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Online ISSN (2789-3219)

Research Article

Evaluating *TLR4* Gene Expression to Monitor Disease Progression in Iraqi Patients with Rheumatoid Arthritis

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Abstract

Background: Toll-like receptors (TLRs) play a significant role in the activation of adaptive immunity and may have an essential role in the development of rheumatoid arthritis (RA). *Objectives*: To assess the gene expression of *TLR4* in individuals with RA compared to healthy individuals. *Methods*: From July to December 2022. A total of 100 individuals were encompassed in the study, consisting of 50 individuals diagnosed with RA, of whom 42 were females and 8 were males, with an average age of 45.22 years. Additionally, there were 50 healthy control participants, 40 of whom were females and 10 were males, with an average age of 45.64 years. To assess the TLR4 transcript levels, 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 researchers also assessed the clinicopathological characteristics of the patients. *Results*: The serum TLR4 gene was significantly overexpressed in RA patients (fold change 2.59) compared to the controls (fold change 1.07). The expression level of the TLR4 gene was correlated with the clinicopathological characteristics of the patients, including erythrocyte sedimentation rates (ESR), RF, anti-CCP antibody, and DAS28. *Conclusion*: TLR4 was overexpressed in RA patients and was correlated with disease activity. It might be a therapeutic target and may contribute to the pathogenesis of RA.

Keywords: DAS28, Rheumatoid arthritis, Real time PCR, TLR4 gene.

تقييم التعبير الجيني عن مستقبلات شبيهة التول لرصد تطور المرض في عينة من المرضى العراقيين المصابين بالتهاب المفاصل الرثوي

الخلاصة

الخلفية: تلعب مستقبلات شبيهة التول (TLR4) دورا مهما في تنشيط المناعة التكيفية وقد يكون لها دور أساسي في تطور التهاب المفاصل الرثوي. **الهدف**: صممت هذه الدراسة لغرض تقييم التعبير الجيني لمستقبلات TLR 4 في الأفراد المصابين بالتهاب المفاصل الرثوي ومقارنته مع الأفراد الأصحاء بهدف استكشاف العلاقة بينه وبين بداية وتطور المرض وتقييم بعض المؤشرات الحيوية المناعية التي لها علاقة بالمرض. **الطرائق**: شملت هذه الدراسة مئة شخص قسموا إلى مجموعتين. مجموعة الأصحاء و مجموعات المرضى، تم جمع 50 عينة دم من المرضى الذين تم تشخيصهم في قسم أمراض الروماتيزم، مستشفى بغداد التعليمي وكانوا تحت العلاج في الفترة من تموز 2022 إلى تشرين ثاني 2022. وشملت الدراسة أيضا خمسون عينة من الاشخاص الاصحاء الغير مصابين بالمرض. في الجزء الأول من الدراسة قيست بعض المتغيرات بما في ذلك البروتين المتفاعل، العامل الرثوي و الببتيد الستروليني المضاد بواسطة تقنية ELISA. **النتائج**: أظهرت نتائج الاختبارات وجود فروق ذات دلالة إحصائية في قيم كل من الواسمات الرواتيزمية بين المرضى ومجموعة السيطرة حيث كانت لدى المرضى بمستويات عالية جدًا. الجزء الثاني هو الدراسة الجزيئية التي تتضمن تقدير التعبير الجيني لمستقبلات TLR4 في كل من المرضى ومجموعة السيطرة. **النتائج**: يظهر تحليل النتائج للتعبير الجيني لمستقبلات TLR 4 تعبيرا أعلى لدى مرضى التهاب المفاصل الرثوي بمقدار 1.86 مقارنة مع مجموعة السيطرة (1.12). **الاستنتاج**: هنالك تعبيرا عاليا لمستقبلات التول TLR لدى المرضى المصابين بالتهاب المفاصل الرثوي مقارنة بالاشخاص الاصحاء وكان مرتبطا بنشاط المرض وقد يكون ايضا هدفا علاجيا أو قد يساهم في التسبب بالمرض ايضا.

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INTRODUCTION

Rheumatoid arthritis (RA) is classified as a long-term autoimmune inflammatory disorder that arises from unknown origins [1]. RA is present in the regions of the Middle East and North Africa, where limited understanding of epidemiology and insufficient data concerning the occurrence and intensity of the disease among Arab nations still persist [2]. Causing joint pain and stiffness, with often debilitating and life-limiting consequences [3]. Toll-like receptors (TLRs) play a vital role in recognizing diseases caused by harmful microorganisms invading the body. Moreover, they are considered to be pivotal elements in the development of RA. The impacts of RA, characterized by joint discomfort and limited mobility, often have severe and potentially fatal consequences [4]. Within the human body, 13 distinct structural components make up the TLR family. One of these components, Toll-like Receptor 4 (TLR4), holds great importance. TLR4 can form connections with lipopolysaccharide (LPS), a substance that has been found to contribute significantly to the emergence of rheumatic diseases. Additionally, TLR4 initiates the nuclear factor-B (NF-κB) signaling pathway, which plays a crucial role in the innate immune response [5]. TLR4 is among the TLRs that can detect lipopolysaccharide (LPS), an essential component found in the outer membranes of gram-negative bacteria [6]. Recent research has shown increased expression of TLR2 and TLR4 receptors in cartilage lesions during osteoarthritis, suggesting that hematological conditions other than rheumatoid arthritis may also be impacted by abnormal TLR signaling. Additionally, TLR pathway research has provided researchers with an additional viewpoint on the pathogenesis of RA [7].

METHODS

Study design and patient selection

The study consisted of 100 individuals from Iraq, categorized into two distinct groups: patients and controls. The patient group consisted of 50 individuals (42 females and 8 males) with an average age of (45.22±12.9) years. These participants were selected from Baghdad Teaching Hospital-Medical City, specifically the chronic arthritis diseases department, and were diagnosed through clinical examination by a rheumatologist. The selection criteria followed the 1987 American College of Rheumatology criteria for RA and revised principles for the classification of RA. Furthermore, within the patient group, subgroups were formed based on their DAS28 score, which categorized the severity of their disease activity: low (DAS28 ≤ 3.1), moderate (3.1 < DAS28 ≤ 5.1), and severe (DAS28 > 5.1). The control group consisted of 50 healthy individuals (40 females and 10 males) with an average age of (45.64±12.15) years. These participants were

obtained from the National Blood Transfusion Center. They were selected based on specific criteria: no autoimmune disorders, no familial history of autoimmunity, immunodeficiency, or malignancy, and matched in terms of age and sex with the patients in the study.

Exclusion criteria

Subjects under the age of 18 years, pregnant women, those with any other autoimmune disorders such as osteoarthritis, spinal arthritis, and systemic lupus erythematous, patients with severe liver and kidney damage, and individuals with hematopoietic disorders or chronic illnesses were not included in the study.

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 tubes. In order to do an ESR study, sodium citrate was placed in the first tube, and the second tube was placed in gel tubes and allowed to stand at room temperature for half an hour. Following a 10-minute centrifugation, the serum was extracted and separated into 250 µl sections for molecular and serological examination.

Estimation of Erythrocyte Sedimentation Rate

ESR was estimated in mm/1st hour using Wintergreen method.

Estimation of C-reactive protein, Rheumatoid factor, and anti-cyclic citrullinated peptide

Serum samples were performed using the Sandwich enzyme linked immunosorbent assay (ELISA) kits (Dialab, China) and (SunLong Biotech, China) in accordance with the manufacturer's protocols to determine the (RF Cat. No. R97422), (CRP Cat. No. SL0881Hu and ACCP Cat. No. SL01Hu). A plate reader was used to measure the absorbance at 450 nm.

Purification of RNA

RNA was isolated from serum samples using the TRIzol™ reagent protocol. Initially, 300 µL of serum was 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, 0.2 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).

Reverse transcription

The process involved in this study was the conversion of total RNA into complementary DNA (cDNA) using the Add Script Reverse Transcriptase Kit (Add Bio, Korea). The manufacturer's instructions were followed, and the reaction took place in a volume of 20 µl. All RNA types were transformed into cDNA during this procedure. The thermal cycler steps for the cDNA reverse transcription process were carried out according to the details provided in Table 1.

Table 1: Program PCR converted RNA to cDNA

Step	Temperature (ºC)	Time (min)	No. of cycles
Annealing	25	10	1
Extension	50	60	1
Enzyme inactivation	80	5	1
Hold	4 ∞		

Quantitative real time PCR

After converting RNA to cDNA, a real-time PCR reaction was performed in which cDNA served as the template. RNA-specific primers listed in Table 2 were used to detect RNA [8,9]. The reaction mixture components and their quantities are mentioned in Table 3, while the thermal cycling conditions for TLR4 are outlined in Table 4, following the guidelines cited in AL-Faisal and Alfartusi (2017) [10].

Table 2: Primers that used for gene expression of *TLR4* and *GAPDH* genes

Primer	Sequence (5′→3′ direction)	Primer size bp	Annealing Temp (ºC)	Reference
TLR-4				
Forward	CTTATAAGTGTCTGAACTCCC	21		
Reverse	TAC CAGCACGACTGCTCAG	19	59	[8]
GAPDH- Glyceraldehyde 3-phosphate dehydrogenase				
Forward	TGCACCACCAACTGCTTAGC	20		
Reverse	GGCATGGACTGTGGTCATGAG	21	60	[9]

The expression of GAPDH was used as an internal control to normalize the data. The relative quantification (fold change) of TLR4 expression between the patients and the seemingly healthy individuals was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 3: Volumes and concentrations of RT-PCR reaction mix

Components	20 µl
Luna® Universal qPCR Master Mix	10
Nuclease free water	6
Forward Primer (10 µM)	1
Reverse Primer (10 µM)	1
cDNA	2

Table 4: RTPCR cycling program

Step	Temperature °C	Time m: s	Cycle
Initial Denaturation	95	03:00	1
Denaturation	95	00:20	45
Annealing	60	00:30	
Extension	72	20:00	1

Data analysis of RT-qPCR

The expression of Toll-like receptors genes (TLR-4 genes) was assessed using the double delta Ct (threshold cycle) (ΔΔCT) analysis (folding change), with the GAPDH serving as the housekeeping reference gene [11]. The following were the calculations:

$\Delta CT = CT$ target gene – CT of reference gene .... Eq. 1

$\Delta\Delta CT = \Delta CT$ of each sample – average control ΔCT …. Eq. 2

Folding change = 2-ΔΔCt ……………………………… Eq. 3

Statistical analysis

The SAS program was employed to assess the influence of various factors on the variables being examined in the study. The mean, standard deviation (SD), and range were used to describe the quantitative variables. A t-test was used to compare quantitative variables in parametric data, while an LSD test (ANOVA) was used in non-parametric data. Pearson's correlation coefficient test measured the linear correlation between two variables, ranging from +1 to -1. A total positive correlation is represented by +1, no correlation by 0, and a total negative correlation by -1. To evaluate the diagnostic accuracy of the TLR4 gene, a receiver operating characteristic (ROC) curve analysis was conducted. A *p*-value < 0.05 was considered for significant differences.

RESULTS

The age range of the patients in this study varied from 26 to 70 years, with an average age of 45.22±12.90. Similarly, the control group ranged in age from 28 to 65 years, with an average age of 45.64±12.15. The study found no significant differences in the age distribution between the two groups, as detailed in Table 5.

Table 5: Characteristics of the studied participants

| Characteristics	Patients	Controls	*p*-value
Age (years)	45.22±12.9	45.64±12.15	0.86

Values are expressed as mean±SD.

In this study, RA patients had elevated levels of (ESR), (RF), (CRP) and (anti-CCP) biomarkers of 40.94±8.60 mm/h, 45.74±8.76 IU/ml, 11.38±1.06 mg/dl and 122.49±41.67 IU/ml, respectively. While the controls were 14.34±3.89 mm/h, 27.12±5.06 IU/ml, 6.49±1.96 mg/dl, and 81.97±22.56 IU/ml. Table 6 shows a statistically significant difference (*p*=0.001) in the clinical and laboratory characteristics between the patients and controls.

Table 6: Comparison between RA patients and healthy control groups as regards ESR, RF, and CRP anti-CCP

Parameters	RA	Control	*p*-value
ESR (mm/hour)	40.94±8.60	14.34±3.89	0.001
RF (U/ml)	45.74±8.76	27.12±5.06	0.001
CRP (mg/dl)	11.38±1.06	6.49±1.96	0.001
Anti-CCP (U/ml)	122.49±41.67	81.97±22.56	0.001

Values are expressed as mean±SD; ESR: Erythrocyte sedimentation rate; RF: rheumatoid factor; CRP: C-reactive protein.

Based on this research, the level of TLR4 expression among individuals suffering from RA was notably higher than in healthy individuals. The study observed a substantial increase in TLR4 gene expression in a serum blood sample obtained from individuals diagnosed with RA, which was statistically significant (*p*<0.003). The fold change in TLR4 expression in RA patients (1.86±1.28) was considerably higher compared to the controls (1.12±0.47), as depicted in Figure 1. To evaluate the sensitivity and specificity of TLR4, the ROC curve was employed. Figure 2 shows that the cutoff value identified was 1.65, demonstrating a sensitivity of 66% and a specificity of 93%. Furthermore, the calculation of the area under the curve (AUC) yielded a value of 0.83. We used the least significant difference (LSD) test, which is a type of analysis of variance (ANOVA), to look at changes in TLR4 expression levels in different groups of patients based on their DAS28 scores.
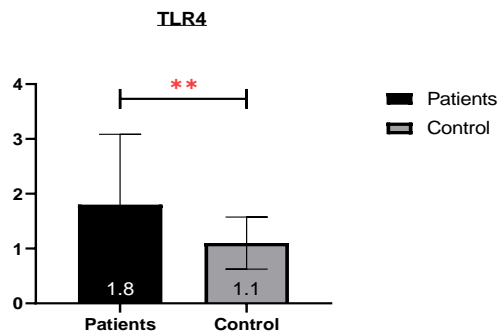
Figure 1: The fold change difference of *TLR4* between RA patients and control groups. ** *p*<0.05.
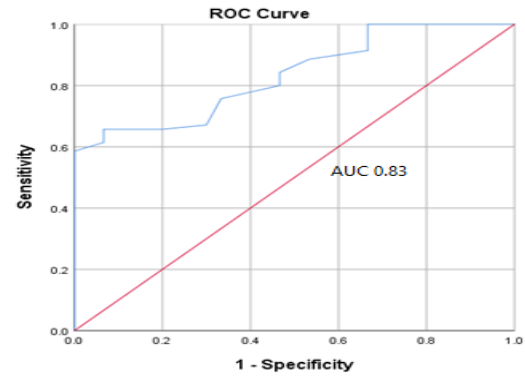
Figure 2: Receiver Operating Characteristic curve of the *TLR4*.

The study discovered a gradual increase in TLR4 expression across these subgroups, although these increases were not deemed statistically significant according to the data presented in Table 7.

Table 7: The association between the level of *TLR4* expression and the course of the disease

DAS-28	TLR4 folding
Low (4)	1.72±0.89
Moderate (18)	1.99±1.37
High (28)	2.00±1.62
LSD value	1.43 NS
p-value	0.941

Additionally, the levels of TLR4 expression were found to have a significant positive correlation with ESR (r =0.30 and p= 0.03), anti-CCP antibodies (r = 0.28 and p= 0.05), RF (r = 0.36 and p= 0.01), and DAS28 (r = 0.29 and p= 0.03). However, no statistically significant correlations were observed between the gene expression levels of TLR4 and age (r = 0.09 and p= 0.5) or CRP (r = 0.28 and p= 0.08), as shown in Table 8.

Table 8: Correlation analysis of the *TLR4* expression levels and clinicopathological properties (ESR, RF, CRP anti-CCP) of the RA patients

		ESR	RF	Anti-CCP	CRP	DAS28	Age
TLR4 folding	r	0.30	0.36	0.28	0.24 NS	0.29	0.09
	p	0.03	0.01	0.05	0.08	0.03	0.5

r: Pearson's correlation coefficient.

DISCUSSION

TLR4 is an essential receptor in the body's natural immune response. It significantly identifies various harmful signals, including those from microbes and within the body itself. This study categorized the outcomes into two groups: patients and controls. Interestingly, no significant age difference was observed between these two cohorts, as indicated in Table 5. This result is consistent with earlier research by Elmalt et al.

[12]. Carefully matching age was done to ensure a fair and unbiased comparison between individuals diagnosed with RA and those in the control group. The current study revealed a significant increase in the average values of ESR, RF, CRP, and ACCP in RA patients compared to controls ($p<0.001$), as presented in Table 6. These elevated ESR, CRP, ACCP, and RF levels result from the production of inflammatory cytokines and immune complexes, which are characteristic of autoimmune diseases. Our findings are in line with several prior studies conducted in Iraq, such as those by [13,14]. In addition, it can be observed from the data presented in Figure 1 that there is a significant rise in the expression of TLR4 among individuals with RA compared to those without the disease. This discovery is consistent with a study in Turkey by Eser and Sahin in 2016 [15], where they identified a higher expression of TLR-2 and TLR-4 in RA patients than in healthy individuals. There are various mechanisms through which the TLR4 gene can be highly expressed in RA: 1) RA is distinguished by chronic inflammation and an overly active immune response, leading to uncontrolled TLR4 expression and the release of pro-inflammatory cytokines; 2) the synovial tissue in the joints becomes activated in RA, resulting in increased production of pro-inflammatory substances that activate TLR4 on synovial cells. 3) TLR4 activation contributes to releasing enzymes that cause tissue degradation and joint erosion. Genetic variations in the TLR4 gene can impact its expression levels and increase the susceptibility to developing RA. The receiver operating characteristic (ROC) curve shown in Figure 2 illustrates that the TLR4 gene expression has an area under the curve (AUC) of 0.83, indicating favorable diagnostic and monitoring capabilities for detecting inflammation in patients with RA. In addition, this study found a noteworthy distinction in the levels of TLR4 expression between RA patients with severe disease activity and those with mild disease activity. Contrary to what was expected, the results presented in Table 7 revealed higher levels of TLR4 in patients with severe disease than in those with low-grade disease activity. Radstake *et al*. [16] have discussed that TLR4 acts as a receptor located on the cell membrane that triggers NF-kB activation when stimulated by internal and external ligands. As a result, the activation of this process results in the generation of different pro-inflammatory cytokines and chemokines, ultimately contributing to disease activity during the progression of RA. Similarly, Salim *et al*. [17] observed significantly raised TLR4 expression in RA patients with high disease activity compared to those with low disease activity, which aligns with the findings of this analysis. There was a strong link between the amount of TLR4 expressed in RA patients and measures of disease activity like DAS28, ESR, anti-CCP antibody, and RF titers. These findings suggest that TLR4 may play a role in the development of RA. Previous research has indicated that impeding the activation of TLR signaling has the potential to be a viable treatment strategy for RA. Arleevskaya *et al*. [18] emphasized the importance of controlling TLR activation in RA patients, identifying individuals who respond to TLR signals, and gaining a comprehensive understanding of the innate immune mechanisms involved. Excitingly, ongoing preclinical trials are investigating antagonistic antibodies targeting TLR2 and TLR4 and a blocking antibody that effectively suppresses TLR activation and the release of pro-inflammatory cytokines [19].

Conclusion

In RA patients, TLR4 was highly expressed and correlated with various clinical and laboratory measures, including DAS28. These values are known to be associated with the progression of the disease, chronic inflammation, joint damage, and increased immune activation.

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