.05$), but the difference between the benign tumor and healthy control groups means was not significant (Table 3).

DISCUSSION

In this study, the roles of IL-22, IL-23, and TLR9 were investigated for the first time in the development of breast cancer as a significant increase in its levels was shown in BC patients who have not received chemotherapy in comparison with the control group.

For the confirmation of the IL-22 role in breast cancer, in our study, the expression level of IL-22 was detected in the sera of breast cancer patients. The current results showed highly significant levels of mean IL-22 in BC patients. Similar increased IL-22 levels were detected in the sera of BC patients compared to the healthy controls.[6] IL-22 can function on cancer cells to enhance the aggressiveness, tumor growth, and treatment resistance.[21] While another study observed that high IL-22 expression was observed in many human tumours, such as, breast, ovarian, prostatic, gastric, hepatocellular, esophageal, and non-melanoma skin carcinomas.[22] The role of IL-22 in cancer progression and development was identified in many epithelial cancers, such as, lung and breast cancers.[2,23]

Although many studies affirmed the key role played by IL-23 and Th17 cells in the pathogenesis of human chronic inflammatory and autoimmune diseases, and that previous studies showed the presence of these cells in human tumors, their functional role in cancer's progression and growth is still unclear. IL-23, which is the heterodimeric cytokine discovered in the year 2000, contributes to the Th17 cell induction.[24]

Our results were in concordance with several studies, which confirmed that IL-23 was highly significantly expressed in the sera of breast cancer patients compared with the healthy controls.[9,10,25] On the other side, serum concentrations of IL-23 were significantly elevated, especially in the advanced stages,[26] while another study showed that the low levels of IL-23, which is related to human tumors, seems to promote inflammation and increasing angiogenesis.[27] Our findings did not agree with Sarin *et al.* who found that the depletion of IL-23 leads to infiltration of cytotoxic T cells into the tumour tissues. However, Sarin showed that IL-23 expression in tumour cells induced tumor suppression.[28] Endogenous IL-23 expression was shown to promote tumor growth and development, through the activation of STAT3 by induction of inflammatory responses, including the production of IL-17.[29]

Finally, to confirm the role of the TLR9 in breast cancer, expression levels of IL-22 in the sera of breast cancer patients were detected in this study. This finding agreed with another study, which referred that the higher circulating levels of TLR9 were found in breast cancer compared to healthy control groups.[30] Also, Ivesaro *et al*. and Klinman *et al*. found that TLR9 concentration is increased in breast cancer cells compared to the benign tumor.[31,32]

A previous study found that TLR9 concentration was elevated in serum breast cancer and ovarian cancer.[18] According to Qiu *et al*., TLR9 assists in the progression of BC.[33] Thus, altered TLR9 levels among benign, malignant, and healthy controls suggested an association of TLR signaling in the disease progression among BC patients.[34]

TLR9 expression and its prognostic role in breast cancer were reported by many studies with conflicting results. However, it seems that there is a higher-level expression of TLR9 in ER negative and in high-grade tumors. In regard to the prognostic significance of TLR9 expression, three studies have related the high expression with better consequences,[20,35] while, two other studies reported worse survival.[33,36]

Interestingly, Karki *et al.* showed that BC patients had lower serum TLR9 levels in comparison to patients with benign lesions and healthy individuals, assuming it as a potential diagnostic biomarker.[34] Moreover, triggering TLR9 on cancer cells was shown to protect cancer cells from TRAIL-induced apoptosis and promote the proliferation of tumor cells.[37]

CONCLUSION

On the estimation of IL-22, IL-23, and TLR9 parameters, the results showed the concentrations of IL-22, IL-23, and TLR9 in the sera of breast cancer were significantly elevated compared to those in the controls. Expression of IL-22, IL-23, and TLR9 could be used as a diagnostic tumor biomarker.

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