Rheumatol Int (2011) 31:1369–1374
DOI 10.1007/s00296-010-1472-8

ORIGINAL ARTICLE

The investigation of toll-like receptor 3, 9 and 10 gene polymorphisms in Turkish rheumatoid arthritis patients

Ebru Onalan Etem · Halit Elyas · Salih Ozgocmen · Arefe Yıldırım · Ahmet Godekmerdan

Received: 20 July 2009 / Accepted: 27 March 2010 / Published online: 27 April 2010
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Abstract Toll-like receptors (TLRs) play an important role in the induction and regulation of the innate immune system or adaptive immune responses. Genetic variations within human TLRs have been reported to be associated with a range of immune-related diseases. This study was conducted to investigate the frequencies of TLR3 rs3775290, TLR9 rs187084, and TLR10 rs4129009 polymorphisms and to detect between polymorphisms and autoantibody positive as RF, collagen type II, anti-RNP, and anti-CCP in patient group. We performed a case–control study of 100 rheumatoid arthritis (RA) cases and 100 healthy controls matched on age, sex, and residence. All polymorphisms in TLRs were determined by polymerase chain reaction-based restriction fragment length polymorphism. Serum autoantibody level was measured using quantitative ELISA. SNPs were genotyped in all samples. Our results showed that TT genotype for SNP 1237 T/C increased the RA risk significantly ($p < 0.05$). No statistically significant differences were found in the TLR3 and TLR10 genotypes or allele distribution between RA patients and control individuals. No associations were noted with autoantibody production and TLR3, TLR9, and TLR10 polymorphisms genotypes ($p > 0.05$). Our study suggests that a single nucleotide polymorphism (rs187084) in TLR9 gene may be a susceptibility factor for RA in Turkish population. Further studies are required to explore the role of TLRs gene polymorphisms in the risk of RA, especially in ethnically different populations to confirm our results.

Keywords TLRs · Polymorphism · Rheumatoid arthritis · PAMP

E. O. Etem (✉) · H. Elyas
Medical Biology and Genetic Department, Firat University Hospital, Firat University, Elazig, Turkey
e-mail: ebruetem@gmail.com

S. Ozgocmen · A. Yıldırım
Physical Medicine and Rehabilitation, Firat University Hospital, Firat University, Elazig, Turkey

A. Godekmerdan
Immunology, Firat University Hospital, Firat University, Elazig, Turkey

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease mainly characterized by chronic inflammation of the synovial joints and Th1 immune-response. It has been suggested that viruses and bacteria may contribute to initiate or exacerbate RA by binding to Toll-like receptors (TLRs) [1–5]. TLRs recognize a wide variety of pathogen-associated molecular patterns (PAMPs) from bacteria, viruses, and fungi, as well as some host molecules [6–9]. Evidence has shown that TLRs control multiple dendritic cells capable of sensitizing naive T cells and also activate signals that are critically involved in the initiation of adaptive immune responses [10]. Due to their ability to modulate adaptive immunity, TLRs may serve as one of the promising strategic therapeutic targets for diseases related to autoimmune disorders [11]. TLRs are expressed by a variety of immune cells, including B lymphocytes and T lymphocytes, antigen-presenting cells, regulatory T cells, and non-immune cells such as fibroblastic synoviocytes [1–5]. All of these cell populations that are found in the rheumatoid synovium include TLR ligands such as peptidoglycans and double-stranded DNA [12].

TLR9 is mainly expressed by plasmacytoid dendritic cells (pDC), B cells, and neutrophils, and recognizes unmethylated CpG motifs present in bacterial DNA and intracellular viral antigens [12–14]. Activation of TLR9 in pDC induces Th1 cytokines such as interferon-α or interleukin-12 (IL-12), and Th1-biased immune response [15]. TLR9 overexpression plays an important role in the development and perpetuation of RA [16]. Interestingly, TLR9-transduced signaling pathway contributes to the activation of autoreactive, rheumatoid factor-producing B cells [15]. Moreover, experimental data suggest that TLR9 and its ligands participate in the pathogenesis of RA. Intra-articularly injected bacterial DNA containing CpG motifs induces transient arthritis and aggravates collagen-induced arthritis in mice [17, 18]. The data suggest that DNA from dead cells circulating in the peripheral blood is capable of interacting with TLR9 receptors and enriched with sequences possessing potent immunostimulatory properties [19, 29, 30]. Recently, the increased expression of TLR9 in the peripheral blood B cells of RA patients versus healthy donors was reported, suggesting that TLR9 plays a role in the hyperreactivity of RA B lymphocytes [31]. Rudnicka et al. [32] suggested that TLR9 may participate in direct activation and proliferation of B cells in bone marrow, and therefore could play a role in the pathogenesis of RA. Stimulation of TLR9 by ligands trigger signaling pathway common for all TLR that results in NF-kB transcription factor activation and production of proinflammatory cytokines, including TNF-α, IL-1b, and IL-6 [33, 34].

TLR3 plays an important role in antiviral responses and expressions in conventional dendritic cells (DCs), a variety of epithelial cells including airway, genital tract, biliary and intestinal epithelial cells, and in the brain [35]. TLR3 is activated by the dsRNA analog poly-rI:rC as well as by host cell-derived mRNA and dsRNA purified from reovirus [36, 37]. Because no specific artificial or natural ligand is currently known for TLR10, a more complex picture evolves with this receptor. TLR10 is capable of homodimerization but can also form heterodimers with TLR1 and TLR229 [38]. It is predominantly expressed in immune cell-rich tissues, including spleen, lymph node, and lung [39]. TLR10 expression is barely detectable in naive human B cells, but is rapidly induced after B cell receptor triggering [40].

Recent studies on the etiopathogenesis of RA have focused on TLR receptors responsible for innate and adaptive immune system. Genetic variations in TLR have been reported to be associated with a range of inflammatory disorders, including Th1-dominated RA (15). The aim of the present study was to further clarify the role of TLR3 **rs3775290** (1377 T/C), TLR-9 rs187084 (−1237 T/C), TLR10 **rs4129009 (2322 A/G)** polymorphism in the development of RA. This study was conducted to investigate polymorphism frequencies and to detect the association between genotype and autoantibody positive as RF, collagen tip II, anti-RNP, and anti-CCP in patient group.

Materials and methods

Study population

In this study, 100 RA subjects according to the American College of Rheumatology remission criteria (ACR), ranging with varying degrees of disease activity and a control group of 100 healthy subjects matched by age and sex, without any joint diseases were evaluated. Blood samples were obtained with informed written consent. Ethical approval was obtained from the University of Firat Committee on the Ethics of Research on Human. All subjects were living in Elazig Province. Patients were recruited from December 2006 to October 2007 with confirmed diagnosis according to American College of Rheumatology (ACR) criteria.

Genotyping

Genomic DNA was prepared from the peripheral blood using the Wizard Genomic DNA Exraction Kit (Promega, USA), according to the manufacturer's instructions. Genotyping for the all three polymorphisms was performed using PCR-RFLP. Detection of TLR3 polymorphism was performed according to Noguchi et al. [41]. We designed the new primers for detection TLR9 and TLR10 gene polymorphism. PCR reactions were run at 95 for 5 min followed by 35 cycles at 95°C 30 s, at 60°C for 30 s, at 72°C for 30 s, and a final incubation at 72°C for 5 min. Briefly, polymerase chain reactions (30 μl final volume) contained 20 ng of genomic DNA, 20 pmol of each primer (IDT 25 pmol/ml), 3 μl ml of dNTPs (Fermentas 2.5 mM), 3 μl of MgCl2 (Fermentas 25 mM), 1 ml of NH4 buffer (Fermentas 106), and 0.2 μl of Taq polymerase (Fermentas 5 U/ml) using the following primers, all written forward: 5′-ACTATGGAGCCTGCCTGCCATGATACC-3′, reverse: 5′- ATCCAGCCTTCTTACAAACCTCCCACC C-3′ for TLR9 1237 T/C and forward: 5′-CTTACTGG AACCCATTCCATTCTATTGC-3′, reverse: 5′-TCAAT GTACATCCCAACAGTGTATGTGG-3′ for TLR10 rs412 9009 2322A/G. A 15-μl aliquot of the product was digested with the 0.5 U TagI (Fermentas, USA) for TLR3, 0.5 U BspTI (Fermentas, USA) for TLR9, and 0.5 U VspI (Fermentas, USA) for TLR10, at 37°C overnight for TLR9 and TLR10, at 65°C overnight for TLR3 to minimize partial digests. The presence of a wild-type allele (C allele) for TLR9 resulted in an intact 423-bp band, whereas the RFLP profile of the T variant was characterized by two bands of 172 and 251 bp. 309-bp amplicons for TLR10 resulted in

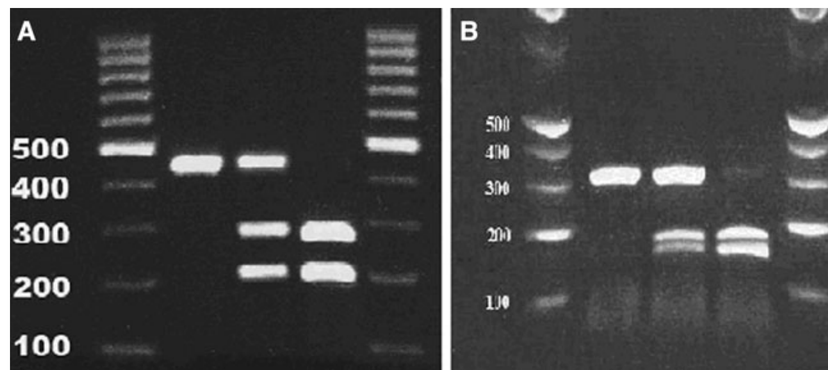

Fig. 1 **a** Genotyping of the TLR9 gene polymorphism by BspTI RFLP: *lanes 1* and *5* 100-bp DNA marker (Fermentas, USA), *lane 2* CC are undigested (423 bp), *lane 3* CT are digested into three fragments (172, 251, and 423 bp), *lane 4* TT are digested into two fragments (172 and 251 bp). **b** Genotyping of the TLR10 gene polymorphism by VspI RFLP: *lanes 1* and *5* 100-bp DNA marker (Fermentas, USA), *lane 2* GG are undigested (309 bp), *lane 3* AG are digested into three fragments (141, 168, and 309 bp), *lane 4* AA are digested (141 and 168 bp)

two fragments of 141 + 168 bp (allele A) or 309 bp (allele G). PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. The results obtained for the genotypes in TLR9 and TLR10 of the Turkish population by RFLP were further validated by sequencing analysis of three randomly selected samples.

Autoantibody measurements

Anti-RNP, anti-CCp, anti-collagen type II autoantibody were assayed using commercially available ELISA kits. The autoantibody detection were performed using REAADS anti-RNP kit (Corgenix, Colorado, USA) for anti RNP, Aeskulısa RA/CP-Detect kit (Aesku, Wendelsheim, Germany) for anti-CCP, collagen Type II, Human IgG, anti-Human BioAssay ELISA kit (USBiological, Massachusetts, USA) for anti-collagen type II.

Statistical analysis

Data are presented as means with 95% confidence intervals (CI). Mann–Whitney *U* tests were used to compare continuous values. Fisher's exact tests were used to determine genotype distribution and dichotomous values. Student's *t* tests, and when necessary Mann–Whitney *U* tests, were used in the comparison of the means between the groups. After the assessment of normality assumption, a *p* value <0.05 was considered to be statistically significant. For all statistical tests, SPSS version 12.0 was used (SPSS Co. LEAD Techn., IL, USA).

Results

We screened in the TLR3, 9, and 10 genes, and examined the possible associations of these polymorphisms with RA in Turkish population. RFLP method is quite appropriate to determine the polymorphisms on the TLR9 and TLR10 genes, and was successfully genotyped in the patient and control groups (Fig. 1a, b). The principal characteristics of patients in our studies are presented in Table 1. The genotypes observed were in Hardy–Weinberg equilibrium. The frequency of the TLR9 −1237 TT genotype carriers was significantly higher in patients with RA than controls ($X^2 = 8{,}698$, $df = 2$, $p = 0{,}013$) (Table 2). A significant difference was also found in the comparison of the patient and control groups in terms of TLR9 −1237 allele frequencies ($X^2 = 9{,}014$, $df = 1$, $p = 0.003$). Genotype frequencies of the patient and control groups are presented in Table 2. The genotype and alleles frequency of the TLR3 and TLR10 were not statistically significant in patients with RA than controls (Table 2). No significant association was observed between personality traits, DAS28, autoantibody levels or positive, and TLR3, 9, and 10 genotypes ($p > 0.05$).

Table 1 Demographic and clinical features of patients with RA

Demographic features	Mean ± SD ($n = 100$)
Age in years (min–max)	51.03 ± 12.4 (24–75)
Sex (female/male)	94/6
Year of RA diagnosis	8.96 ± 7.11
Morning stiffness (min)	0.98 ± 1.15
ESR (mm/h)	29.81 ± 24.20
C-reactive protein (CRP) (mg/L)	17.55 ± 24.96
Hemoglobin (g/dl)	12.42 ± 1.47
Rheumatoid factor (IU/ml)	186.85 ± 195.37
Ritchie articular index (RAI)	16.23 ± 13.74
DAS28	4.04 ± 1.41

Table 2 TLR3 rs3775290 (1377, T/C), TLR-9 rs187084 (−1237 T/C), and TLR10 rs4129009 (2322A/G) genotype distributions in RA and controls

Gene	Patients ($n = 100$)	Controls ($n = 100$)	X^2	p value
TLR3				
TT	11	8	0.56	0.75
TC	43	43		
CC	46	49		
TLR9				
TT	37	20	8.6	0.01
TC	45	49		
CC	18	31		
TLR10				
AA	55	48	1.33	0.51
AG	36	44		
GG	9	8		

Discussion

In the present study, we examined the effects of the TLR3, 9, and 10 polymorphisms on autoantibody positive and clinical features in RA patients. It is the first study to observe the association between TLR3, TLR10, and RA in Turkish population. The major finding of the study was that 1237 T/C polymorphism in TLR9 appears to be associated with the development of RA in TT genotype and increased CRP level in CC genotype.

Lazarus et al. sequenced regions of TLR9 in DNA from three different ethnic groups, African-Americans, Caucasians, and Hispanic Americans, and performed case–control studies of asthma, deep venous thrombosis, and myocardial infarction. They found an increased risk for asthma with the TLR9 −1237 C allele (odds ratio 1.85) in European Americans [20]. The variation −1237 T/C in the promoter region of TLR9 has been shown to be associated with an increased risk for Crohn's disease [21]. Nevertheless, some studies also report no association between this polymorphism and susceptibility to systemic lupus erythematosus [22] and atopy [23]. Ito et al. [24] reported that TLR9 gene polymorphisms were not significantly associated with the susceptibility to Behcet's disease in Japanese patients. Carvalho et al. [25] stated that no significant association with multiple sclerosis (MS) and no protective effect of −1237 T/C concerning age of onset, disease severity or disease subtype in MS patients was found. Jaen et al. [26] reported that no significant association was found between the TLR9 −1237 T/C promoter polymorphisms and increased risk of RA. Our results show statistically significant association between TT genotype and RA diseases.

TLR9 have just recently started to be characterized, and their functional importance elucidated. Among these, the most studied is −1237 T/C polymorphism located within the putative promoter region that may influence transcriptional regulation of the TLR9 gene. Analysis of variant sequences predicted the creation of a potential transcription factor binding site for c-rel/NFkB by the T/C transition implying a potential influence on the transcriptional regulation, which seems to be a good candidate for a functional SNP. Hamann et al. [27] revealed a higher transcriptional activity of the TT allelic variant at −1237 T/C. Novak et al. [28] reported that Luciferase reporter gene assays revealed significantly higher promoter activity of the TT allelic variant at this single nucleotide polymorphism site. Changed expression or co-segregation with other TLR9 gene polymorphism, effected gene over-expression or regulation, of the TT genotype of the gene in RA patients may cause TLR9 to be secreted at higher than normal levels.

The association between genetic variation of TLR3 and human diseases has been suggested. Polymorphisms in the TLR3 gene may be associated with type 1 diabetes, Stevens–Johnson syndrome, and toxic epidermal necrolysis [42, 43]. Korrman et al. [44] reported that TLR10 gene polymorphism was associated with atopic asthma. Stevens et al. [45] suggest that a common haplotype in the TLR10–TLR1–TLR6 gene cluster influences prostate cancer risk. Primary cells derived from carriers of protective TLR10, capable of TLR2 heterodimer, variants showed augmented inflammatory responses, increased TH1 cytokine expression, and reduced TH2-associated IL-4 production after specific stimulation. But, we do not find any association between TLR 3 and TLR10 polymorphism genotypes and RA.

Our study provides the evidence of an association between a TLR9 sequence variant and RA risk in the Turkish population. Although the contribution of the −1237 T/C polymorphism in *TLR9* is modest, these sequence variations, together with polymorphisms of other "minor-effect" genes, may define a genetic susceptibility background for RA. We suggest that there is no evidence for an association of TLR3 and TLR10 genotypes with the clinical parameters of the chronic disease. Because cases and controls were matched in age and gender, the difference between normal control and patients was not likely caused by these factors. In addition, because of the population-based design of the study, these results may not be generalizable to the general population. Genetic polymorphisms often vary between ethnic groups. Furthermore, the sample size of the RA cases was not large enough to detect a small effect from very low penetrance genes or SNPs. If our findings are replicated, it will be valuable to further investigate the pathological role of TLR3, TLR9, and TLR10 polymorphisms in RA etiopathogenesis.

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