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The frequency of toll-like receptor 4 gene polymorphism in ankylosing spondylitis and its relationship between disease activity

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ABSTRACT

Objective. We aimed to evaluate the frequency of toll-like receptor 4 (TLR4) gene polymorphism and its relationship between disease activity in patients with ankylosing spondylitis (AS). ***Methods.*** Forty-one AS patients (25 male/16 female) fullfilling the 1984 Modified New York Criteria and 41 healthy controls (25 male/16 female) were included in this study. Disease activity of the AS patients was assesed by Bath Ankylosing Spondylitis Disease Activity Index (BASDAI). The TLR4 gene polymorphism of AS patients and healthy controls were analyzed by Real-Time Polymerase Chain Reaction (PCR) System. ***Results.*** Three (7.3%) patients with AS had TLR4 gene polymorphism compared with healthy controls (0/41; 0%). Two of these patients had heterozygous mutation and one had homozygous mutation. Significant correlation was not found between TLR4 gene polymorphism and BASDAI score ($p > 0.05$). ***Conclusions.*** In our study, TLR4 gene polymorphism was higher in patients with AS compared with control group. But, this polymorphism was not associated with disease activity, erythrocyte sedimentation rate and C-reactive protein levels.

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Keywords: Ankylosing spondylitis, disease activity, mutation, polymorphism, toll-like receptor 4

Introduction

Ankylosing spondylitis (AS) is an autoimmune, chronic inflammatory disease which is characterized by axial skeletal ankylosis, inflammation at the entheses and arthritis of the peripheral limbs [1]. Although the exact cause of AS remains unclear, the role of genetic susceptibility and epigenetic modifications caused through environmental factors have been respected in the pathogenesis [2]. Toll-like receptors (TLRs) defined as type I integral membrane glycoproteins play important roles in innate immune system [3]. TLR ligands are released from inflammatory cells and activates the TLR signaling pathway. With the activation of this pathway; cytokine, growth factors and anti-apoptotic proteins are expressed. Tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-12, IL-18, etc., and some

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other proinflammatory cytokines are activated with the gene activation [4].

In our study we investigated the frequency of TLR 4 (TLR4) gene polymorphism and its relationship between disease activity in patients with AS.

Methods

The study was carried out at Department of Physical Medicine and Rehabilitation, Çanakkale Onsekiz Mart University. The ethics Committee of the Institution approved the study. Written informed consent was taken from all participants. Fourty-one AS patients who were diagnosed with AS according to the 1984 Modified New York criteria and 41 healthy controls age and sex-matched were included in this study. Patients with AS and healthy controls were not taking any treatment. Healty controls were selected from hospital staff. Exclusion criteria for the study were presence of additional co-morbidities (rheumatologic diseases, malignities, systemic diseases, infections, neuromuscular diseases, etc.).

The all medical records of these patients in our hospital were analysed and later ages, gender, Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score were recorded to the research form. All blood samples were taken for hemogram, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). Hemogram parameters were stored in the tubes in which ethylene diamine tetra acetic acid was used as anticoagulant. ESR (normal range: 0-20 mm/h) was measured by Westergren method [5, 6] (Eventus Vacuplus ESR100, Turkey), CRP (normal range: 0-5 mg/L) was measured by immunoturbidimetry [7] (Prestige 24i CRP Ultra, P.Z. Cormay, Lublin, Poland).

About 10 ml of venous blood were obtained from each subject for TLR4 gene polymorphism and then stored at 4 oC until analysis. For TLR4 gene polymorphism analysis, total cellular RNA was extracted using the High Pure RNA Isolation Kit (Roche, Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from the RNA using the Magna Pure Compact Isolation Kit (Roche, Germany) and Invitek Kit (Roche, Germany) according to the manufacturer's instructions [8]. HLA-B27 was determined by PCR [9] and TLR4 gene was genotyped by Real Time Polimerize Chain Reaction (PCR) using the TaqMan Gene Expression Assays containing the FAM dye-labeled probes (TaqMan Pre-designed Gene Expression Products, Applied Biosystems, Foster City, CA, USA) and Light Cycler Probes Master 480 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) [10]. After PCR analysis A and G alleles of TLR4 were determined using melting point analysis [11].

Statistical Analysis

TLR4 A896G gene polymorphism and the frequency of allele and genotype was tested by descriptive statistics in AS patints and healty control groups. To compare the association between TLR4 gene polymorphism and HLA-B27, Fisher's exact test was used. Kolmogorov-Smirnov test was used to determine whether sample data is normally distributed. The Mann-Whitney nonparametric test was used to determine the difference between ESR and CRP in TLR4 gene polymorphism positive and negative groups. Statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS), version 19.0 (IBM Corp; Armonk, NY, USA) and $p < 0.05$ was considered to be statistically significant.

Results

There were 25 males and 16 females in the both groups. The mean age was 40.24 ± 9.44 years (range; 25-60 years) for AS group and 40.76 ± 8.41 (range; 27-57) for healty controls. There were no significant difference between groups in terms of age, and gender. Three (7.3%) patients with AS had TLR4 gene

Table 1. The frequency of toll-like receptor 4 gene polymorphism

TLR4 gene polymorphism	AS group n (%)	Healty controls n (%)
Positive	3 (7.3)	0 (0)
Negative	38 (92.7)	41 (100)
Total	**41 (100)**	**41 (100)**

TLR4 = toll-like receptor 4, AS = ankylosing spondylitis

Table 2. The association between TLR4 gene polymorphism and HLA-B27

HLA-B27	TLR4 gene polymorphism Negative n (%)	Positive n (%)	*p* value
Positive	31 (93.9)	2 (6.1)	0.488
Negative	7 (87.5)	1 (12.5)	

TLR4 = toll-like receptor 4

Table 3. The association between TLR4 gene polymorphism and disease activity

	TLR 4 gene polymorphism Positive	Negative	*p value*
ESR (mm/h)	31.82 ± 19.69	34.00 ± 32.08	0.860
CRP (mg/L)	1.17 ± 1.10	1.26 ± 1.02	0.817
BASDAI score	3.46 ± 1.87	2.67 ± 1.32	0.477

Data are shown as mean ± standard deviation. BASDAI = Bath ankylosing spondylitis disease activity index, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, TLR4 = toll-like receptor 4

Table 4. The frequency of allele genotype and the TLR4 A896G polymorphisms in AS patients and control group

TLR4 A896G	AS group (n)	Healthy control group (n)
GENOTYPE		
AA	38	41
AG	2	0
GG	1	0
ALLELE		
A	78	82
G	4	0

TLR4 = toll-like receptor 4, AS = ankylosing spondylitis

polymorphism (2 heterozygous and 1 homozygous) (Table 1) and 2 patients with AS had HLA-B27 positivity (Table 2). In healty controls, there was no TLR4 gene polymorphism and 31 of them had HLA-B27 positivity. Significant difference between groups in terms of TLR4 gene polymorphism and HLA-B27 positivity ($p = 0.488$) (see Table 2).

The association between TLR4 gene polymorphism, ESR, CRP and BASDAI score were presented in Table 3. Significant difference was not found between TLR4 gene polymorphism and ESR ($p = 0.860$), CRP ($p = 0.817$) and BASDAI score ($p = 0.477$). The frequency of allele and genotype and the TLR4 A896G polymorphisms in AS patients and controls were presented in Table 4. Melting point analysis was presented in Figures 1.

Discussion

TLR4 gene polymorphism and its relationship between disease activity in patients with several autoimmune diseases has been investigated in previous studies [12-22]. TLR ligands are primarily involved in innate immune responses [12]. With the activation of the innate immune system proinflammatory cytokines (TNF-α, IL-1 , IL-6, IL-12, IL-18, etc) are expressed [4].

Gergely *et al*.'s study [13] included 138 patients with AS and 140 healthy controls for TLR4 Asp299Gly and Thr399Ile polymorphisms. Their study showed no significant differences in allele or genotype frequencies between controls and AS patients. They pointed out that TLR4 signalling pathway seem not to be genetically determined by

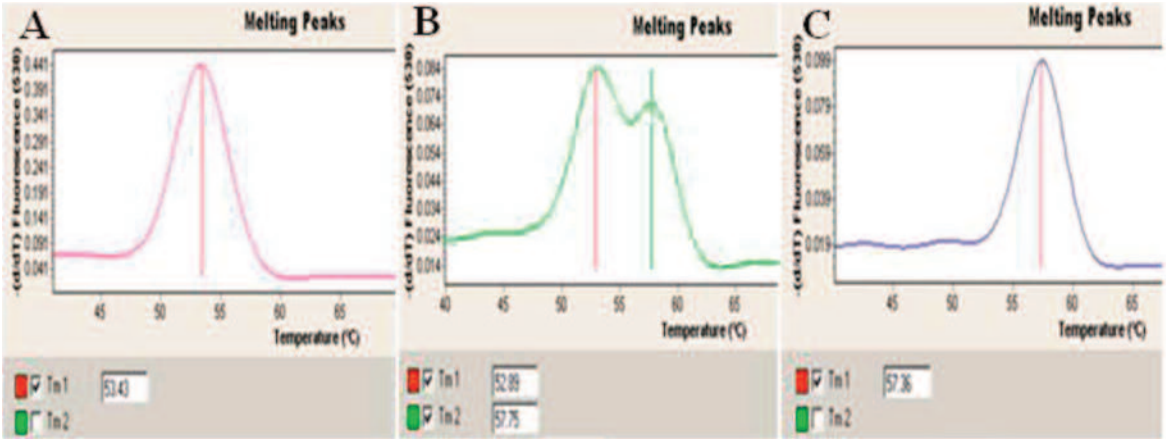

Figure1. Homozygous wild type alleles (A896A) (A); Heterozygous mutated alleles (A896G) (B); Homozygous mutated alleles (G8946G) (C).

these two common polymorphisms. Pointon *et al*. [14] assesed 522 United Kingdom probands and 516 sex-matched controls. No association was found about TLR4 with AS. In a meta-analysis, Xu *et al.* [15] reviewed all of the studies discussing the relationship between TLR4 D299G/T399I and rheumatoid arthritis/AS. Their study suggested that TLR4 D299G/T399I polymorphisms are not associated with rheumatoid arthritis/AS. Na *et al*.'s study [16] included 200 Korean AS patients and 197 healthy controls. All subjects were genotyped for two functional single necleotide polymorphisms (SNPs) in the TLR4 gene: Asp299Gly (A/G) and Thr399Ile (C/T). They indicated that TLR4 gene polymorphisms cannot be regarded as major contributors to AS in Korean population. Adam *et al.* [17] compared the frequency of two TLR4 mutations (Asp299Gly and Thr399Ile) in 193 AS patients and 125 HLA-B27 healthy controls. The results of their study showed that 29/193 (15%) patients with AS had a polymorphism in the Asp299 site compared with 18/125 (14.4%) healthy HLA-B27 controls and 29/184 (15.8%) patients with AS had a polymorphism in the Thr399Ile site compared with 19/113 (16.8%) HLA-B27 controls. No significant difference was found in allele frequency in AS and healthy HLA-B27 controls. Van der Paardt *et al.* [18] investigated the distribution of the CD14C-260T and TLR4 A896G polymorphisms in 113 unrelated white Dutch AS patients and 170 healthy controls. The results of their study showed no significant differences between patients and controls in the frequencies of the TLR4 896G allele.

Yang *et al.* [19] investigated TLR4, TNF-α, IL-12 and soluble tumour necrosis factor-related apoptosis-inducing ligand (sTRAIL) in 60 patients with AS (38 HLA-B27 positive and 22 HLA-B27 negative), 20 patients with rheumatoid arthritis and 30 patients with healthy volunteers. Their results showed that TLR4 levels were significantly higher in AS patients than healthy controls. They reported that TLR4 plays an important role in the pathogenesis of AS, as independent of HLA-B27. De Rycke *et al*. [20] investigated TLR2 and TLR4 gene polymorphisms in 23 patients with spondylarthropathy, 15 patients with rheumatoid arthritis and 18 patients with osteoarthritis. As a result of their study TLR4 expression was increased in patients with spondylarthropathy. They suggested that inflammation in spondylarthropathy is characterized by increased TLR4 expression and is reduced by tumour necrosis factor-α (TNF-α) blockade. Snelgrove *et al*.'s study [21] included 101 AS patients and 100 healthy controls for 2 variants in the TLR4 gene: Asp299Gly (A/G polymorphism) and Thr399Ile (C/T polymorphism). As a result of their study, the frequency for Asp299Gly variant (G) was significantly higher in AS cases compared to controls (7.5% vs 2.6%, respectively; OR: 3.10, $p = 0.037$). The minor allele frequency for the Thr399Ile variant (T) for cases and controls was 7.4% vs 3.0% ($p = 0.071$). They suggested that TLR4 has a minor risk factor for AS.

Assassi *et al*.'s study [22] is the only study about the relationship between TLR4 gene polymorphism and disease activity in AS patients. Their study included 16 patients with AS, 14 matched controls, 74 patients with systemic sclerosis, 21 matched controls and 17 patients with systemic lupus erythematosus. Also these genes were investigated in 27 patients with AS (before and after anti TNF-α treatment) and 27 matched controls. Disease activity of the AS patients

was evaluated with BASDAI score and CRP were measured from all AS patients. As a result of their study over expression of TLR4 and TLR5 was found in AS patients in comparison to controls ($p = 0.012$ and $p = 0.006$, respectively). TLR4 and TLR5 were significantly upregulated among the AS patients before anti TNF-α treatment ($p = 0.007$ and $p = 0.012$, respectively) and were decreased significantly after anti TNF-α treatment ($p = 0.002$ and $p = 0.025$, respectively). CRP levels were correlated with TLR4 and TLR5 levels ($p = 0.015$, $p = 0.001$, respectively). BASDAI scores did not correlate with TLR4 and TLR5 levels. Their study supported the importance of TLR subtypes in the pathogenesis of AS.

As a result in some studies no significant differences was found in TLR4 gene polymorphism between controls and AS patients [12-17]. But we confirmed the overexpression of TLR4 gene polymorphism among patients with AS, similar to some previous studies [18-21]. This can be due to the activation of this TLR4 cytokine pathway, TNF-α and other proinflammatory cytokines are expressed. These proinflammatory cytokines are expressed also in AS [4]. We didn't find any association between TLR4 gene polymorphism and ESR, CRP levels and BASDAI score. In Assassi *et al*.'s study [22], CRP levels were correlated with TLR4 and TLR5 levels but BASDAI scores did not correlate with TLR4 and TLR5 levels. Our study differs in this respect.

The Limitations of the Study

The limitations of our study were that we evaluated the frequency of TLR4 gene polymorphism and its relationship between TLR4 gene polymorphism and disease activity in a small number of patients. As a result of this limitation more studies with more patients are warranted to elucidate the frequency of TLR4 gene polymorphism in AS and its relationship between disease activity.

Conclusions

Our study revealed that TLR4 pathway is important in AS. We confirmed the overexpression of TLR4 gene among patients with AS. But, this polymorphism was not associated with disease activity, ESR, CRP levels.

Authorship declaration

All authors listed meet the authorship criteria according to the latest guidelines of the International Committee of Medical Journal Editors, and all authors are in agreement with the manuscript.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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