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Expression of Toll-like receptor4 (TLR4) in a sample of Iraqi women with Breast Cancer

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ABSTRACT

Toll-like receptor 4 (TLR4) is a key receptor involved in the innate immune response and has been implicated in various diseases, including cancer. To assess the gene expression of TLR4 in women with breast cancer compared to healthy individuals. From the end of September 2022 to the beginning of August 2023, a total of 100 subjects, only women. It included seventy patients with breast cancer, divided into two groups: 35 untreated patients and 35 treated patients by chemotherapy were admitted to the Oncology Unit of Al-Yarmouk Teaching Hospital in Baghdad City, and 30 apparently healthy volunteers, whose ages similarly matched the patient group. To assess the concentration of TLR4 in sera, the ELISA technique was used, and for the estimation of transcript levels of TLR4 gene expression, blood samples were collected from each participant, and RNA extraction was performed. cDNA synthesis was carried out, and real-time PCR was utilized for the analysis. The TLR4 serum concentration was found to be significantly lower in treated and untreated patients than in the control group, with mean values of 2.4777, 2.3269, and 3.5163, respectively. Also, both treated and untreated groups showed lower levels of TLR4 expression compared to healthy controls, with a significant difference between these groups. The gene expression and serum concentration of TLR4 was lower in patient with breast cancer than healthy individuals.

Keywords: TLR4, Breast Cancer, Gene Expression

1. INTRODUCTION

Breast cancer (BC) is a disease that includes several molecular kinds and subtypes [1]. BC is the most common malignancy in women worldwide, ranking second in terms of cancer diagnoses and a major cause of cancer-related deaths among Iraqi women [2][3]. Many factors, either individually or collectively, contribute to the onset of breast cancer, especially in individuals with a genetic predisposition to the disease or who have been exposed to high-risk factors [4]. Advanced age, early menarche, late menopause, first full-term pregnancy at a late age, use of hormonal therapy, such as oral contraceptives, obesity, lack of exercise, diet, smoking, alcohol consumption, low physical activity, and early life exposure to high doses of radiation are all risk factors [5]. Disease pathogenesis is heavily influenced by the immune response [6]. Innate immunity serves as the body's

initial line of protection against infections. The non-specific immune response, which includes pathogen recognition receptors (PRR), such as toll-like receptors (TLRs), is the first line of defense for humans against invading pathogens [7]. These receptors play a significant role in linking innate and adaptive immunity by directing the activation of antigen-presenting cells and important cytokines [8][9]. TLRs play two regulatory roles in the tumor microenvironment (TME) [10]. Chemokines and TLRs work together to promote the initiation and progression of breast cancer [11]. TLR4 is implicated in the invasion of cancer cells. TLR4 is an innate immune receptor that is widely expressed in living organisms; when it is out of balance, it contributes significantly to the development of a variety of ailments, including heart issues, cancer, and inflammatory conditions [6]. The gene is expressed by immune cells such as dendritic cells, macrophages, endothelial cells, neutrophils, and T cells. It has four exons and is located on chromosome 9q32-q33 [12].

2. METHODS

2.1. Study design and patient selection

This study included 100 volunteers, all of whom were women between the ages of 25 and 75, as well as 70 patients who were diagnosed with BC for the first time and had no past infection. These participants were selected from the Oncology Unit of Al-Yarmouk Teaching Hospital, which admitted all patients. The control group consisted of 30 apparently healthy volunteers. They were selected based on specific criteria: exclusion of men, triple-negative breast cancer (TNBC), pregnant women, patients treated with other types of cancer therapy like radiotherapy, those with any other cancer, and those who have had a previous infection with BC. The study matched the controls in terms of age and sex with the patients.

2.2. Sample collection

Every participant in this study had a venipuncture performed to obtain blood samples, with a volume of 5 ml extracted using disposable syringes. After that, each sample was divided and kept in two parts. The first part will be put in an EDTA anticoagulant tube after gentle mixing for estimation of *TLR4* gene expression, and the second part was placed in a sterile gel vacuum tube and left to stand for 10–20 minutes at room temperature (25°C). Following a 10-minute centrifugation, the serum was extracted and separated into 250 ml sections for serological examination.

2.3. Assessment of TLR4 serum levels by enzyme-linked immunosorbent assay

Serum samples were performed using the sandwich enzyme linked immunosorbent assay (ELISA) kit (YL Biont, Shanghai) in accordance with the manufacturer's protocols to determine the (TLR4 Cat. No. R97422). A plate reader was used to measure the absorbance at 450 nm.

2.4. Purification of RNA

RNA was isolated from blood samples using the TRIzol™ reagent protocol. Initially, 300 µL of serum were promptly mixed with 500 µL of TriQuick Reagent (SolarBio, China). The cells in the sample were lysed by vortexing multiple times, followed by a 10-minute incubation at room temperature. To extract the RNA containing aqueous phase, 0.25 mL of chloroform was added. Subsequently, 0.5 mL of isopropanol was introduced to precipitate the RNA, resulting in a white gel-like pellet. The washing of the RNA was carried out by adding 0.5 mL of 70% ethanol. Lastly, the pellet was rehydrated in 50 µL of nuclease-free water and incubated in a water bath at 55–60 °C for 10–15 minutes. The purity and concentration of the extracted RNA

were measured using a Quintus Fluorimeter (Promega, USA).

2.5. Reverse transcription

Reverse transcription is the procedure used in this work to convert total RNA into complementary DNA (cDNA) using the Kit EntiLinkTM Reverse Transcriptase (ELK Biotechnology, China). The instructions provided by the manufacturer were adhered to, and the reaction occurred within a volume of 20 µL. The thermal cycler procedures for the cDNA reverse transcription process were performed in accordance with the specifications stated in Table 1.

Table 1. Program PCR converted RNA to cDNA

Step	Temperature (°C)	Time (min)	No. of cycles
Annealing	25	05:00	1
Extension	42	60:00	
Enzyme inactivation	70	15:00	
Hold	4	10:00	

2.6. Quantitative Real-Time PCR

A real-time PCR experiment was conducted using cDNA as the template after converting RNA to cDNA. RNA-specific primers were created and employed for the purpose of identifying RNA (Table 2). The components and quantities of the reaction mixture are specified in Table 3, whereas the thermal cycling conditions for *TLR4* are defined in Table 4.

Table 2. presents the primers employed for gene expression analysis of *TLR4* and *GAPDH* genes.

Primer	Sequence (5′→3′ direction)	Primer size bp	Annealing Temp (°C)
TLR4 (Toll Like Receptor 4)			
Forward	GATGAGGACTGGGTAAGGAATG	22	60
Reverse	GGCCACACCGGGAATAAA	18	

GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)			
Forward	GAGTCAACGGATTTGGTCGT	20	59
Reverse	TTGATTTTGGAGGGATCTCG	20	

Table 3. presents the volumes and concentrations of the RT-PCR reaction mix

Master mix components	20 µL
2 x SYBR Green PCR Master Mix	10 µL
ROX Dye	0.4 µL
Forward primer	0.4 µL
Reverse primer	0.4 µL
Nuclease Free Water	5.8 µL
cDNA	3 µL

Table 4. presents the thermal cycling program used for RT-qPCR

Steps	Temperature °C	Time (min:sec)	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:20	45
Annealing	59	00:20 Acquiring on Green	
Extension	72	00:20	

The expression of *GAPDH* was used as an internal control to normalize the data. The 2−ΔΔCt approach was

used to calculate the relative quantification (fold change) of *TLR4* expression between the patients and the apparently healthy women.

2.7. Data analysis of RT-qPCR

The expression of Toll-like receptors genes (*TLR4* genes) was assessed using the double delta Ct (threshold cycle) (ΔΔCT) analysis (folding change), with the *GAPDH* serving as the housekeeping reference gene. The following were the calculations:

ΔCT= CT target gene – CT of reference gene ..... Eq. 1

ΔΔCT= ΔCT of each sample – average control ΔCT …. Eq. 2

Folding change = 2-ΔΔCt …… Eq. 3

2.8. Ethical approval

This study was conducted in compliance with the ethical guidelines derived from the Declaration of Helsinki. The study was conducted after obtaining verbal and analytical consent from the patients before their recruitment. The study protocol and the subject information and consent form underwent evaluation and received approval from the institutional ethics committee (H.42765, 2022/8/15).

2.9. Statistical Analysis

The process of analyzing data using statistical methods. The IBM SPSS Statistics 29 software was utilized to identify the impact of various factors on research parameters. A one-way analysis of variance (ANOVA) and t-test were employed to statistically compare the means and determine their significance. A chi-square test was employed to statistically compare percentages at significance levels of 0.05 and 0.01. Requesting an estimation of the odds ratio and confidence interval in this study.

3. RESULTS

3.1. Baseline characteristics of BC patients

The present study was conducted on 100 subjects that were classified into two groups: group I: n = 70 (treated and untreated) BC patients and group II: (n = 30) apparently healthy controls. The age of the 100 subjects in this study, which included both treated and untreated patients as well as controls (seventy patients and thirty controls), varied between 25 and 75 years. The individuals in each group were further separated into five subgroups based on age ranges: 25–34, 35–44, 45–54, 55–64, and 65–75 years. Three-quarters of the cases diagnosed with breast cancer during sample collection were in the II stage, and the rest were diagnosed within the III stage. It has been classified BMI into three main groups: the first group has fewer numbers; only those between 18.5 and 24.9 are related to a healthy range; 30 women in the second group have a range between 25 and 29.9, described as overweight; whereas in the in the last group, about 35 individuals are described as obese (Table 5).

Table 5. Demography of Breast cancer patients

Parameter	*Number*	*Percentage*
1. *Age*		

25-34	1	1.4
35-44	14	20
45-54	30	42.9
55-64	15	21.4
65-75	10	14.3
2. *Stage*		
II	53	75.14
III	17	24.28
3. *BMI*		
18.5-24.9	5	7.1
25-29.9	30	42.9
> 30	35	50

In serum of each group level of TLR4 has been measured. Table 6 shows the highest level of TLR4 was found in sera of control group (3.51 ± 1.58 ng/ml) with a significantly increased (p = 0.001) compared to other groups, and there was no significant difference between the treated and untreated patient groups.

Table 6. Comparison between patients (treated and untreated) and control groups in TLR4

Groups	Mean + SD of TLR-4
Treated	2.47 ± 0.66b
Untreated	2.32 ± 0.28 b
Control	3.51 ± 1.58 a
P-value	0.001**

* Means having different letters in the same column differed significantly.

** (P≤0.01).

The present study included two stages (II and III) of breast cancer, as shown in Table 7 the serum level of TLR4 in the treated group exhibited the highest mean value of TLR4 (2.49±0.76 ng/ml), and there was no statistically significant distinction between stages II and III, while in untreated group, the average value of TLR4 was highest at (2.72±0.00), and it showed a significant increase from stage II to stage III (p = 0.04).

Table 7. Comparison between patients (treated and un treated) in stage and TLR4

Groups	Stage	Mean + SD of TLR-4	P-value
Treated	II	2.49±0.76	0.8 NS
	III	2.45±0.52	
Untreated	II	2.30±0.27	0.04*
	III	2.72±0.00	

In this work, as shown in Table 8, the comparison of the *TLR4* gene expression data in the blood of the studied group was normalized by utilizing *GAPDH* as a reference gene. The amplification plots for both *TLR4* and *GAPDH* were observed, allowing for the determination of the threshold cycle (CT) values for each gene. The folding of *TLR4* expression in treated and untreated patients and control (0.059, 0.21, and 1.00), respectively, was lower in patients than in control.

Table 8. Measurement of *TLR4* gene expression normalized using *GAPDH*

Groups	Means Ct of *TLR4*	Means Ct of *GAPDH*	ΔCt (Means Ct of *TLR4*)	$2^{-\Delta Ct}$	experimental group/ Control group
Treated	35.67787	31.48636	4.19150	0.05473	0.05473/0.92592
Untreated	34.00961	31.65227	2.35733	0.19515	0.19515/0.92592
Control	31.42583	31.3148	0.11103	0.92592	0.92592/0.92592

Table 9 shows that there was no statistically significant difference (p = 0.5) in *TLR4* expression between stages II and III in the treated group. The mean values were 0.1946 and 0.121, respectively. Additionally, there was a

downregulation seen. In the untreated group, the expression of *TLR4* was seen to be reduced in stages II (0.6469) and III (0.5117), with no statistically significant difference (p = 0.8).

Table 9. Comparison between *TLR4* expression in stages of patients (treated and un treated)

Groups	Stage	Mean ± SD of *TLR-4* expression
Treated	II	0.19± 0.14
	III	0.14± 0.12
p-value	0.5 NS	
Untreated	II	0.64±0.58
	III	0.51±0.49
p-value	0.8 NS	

*NS: Non-Significant.

The correlation of the of the serum levels of TLR-4 and TLR-4 expression reveled that no significant correlations were observed between serum levels of TLR-4 and TLR-4 expression folding (r = 0.081 and p = 0.466), as shown in Table (10).

Table 10. The correlation between serum TLR-4 concentration and *TLR-4* expression of (treated and un treated) patients.

		TLR-4 expression
TLR-4	Pearson Correlation (r)	0.081
	Sig. (2-tailed) (p)	0.466

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

3.2. Discussion

Toll-like receptor 4 (TLR-4) is a vital receptor that plays a pivotal role in the immune system by recognizing pathogens and initiating immunological responses [13][14]. An important link exists between toll-like receptors (TLRs) and the start and growth of breast cancer [15]. The current study categorized the results into three groups: treated, untreated patients and controls. The most cases were in middle and elderly age Table 5. All patients there was in II and III stages approximately 75% of them diagnosed in early stage (II) it was similar to study prepared by Nasser & Behadili (2022) [16], rising awareness and demand for breast cancer diagnosis [17]. Only 7% of cases within ideal weight and rest commonly were have overweight and obese described in Table 5. The previous Iraqi study highlights the significant correlation between high BMI and crucial breast cancer characteristics, emphasizing the clinical significance of BMI in influencing breast cancer profiles [18]. As general in the results found the TLR4 concertation in serum and TLR-4 gene expression lower in patients than controls Tables 6, 7, 8 and 9, these results consistent with study it was compared with the normal control tissues, the expression levels of many of TLRs included TLR4 were significantly decreased in breast cancer tissues [15]. Whereas these findings differ from other studies who found the upregulation of TLR4 level and gene expression of TLR4 when comparing breast tumor patients to controls [11][19][20]. The prognosis of breast cancer patients can be determined by assessing the hormone receptor status and HER2 status, which are of crucial significance [21], although the cases in our study are ER/PR positive breast cancer, there are studies like a study their findings indicate that TLR4 is present and functional in breast cancers that are negative for ER/PR [22]. Also, it was found the TLR4 plays a vital role in the movement and spread of cancer cells, angiogenetic potential, as well as in the metastasis of cancer cells in lymph nodes and local tumor growth [22][23][24]. Da Cruz et al. (2021) found that TLRs expression in tumor and immune-associated cells may affect tumor microenvironment (TME) cell communication, modeling cancer progression and responsiveness to treatment [25]. TLR4 expression is downregulated in chemoresistant glioblastoma (GB) and macrophages co-cultured with GB cells. GB-associated immune escape may be achieved by downregulating TLR4 expression and activation [25]. TLRs may have two functions in cancer. Key chemical TLR4 may fight breast cancer both friend and foe. DAMP detection by TLR4 kills breast cancer cells and stimulates immunological responses [26]. TLR4 selectively detects microbial lipopolysaccharide (LPS) and triggers both conventional and non-canonical signaling pathways of NF-κβ activation, leading to the production of pro-inflammatory cytokines and chemokines [27][28]. Some hypotheses suggest that obesity causes a condition of ongoing, mild inflammation, particularly in white adipose tissue. This leads to immunological dysfunction, characterized by increased production of pro-inflammatory cytokines, activation of alternative macrophages, and reduced T-cell activity. The development of breast cancer requires direct contacts and signals between cells in the breast and the adipose tissues, which are mostly composed of white fat. These interactions are regulated by the individual's obesity status [29][30][31]. It is possible to hypothesize that the decreased presence of TLR4, it was also the elevated levels of lipopolysaccharides (LPS) have been seen in obesity due to heightened intestinal permeability caused by a high-fat diet and the escape of gut microbiota [32]. The continuous existence of lipopolysaccharide (LPS), even in small amounts, can lead to endotoxin tolerance. This condition is characterized by a decrease of cell-surface TLR4, which aims to inhibit additional inflammatory reactions in individuals who are already prone to developing inflammatory responses due to their obesity [33][34][35].

4. CONCLUSION

We concluded the toll like receptor 4 concentration in patient's sera who has breast cancer (treated and untreated) was lower than controls, also, it was observed *TLR4* gene expression in patients reduced than healthy women.

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