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RESEARCH ARTICLE

The Association between Toll-like Receptor 7 and Hepatitis C Virus in a Sample of Iraqi Rheumatoid Arthritis Patients

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Abstract

Background: Rheumatoid arthritis (RA) is one of the chronic inflammatory autoimmune diseases that included articular and joints infections. Viral infection and immunity had been suggested to play a role in the pathogenesis of autoimmune diseases. Objectives: The present study designed to estimate the association among RA, Hepatitis C virus (HCV) infections and the role of innate immunity expressed by toll-like receptor-7 (TLR-7) as a possible triggering factor in the sera and blood samples of 47 RA patients. Results: The findings of the present study demonstrated that 18 of the 47th RA samples were positive for anti-HCV IgM and IgG antibodies, and 13 of the 18th HCV IgG and IgM seropositive samples were positive for HCV-RNA by using RT-PCR technique. Also, the findings revealed a significant association between RA, HCV infections and TLR-7 serum level in the studied groups. There was a significantly increased level of TLR-7 in the RA group (103.0±73.8 ng/ml) compared to controls (72.9±36.8 ng/ml). Conclusion: These findings concluded that hepatitis C virus infection and TLR-7 might have a role in RA pathogenesis.

Keywords: *Autoimmune disease, Rheumatoid arthritis, Hepatitis C virus, Innate immunity, Toll-like receptor-7.*

Introduction

Rheumatoid arthritis (RA) is a progressive autoimmune disease of the unknown etiology that affecting 2-3 times more prevalent in females than in males [1]. The disease characterized by long-lasting inflammation primarily affects joints rendering them swollen, deformed, and painful with movement limitation especially wrists, fingers, ankles and feet typically in a symmetrical pattern, a characteristic not found in other forms of arthritis [2].

RA is a multi-factorial disease, resulting from the interference between both the genetic (50-60%) and the environmental factors [3]. Several environmental factors had been involved in RA pathogenesis include socioeconomic status, smoking, diet, infectious agents and hormonal factors [4, 5].

Viral infection has been proposed to be a trigger factor of autoimmune diseases such as hepatitis C virus (HCV). HCV is a single-stranded RNA without a DNA intermediate in its replicative cycle; therefore, the viral genomic sequences cannot be combined with the host genome. Much evidence mentions that the HCV infection results from the chronic stimulation of the immune system that assists in increasing the clonal B-lymphocyte [6, 7]. HCV infections have been associated with several autoimmune and rheumatologic disorders. The most common extrahepatic rheumatologic symptoms that include arthralgia, pruritus, myalgia, paresthesia, and sicca syndrome [8].These extrahepatic manifestations of HCV infection are significant in disease diagnosis and treatment [9].

Much of extra-hepatic symptomatology in HCV can be due to chronic immune complex disease [10]. Toll-like receptors (TLRs) are transmembrane protein structures expressed by innate immune system cells; these transmembrane protein structures recognize the invading microbes, such activate signaling pathways that commence the immune and the inflammatory responses to destroy the invaders. In addition, TLRs belong to the family of pathogen recognition receptors, consider an essential part of the

innate immune response, and detects conserved pathogen-associated molecular patterns (PAMPs) of the invader's components such as bacteria, viruses, parasites, fungi, and protozoa [10]. TLRs have been involved in the pathogenesis of several autoimmune diseases such as RA, systemic lupus erythematosus (SLE), and systemic sclerosis (SSc) [11, 12]. The abnormal expression of TLRs may be associated with sepsis and autoimmune diseases such as (rheumatoid arthritis, lupus erythematosus, and type 1 diabetes), TLR7 is the receptor that recognizes single-stranded viral mRNA, and such finding highlights the possible involvement of viruses in RA pathogenesis [13].

Although not fully investigated, the RNA of invaders occur in the synovial compartment and it was demonstrated that there is an increased expression level of TLR-7 in the synovium of RA patients compared to healthy controls [11]. HCV considered as one of the immunogenic triggers the immune responses.

The intrinsic components of the virus presumably lead to activate the innate immune system that effectively limits viral replication. The earliest response to HCV is by interferon (IFN) type's production, which is a critical cytokine, establish an antiviral state and link the innate and adaptive immune systems [14, 15].

TLR-7 is expressed primarily in the endosome-lysosome membrane of each of B lymphocytes, plasmacytoid dendritic cells (pDCs), hepatic natural killer cells, and virally infected hepatocytes. The endosomal proteases in these cells digest HCV molecules such as uncoated and released viral ssRNA that is generally recognized by TLR7 [15, 16]. Therefore, the aim of the present study is to assess the presence of viral infection and part of innate immunity represented by TLR-7 serum level in the pathogenesis of RA patients.

Materials and methods

Patients

Forty-Seven Iraqi RA patients (35 females and 12 males) were enrolled in this study with age mean ± standard deviation (SD) (49.9 ± 13.3 years). They referred to the Consultant Clinic at the Department of Rheumatology, Baghdad Teaching Hospital during the period October 2016 -- June 2017 for diagnosis and treatment. The diagnosis depended on the clinical symptoms and the clinical laboratory examinations according to the 2010 American College of Rheumatology/ European League against Rheumatology for RA [17]. In addition to patients, 28 apparently healthy individuals (20 females and 8 males) with no clinical symptoms or a family history for RA were enrolled in the study as a control group with the age mean ± SD matched patients group (44.7 ± 11.6 years). The study was permitted by the local ethics committee of the Health ministry, Baghdad Teaching Hospital. In addition to the informed permission that was obtained from the study volunteers.

Methods

Five milliliters of venous blood was dropped from all the study volunteer groups. The withdrawn blood amount was divided into two parts, the first part (4 milliliters) was separated in a polyethylene test tubes to collect serum for serological examination, the blood left to clot at room temperature (25ºC), then the tubes were centrifuged at 1207 ×g for 10 minutes, and the sera were separated in Eppendorf tubes.

While the second part (1 milliliter) was kept in an ethylenediaminetetraacetic acid tube (EDTA) for molecular examination. The sera of studied groups were tested to detect the presence of anti-CCP IgG antibody (EUROIMMUN company, Germany), anti-Hepatitis C Virus IgG and IgM antibodies (Foresight / USA, CUSABIO Biotech Co./ China), and to assess the level of TLR-7 (Shanghai Yehua Biological Technology Co. / China) by using ELISA technique.

Confirmatory test was also performed by using conventional PCR technique; the amplification of HCV-RNA was performed to confirm the virus existence in HCV seropositive samples for RA patients. The detecting RNA of Hepatitis C Virus for the seropositive for IgG and IgM antibodies samples was by using a reverse-transcriptase PCR technique. The HCV-RNA was extracted by using the commercially ZR Viral RNA Kit™ (Zymo Research Corp./ USA) according to the manufacturer's protocol.

The concentration and purification of extracted RNA were quantified by using the

Nanodrop spectrophotometer (Quawell technology Inc. / USA) with the wavelength (230, 260 and 280 nm). Specific forward and reverse oligonucleotide primer sequences for RT-PCR were designed for the amplification of HCV-RNA by conventional RT-PCR (forward primer: GTG AGG AAC TAC TGT CTT CAC G; reverse primer: ACT CGC AGG CAC CCT ATC AGG) (Integrated DNA Technologies company/ USA) [18], Primers compatibility search was performed by using the Basic Local Alignment Search Tool (BLAST) of the National Center Biotechnology Information (NCBI) online program. For producing a dsDNA frequent of 262 base pair (bP), the primers were dissolved and diluted according to the manufacturer's protocol.

A PCR mixture was set up in a total volume of 25µl for the reaction mixture that involved 1.5µl of a previously extracted RNA (~50 ng/µl), 0.5µl of RT master mix, 5µl of a PCR master mix, 2µl of (10 Pmol/µl) of each primer, 0.5µl of dNTPs, and 15.5µl of sterile de-ionized free nuclease water. The HCV-RNA was converted to cDNA and then to dsDNA in the same reaction, the conditions of an RT-PCR for an HCV-RNA were one cycle at 50 ºC for 30 minutes to convert the ssRNA to a ssDNA (cDNA), once cycle at 95 ºC for 15 minutes to complete the other strand of the DNA that result as a dsDNA, 40 cycles at (95 ºC for 1 minute, 55 ºC for 1 minute, 72 ºC for 1 minute), and one cycle at 72 ºC for 10 minutes. The PCR products were separated to 2% agarose gel (Promega/ USA) electrophoresis stained with 0.02 µl/ml of Red Safe® stain (Intron biotechnology com./ Korea) and 5 µl of 50-10000 bp DNA ladder (Kappa biosystem/ USA), then visualized by using an ultraviolet light transilluminator.

Statistical Analysis

The data were analyzed by using the IBM SPSS computer program version 24 and expressed as mean ± SD, one-way ANOVA table, and Duncan test. OR, 95% CI, Pearson's X^2 and two-tailed Fischer exact probability were calculated by using WinPepi computer program V. 3.08. The statistical significance level was set at $P < 0.05$ [19].

Results

In the present study, Table 1 showed the level of anti-CCP IgG antibody in the sera of RA patients and healthy control groups.

Table 1: Anti-CCP value in the studied groups

Anti-CCP antibody level RU/ml (mean ± SD)	
RA patients	**Healthy control**
31.9±15.2 [a]	2.3±0.9 [c]

Similar letters referred to non-significant differences ($P > 0.05$).
Different letters referred to significant differences ($P < 0.05$).

Such, Table 2 showed the level of TLR-7 in the sera of RA patients and the healthy control groups.

Table 2: Toll-like receptors7 level in the studied groups

TLR-7 level ng/m (mean ± SD)	
RA patients	**Healthy control**
103±73.8 [a]	72.9±36.8 [b]

Similar letters referred to non-significant differences ($P > 0.05$).
Different letters referred to significant differences ($P < 0.05$).

According to the present study, the results of anti-HCV IgM antibody assessment demonstrated that 11(23.4%) was seropositive in RA patients and 36 (76.6%) were seronegative for anti-HCV IgM antibody. In contrast, 0(0%) was seropositive and 28(100%) was seronegative in the healthy control group for anti-HCV IgM antibody, as shown in Table 3.

Table 3: Anti-HCV IgM antibody percentage distribution in the studied groups

Anti-HCV IgM	Groups				OR (95% CI)	X^2	P
	Healthy control		RA patients				
	Observed subjects	%	Observed subjects	%			
Positive	**0**	0	**11**	23.4	**18.0**	7.6	**$5.2x10^{-3}$**
Negative	28	100	36	76.6			
Total	28	100	47	100			

OR: Odd ratio, 95% CI: 95% confidence interval, X^2: Pearson's chi-square test, P: Two-tailed Fischer exact probability.

Anti-HCV-IgG antibody was also determined. In the RA patient group, 7(14.9%) was seropositive and 40(85.1%) was seronegative. In contrast, 0(0%) was seropositive and 28(100%) was seronegative in the healthy control group, as shown in Table 4.

Table 4: Anti-HCV IgM antibody percentage distribution in the studied groups

Anti-HCV IgM	Groups				OR (95% CI)	X^2	*P*
	Healthy control		RA patients				
	Observed subjects	%	Observed subjects	%			
Positive	**0**	0	**7**	14.9	**10.6**	4.6	**0.041**
Negative	28	100	40	85.1			
Total	28	100	47	100			

OR: Odd ratio, 95% CI: 95% confidence interval, X^2: Pearson's chi-square test, *P*: Two-tailed Fischer exact probability.

In addition, the results of molecular detection of HCV in the sera of RA group in the current study demonstrated that 13 samples from the 18th seropositive samples for anti-HCV IgG and IgM antibodies were positive for HCV-RNA by using RT-PCR technique. Figure 1 showed the electrophoresis fractionation of the amplified RT-PCR products of the 5'untranslated region (UTR) of HCV genome that was located at (262 bp) on a 2% agarose gel stained with Red Safe® stain at 10 volt/cm2 for 1 hour in 1x Tris-Borate-EDTA buffer and visualized under UV. light. Thus, there may be a causal association might exist between HCV and RA in some patients [20].

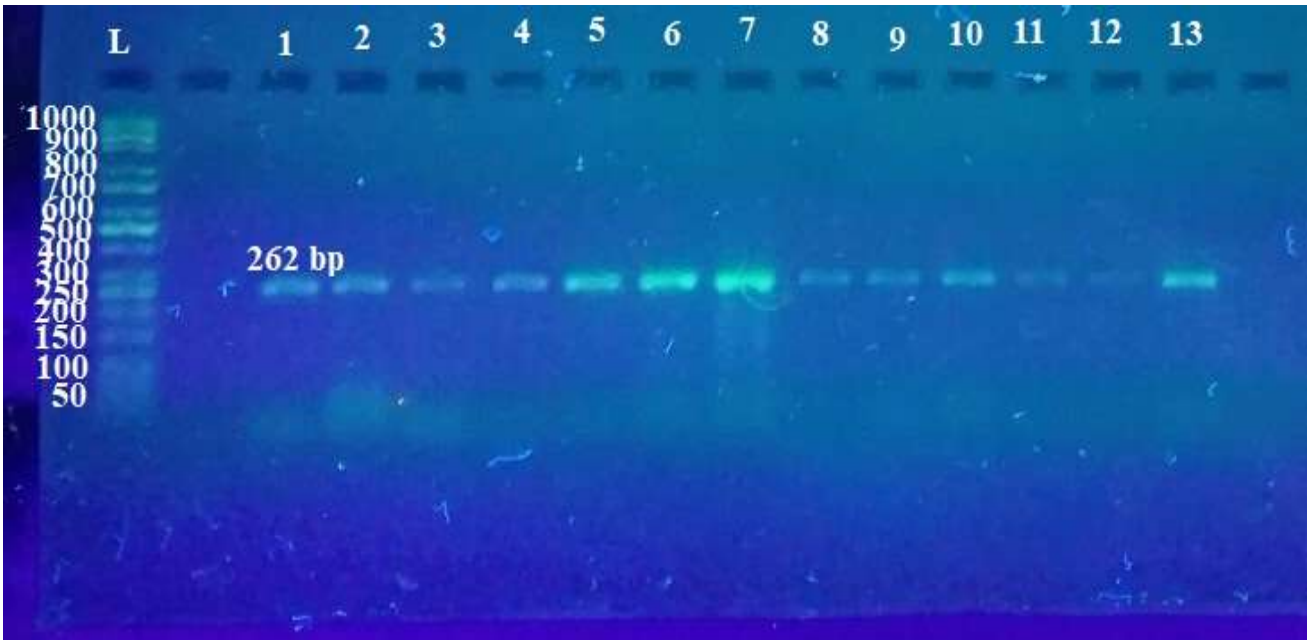

Figure 1: Agarose gel electrophoresis of the PCR product for detecting the 5'UTR of HCV-RNA (the band size is 262 bp) that fractionated on a 2% agarose gel (Promega/ USA) stained with Red Safe® stain (INTRON Biotechnology com./ Korea) at 10 volt/cm² for 1 hour in 1x Tris-Borate-EDTA buffer (Promega/ USA). L: DNA ladder 50-10000bp (Kappa biosystem/ USA), lane 1-13 is the PCR product band (262 bp) that visualized under UV. Light.

Discussion

The results of anti-CCP IgG antibody level in the studied groups showed that there was a significantly increased level ($P < 0.05$) in RA group compared to the healthy control group (31.9 ± 15.2 RU/ml *vs.* 2.3 ± 0.9 RU/ml, respectively). This finding matched the findings of other studies that referred to the significantly increased level ($P < 0.05$) of anti-CCP IgG antibody in RA patients compared to the control group. Therefore, the detection of anti-CCP IgG antibody is important because it is a specific biomarker for RA [21] appears as a result to an epitope response that modified by a hormonally controlled enzyme, peptidyl arginine deiminase that converts arginine to citrulline [22]. Such, the anti-CCP IgG antibody activates the complement system through classical and alternative pathways and leads to a strong indication of their pathophysiologic involvement [23]. The increased synthesis of CCP has been shown to be as a result of apoptosis and inflammation induced cellular death (necroinflammation). This process is depending on inflammation rather than

disease dependent [24]. This contradiction in anti-CCP IgG antibody levels may due to racial and genetic backgrounds [25]. While other authors referred these differences to the presence of different subsets of anti-CCP antibodies directed against different epitopes in citrulline containing molecules [26].

The effect of HLA alleles on the levels of serum anti-CCP IgG antibody in which RA patients with HLA-DRB1 have a higher level than RA patients lacking this epitope [27]. Also, the Results indicate that the serum level of TLR-7 was significantly higher ($P < 0.05$) in RA group compared to control group (103.0 ± 73.8 ng/ml *vs.* 72.9 ± 36.8 ng/ml, respectively).These findings in agreement with the results of a previous study that demonstrated to the high serum level of TLR-7 that expressed in patients with RA [28].

Such, other studies detected that the elevated levels of TLR-7 in the synovium of RA patients were higher than osteoarthritis (OA) patients or healthy controls [11, 29]. Although the mechanisms behind the increased expression levels of TLR-7 have not been clarifying yet; but probably due to the activation of TLR7 by their specific ligand probably single strand RNA (ssRNA) [30, 31] that was demonstrated as an HCV in the current study.

Such, viral infection leads to activate antigen presenting cells (APCs) that are plentiful in the RA synovial compartment, the APCs such as macrophages and DCs have an important role in RA pathogenesis [32]. While Yang *et al*. (2014) [33] reported that the expression level of TLR 3 and 7 was decreased in HCV infections due to the upregulation of microRNA- 758 (miR-758) that inhibit the expression of TLR 3 and 7 in patients with HCV infections. Previous studies demonstrated that TLR7 has a role in inhibiting HCV infection as a part of innate immunity by decreasing the HCV-RNA in HCV infected patients [34].

The decreasing mechanism of TLR7 was by inducing interferon-α production and activating interferon regulatory factors such as INF-ϰB [35]. Also, The findings of HCV IgM and IgG showed a highly OR value (18.0 and 10.0, respectively), these referred that HCV is a strong risk factor triggers RA etiology.

These findings were agreed with previous studies indicated that the prevalence of anti-HCV antibodies in RA patients was 20% higher than the reported prevalence in Egypt (14.7%) [36,39]. The presence of HCV infection in the RA has been well documented, and a genetic background for this association has been postulated. HLA-DR4 is significantly increased in those patients with HCV infection who develop autoimmune diseases [40].

Also, HCV weakens the innate immunity pathways that limit HCV replication such as IFNs and generating a chronic inflammatory state that causes persistent liver injury [41]. In addition, the 5' UTR of the HCV genome is generally used for HCV detection and genotyping assays because it has a highly conserved region contains specific polymorphisms that distinguish the six major genotypes and some subtypes of HCV [42]. Similarly, the detection of HCV genomes by RT-PCR technique was performed by the amplification of a 262 bp of the 5′ UTR domain of the HCV genome by using one-step reverse transcription-PCR [43, 44].

Conclusion

HCV infections and TLR-7 level seem to be related to the pathogenesis of RA.

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