Iraqi Journal of Biotechnology, 2026, Vol. 25, No. 1, 10-16

Study the Expression of IL-18 gene in a sample of Iraqi women with Breast Cancer

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Received: September 16, 2024 Accepted: October 23, 2024 Published March 30, 2026

Abstract

Background. Interleukin-18 (IL-18) stimulates an immune response against cancer cells, which has an anti-tumor effect. **Aim.** To determine the gene expression and serum level of the IL-18 in the blood of women diagnosed with breast cancer. **Methods.** In this research, included of 100 blood samples, 70 from two groups of patients: 35 who were treated with chemotherapy and biology and 35 who were not, who were admitted to Al-Yarmouk Teaching Hospital, as well as 30 of apparently healthy women as a third group. The age of the patient and control groups was matched. All samples were collected from September 2022 through August 2023. ELISA technique was used to measure the level of IL-18 in serum, and the qRT-PCR technique was used to estimate the gene expression of IL-18 after RNA extraction from whole blood. **Results.** It was observed that patient groups had significantly higher levels of IL-18 than the control group (p value = 0.001). The untreated group had a mean of 122.7223 ng/L, the treated group had a mean of 99.9609 ng/L, and the healthy control group had a mean of 93.4469 ng/L. The study found that breast cancer patients showed an overexpression in IL-18 expression compared to controls, with values of fold change 1.14 for the treated group, 1.67 for the untreated group, and 1.0 for the control. **Conclusion.** It was concluded both treated and untreated patients had higher levels of IL-18 sera and gene expression compared to the apparently healthy control group.

Keywords: *IL-18*, Breast Cancer, Gene Expression, RT-PCR, ELISA.

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Introduction

Breast cancer (BC) BC is the most common malignancy in women worldwide, that includes several molecular kinds and subtypes (1, 2, 3). The immune response significantly impacts disease etiology (4). Cytokines are diminutive, physiologically active proteins that modulate cell development, function, differentiation, and facilitate the orchestration of the immune response and inflammation (5). The interleukins are essential for regulating the immune response inside the tumor microenvironment (6, 7). IL-18 is a 1.1 kb cytokine situated on chromosome 11 (11q22.2-22.3), including 6 exons and 5 introns, consisting of 157 amino acids and

has a molecular weight of 18.3 kDa (8). IL-18 is a pro-inflammatory cytokine (9). IL-18 is a key regulator of immune responses, influencing both innate and adaptive responses and influencing immunological-mediated diseases. It is produced by monocytes, macrophages, and dendritic cells and is known for inducing IFN-γ, stimulating inflammatory responses, and enhancing T and NK cells activation (10). The anti-cancer properties of IL-18 via immune system stimulation have been proposed, alongside a potential pro-cancerous effect of IL-18. Elevated serum IL-18 levels in cancer patients correlate with malignancy, cellular migration in gastric and melanoma cancers, and are associated with cancer progression and metastasis, underscoring the significance of tight junction proteins (11). The pro-cancerous effects encompass the increased expression and secretion of IL-18 in breast and gastric cancer patients, which correlates with accelerated tumor progression, metastasis, and adverse outcomes (12). A substantial quantity of IL-18 is expressed in breast carcinoma. Both IL-18 may have a positive correlation with lymph node metastases in breast cancer (13).

Materials and Methods

Description of Samples

In this case – control study was conducted on 100 women with an age range of 25 to 75 years old; embodied 70 patients with BC were 35 untreated and 35 were treated with chemotherapy and biology, admitted at the Oncology Unit affiliated with the Al-Yarmouk Teaching Hospital in Baghdad City. As well as, 30 of women as a control group, who were apparently healthy with no previous history of malignancy. The study collected all the samples between September 2022 and August 2023. Women with BC were included if they had first been diagnosed with invasive ductal carcinoma, and the treated group had chemotherapy and biology without other types of treatments; it was excluded if they had triple-negative BC (TNBC), those with other types of cancer, and pregnant women. From each one (case and control), take about 5 mL of the venous blood sample divided into two parts: the first one to the anticoagulant EDTA tube mixed gently (then directly divided on mini tubes with the addition of TRIzol™ reagent and shaking for 30 time) to use for the expression of IL-18, and the second part of whole blood added to the gel tube for the separation of serum by centrifugation after staying it to clot for 10-20 min at room temperature to use to estimate the concentration of IL-18. All samples were stored frozen (-20 °C) until used for the study.

Estimation serum levels of IL-18

Enzyme-linked immunosorbent assay (ELISA) kit (YL Biont, Shanghai) was used to quantitatively measurement IL-18 in serum samples (IL-18 Cat. No. YLA1254HU). A plate reader measured the absorbance at 450 nm.

RNA Purification

Using the TRIzol reagent methodology, entire blood samples were used for the RNA isolation process. Firstly, 0.3 mL of whole blood mixed with 0.5 mL of TRIzol reagent (Cat. No. 15596026) (Thermo Fisher Scientific, US). The process of cell lysis involves repeatedly vortexing the samples, followed by an incubation period of about 10 minutes at 25 °C, and then adding 0.25 ml of chloroform to create three distinct phases. The RNA-containing aqueous phase was

separated and added to 0.5 mL of isopropanol, which precipitated the RNA into a white pellet. Add 0.5 mL of 70% ethanol to wash the precipitated RNA. Lastly, rehydrate the RNA pellet by adding 50 µL of nuclease-free water, then incubate in a water bath at 55-60 °C for 10 min. Use a Quintus Fluorimeter (Promega, USA) to gauge the concentration and purity of the isolated RNA.

Synthesis of cDNA

In this work, EntiLinkTM Reverse Transcriptase kit (ELK Biotechnology, China) was used to convert total RNA into complementary DNA using a reverse transcription procedure. When the manufacturer's instructions were followed, the reaction happened in a 20 µL volume. The cDNA reverse transcription process's heat cycler operations were carried out in compliance with the given parameters (1st Strand cDNA Synthesis Kit Cat No. EQ003).

Quantitative Real-Time PCR

After converting RNA to cDNA, a real-time PCR technique was carried out with cDNA serving as the template. RNA-specific primers were used to detect RNA expression of *IL-18* and *GAPDH*, as detailed in Table (1). The components and quantities of the reaction mixture was following the manufacturer's instructions of EnTurbo™ SYBR Green PCR SuperMix (ELK Biotechnology, China) (Cat. No. EQ001).

Table (1): Primers used to Analyze the *IL18* and *GAPDH* Genes' Expression.

Primer	Sequence (5′→3′ direction)	Primer size bp	Annealing Temp. (°C)	References
IL-18 (Interleukin 18)				
Forward	CTTCCAGATCGCTTCCTCTC	20	60	(14)*
Reverse	TCAAATAGAGGCCGATTTCC	20		
GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)				
Forward	GAGTCAACGGATTTGGTCGT	20	59	Design for this study**
Reverse	TTGATTTTGGAGGGATCTCG	20		

* (14) Liu *et al*., (2015).

** The primers were designed by using NCBI/Primer3 program (https://www.ncbi.nlm.nih.gov/guide/howto/design-pcr-primers/), Primers obtained from Macrogen company (Korea).

The expression of *GAPDH* is a housekeeping reference gene served as an internal check for data normalization. The 2−ΔΔCt method was used to determine the relative quantification (fold change) of *IL-18* expression between patients and ostensibly healthy women.

and t-test were used. A chi-square test was used to statistically compare percentages at

Statistical Analysis

The IBM SPSS Statistics 29 program was used to ascertain the influence of diverse elements on study parameters. In order to statistically compare the means and determine their significance, an analysis done in one direction of variance (ANOVA)

significance levels of 0.05 and 0.01. Requesting a calculation of the odds ratio and confidence interval for this research. **Results**

A case-control study was conducted on 100 women, specifically 70 patients diagnosed with breast cancer (BC), dividing them into two groups: 35 underwent chemotherapy, 35 remained untreated, and 30 healthy volunteers served as the control group. Each individual's sample consists of whole blood and serum. The study divided samples into five age groups; these groups ranged from 25 years old to 75 years old. The most treated patients were (45–54) years old with a percentage of 31.4%, and the lowest were in the age range (25–34), as the percentage was 2.9%. Also, the most untreated group were (45–54) years old with a percentage of 54.3%, and the lowest in age was between 25 and 34, as the percentage was 0%. The findings correlate with Iraqi research indicating that the largest prevalence of breast cancer occurs in the sixth decade of life, with an incidence of 35.56%, and a mean age of 54.93 ± 14.33 years in the malignant group (3). In this study, the majority of patients (57.1% treated and 94.3% untreated) were in stage II; only stage II and stage III were included. We divided the three groups (control, treated, and untreated) into three classes based on their Body Mass Index (BMI kg/m2); in the treated group, 19 individuals (54.3%) belonged to the obesity class. In the untreated group, 16 (45.7%) were overweight, and another 16 (45.7%) were obese showed in Table (2).

Table (2): Baseline Characteristics of BC Patients.

Parameter		No. (%) of Treated group	No. (%) Untreated group
	Age		
25-34		1 (2.9%)	0 (0.0%)
35-44		9 (25.7%)	5 (14.3%)
45-54		11 (31.4%)	19 (54.3%)
55-64		9 (25.7%)	6 (17.1%)
65-75		5 (14.3%)	5 (14.3%)
	Stage		
II		20 (57.1%)	33 (94.3%)
III		15 (42.9%)	2 (5.7%)
	BMI		
18.5-24.9		2 (5.7%)	3 (8.6%)
25-29.9		14 (40.0%)	16 (45.7%)
> 30		19 (54.3%)	16 (45.7%)

The results of the ELISA evaluation of IL-18 concentrations showed that they were higher in the patient groups than in the control group, with a mean of 122.7223 ng/L for the untreated group, 99.9609 ng/L for the treated group, and 93.4469 ng/L for the apparently healthy control, with a p-value of 0.001 as shown in Table (3).

Table (3): Comparison between Patients (Treated and Un treated) and Control Groups in IL-18.

Groups	Mean (ng/L)	Std. Deviation	P-value
Treated	99.9609 b	26.84753	0.001**
Untreated	122.7223 a	26.87359	
Control	93.4469 b	11.27366	
Total	104.5094	25.57033	
a and b: Different letters mean there is significant difference. Means followed by the same letter are not significantly different.			

In this study, the *IL-18* expression data was standardized by using *GAPDH* as a reference gene marker. The quantitative analysis of the amplification plots for both *IL-18* and *GAPDH* enabled the calculation of the threshold cycle (CT) values for each gene. As shown in Table (4), the fold change of *IL-18* expression in breast cancer patients was considerably greater compared to the controls (1.00), with values of 1.14 for the treated group and 1.67 for the untreated group.

Table (4): Measurement of *IL-18* Gene Expression Normalized using *GAPDH*.

Groups	Means Ct of *IL-18*	Means Ct of *GAPDH*	ΔCt (Means Ct of *IL-18*)	$2^{-\Delta Ct}$	experimental group/ Control group	Fold of gene expression
Treated	38.5024	33.5256	4.9768	0.0318	0.0318/0.0280	1.14
Un treated	38.6420	32.9800	5.6620	0.0465	0.0465/0.0280	1.67
Control	41.2629	33.1871	8.0758	0.0280	0.0280/0.0280	1.00

The levels of IL-18 expression exhibited significant positive connection with its concentration in serum (r= 0.188 and p= 0.147) showed in Table (5).

Table 5. The Correlation between Gene Expression and Serum Concentration of IL 18 of BC patients

		IL 18
IL 18 expression	Pearson Correlation (r)	0.188
	Sig. (2-tailed) (p)	0.147

** Correlation is significant at the 0.01 level (2-tailed)

Discussion

The prior Iraqi research underscores the substantial association between elevated BMI and critical breast cancer features, highlighting the clinical importance of BMI in shaping breast cancer profiles (15). Our findings compatible with the study found the elevation of blood IL-18 levels in cancer patients were associated with malignancy (16) and other study indicated that elevated blood IL-18 levels are a prognostic marker of worse outcomes in individuals with surgically managed breast cancer (17). During tamoxifen treatment, a strong anti-cancer agent, patients may have improved outcomes accompanied by a notable downregulation of IL-18 levels (17). IL-18

has been shown to activate the intracellular NF-κB, PI3K/AKT, and MAPK signaling pathways, facilitating M2 macrophage polarization and subsequently enhancing tumor neoangiogenesis, migration, and metastasis (18). These results were accordance with study found IL-18 is significantly expressed in BC and may have a favorable correlation with lymph node metastases in this disease (19). Interleukin-18 is a multifunctional cytokine that exhibits potent immunomodulatory activity, providing anti-infective, anti-parasitic, and antitumor effects by inducing interferon γ, enhancing perforin and FasL-mediated cytotoxicity, and directly or indirectly inhibiting and eradicating malignant tumors through various mechanisms (13). Overexpression of IL-18 induces aberrant immunological responses that lead to adverse clinical outcomes (20). Besides macrophages, which are a primary source of IL-18, splenocytes and cancer cells also synthesize IL-18 (21). In addition to its established anti-tumor effects within the immune system, it was indicated that IL-18 generated by cancer cells may exhibit pro-cancer properties, including the facilitation of cell proliferation and migration (22).

Ethical Approval

This study was conducted in line with the ethical standards set out by the Declaration of Helsinki. The research was undertaken after the acquisition of verbal and analytical permission from the patients prior to their participation. The research protocol and the subject information and consent form were assessed and approved by the institutional ethics committee (Ministry of Health-Baghdad Karkh Health Department) (H.42765, 2022/8/15).

Conclusion

It was concluded that the gene expression and serum levels of IL-18 was increased in patient with breast cancer than apparently healthy control, and also was found positive correlation between gene expression and serum concentration of IL-18.

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