Promoter Polymorphism (T-1486C) of TLR-9 Gene Is Associated With Knee Osteoarthritis in a Turkish Population

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ABSTRACT: In this study, we aimed to determine whether *TLR-9* T-1486C SNP was associated with susceptibility to OA in the Turkish population. The study group comprised 272 patients with Grade 2-3-4 knee OA according to the Kellgren-Lawrence scoring system and the control group was formed of 296 individuals with Grade 0–1. The *TLR-9* genotype were assessed by real-time polymerase chain reaction. An analysis of *TLR-9* promoter −1486T/C polymorphism revealed that the −1486CC genotype appeared to have a higher risk for OA and −1486TT and −1486CT genotypes have a protective effect against the development of OA (crude OR = 0.473, 95% CI = 0.297–0.754, $p = 0.002$, adjusted OR = 0.531, 95% CI = 0.326–0.864, $p = 0.011$). This study indicate that there is a correlation of *TLR-9* T-1486C gene polymorphism with advanced knee OA in a Turkish population. Changed in *TLR* expression due to different allelles may cause osteoarthrith development outcome cartilage degeneration. © 2017 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 35:2484–2489, 2017.

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Osteoarthritis (OA) is the most common cause of arthritis, which often causes pain, locking and loss of function in the joints. It is the most significant cause of disability in the elderly. It leads to tissue changes in several joints and the knee, hip, and hand joints are usually affected. There is a significant increase in the prevalence of OA between the ages of 40–50 years, especially in females and this increases in a linear manner up to the age of 70 years.[1,2]

Although the development of knee OA is not fully understood, there are many risk factors such as genetics, bone morphology, age, female gender, obesity and knee injury at the beginning of the disease and in its progression.[1]

In the clarification of the pathogenesis of OA, studies conducted on families and twins to research genetic disorders have reported that genetic factors play an important role in the etiology. These genetic studies have stated that OA is a complex disease and many different genetic risk variants together with other factors contribute to the impact.[3,4]

Recently, studies have been conducted on members of the toll-like receptor (*TLR*) family which play an significant role in both innate and adaptive immune response. Studies have identified polymorphism and mutation in the *TLR* gene in diseases such as atherosclerosis, ischemic stroke, sepsis, asthma, cancer, Crohn's disease, acute reactive arthritis, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA).[5,6]

*TLR*s are expressed in important immune system cells such as primarily macrophage and dendritic cells and are key sensors of pathogen invasion.[7] To date 10 *TLR* family members (*TLR*1-10) have been identified in humans and 12 in mice.[8] *TLR* 2 which is formed with heterodimer *TLR* 1 or *TLR* 2, recognizes various bacterial components of gram positive bacteria and microplasma containing peptidoglycan, lipopeptide, and lipoproteins. *TLR* 3 ligand is a double-strand RNA (dsRNA) molecule which is produced by viruses replicating. *TLR* 4 ligand is determined from lipopolysaccharide (LPS) found in gram negative bacteria and *TLR*5 from bacterial flagelli. *TLR* 7 recognizes synthetic imidazoquinoline-like molecules, guanozin analogs (loxoribine, type I human immunodeficiency virus, vesicular stomatitis virus, and influenza viruses originating from ssRNA) and some siRNAs. *TLR* 8 is not functional in mice but is thought to recognize imidazoquinolines and ssRNA. *TLR-9* ligands are DNA containing bacterial and viral CpG.[9] Non-infectious molecules such as cellular fibronectin, heat shock proteins, and endogenous peptides produced in response to tissue damage are recognised by *TLR*s.[10,11] *TLR*2 and *TLR*4 are expressed by articular chondrocytes and expressions have been reported to increase in OA lesions.[12] In a study by Sui-Lung Su et al., *TLR-9* gene polymorphism was determined to have a significant role in knee OA in a Chinese population.[13]

The role of genetic factors is important in the determination of the pathogenesis and etiology of OA. To the best of our knowledge, there has been only one reported study of an association of OA with *TLR* polymorphisms. Therefore, the aim of this case-control study was to determine whether *TLR-9* T-1486C SNP was associated with susceptibility to OA in the Turkish population.

SUBJECTS AND METHODS

This is a level 3 case control study. The study included patients with knee OA according to the diagnostic criteria of the American College of Rheumatology [ACR] (1986).[14] The

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study group comprised 272 patients with Grade 2-3-4 knee OA according to the Kellgren–Lawrence scoring system and the control group was formed of 296 individuals withGrade 0–1.[15] Exclusion criteria were inflammatory arthritis, post-traumatic or post-septic arthritis, skeletal or developmental dysplasia, long-term hormonal therapy, and BMI > 26.

This study was approved by Bozok University medical faculty Ethics Review Board (24.03.2015 no: 24/15). Written informed consent was obtained from each participant. This research complied with the principles of the Declaration of Helsinki.

Kellgren–Lawrence grading scale:

Grade 1: Doubtful narrowing of joint space and possible osteophytic lipping.

Grade 2: Definite osteophytes, definite narrowing of joint space.

Grade 3: Moderate multiple osteophytes, definite narrowing of joints space, some sclerosis, and possible deformity of bone contour.

Grade 4: Large osteophytes, marked narrowing of joint space, severe sclerosis, and definite deformity of bone contour.

Genotyping

Peripheral blood samples were collected from the subjects. Genomic DNA was extracted from all samples of 200 μl whole blood using a commercial kit (High Pure PCR Template Preparation Kit, Roche Diagnostics, Germany). Genotyping of Toll-like receptor 9 single-nucleotide polymorphisms (SNP) rs187084 was performed by real-time polymerase chain reaction with LightSNiP reagents (coupled primer and probe) and FastStart DNA Master HybProbe (Roche Diagnostic, GmBH, Mannheim, Germany) in LightCycler 2.0. The cycling conditions for SNPs was: 10 min at 95°C for activation, followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. For the amplicon identification, the following conditions were used: 30s at 95°C, 2 min at 40°C, and 0 s at 75°C for one cycle. To improve the accuracy of the genotyping methods, positive control (heterozygote genotype) and negative control (double distilled water) were used in each experiment.

Statistical Analysis

While, continuous variables were expressed as mean ± SD, otherwise, number of cases and percentages were used for categorical data. Genotype distribution of polymorphism was calculated for deviation from the Hardy–Weinberg equilibrium (HWE) by using Chi-square (χ^2) tests (one degree of freedom).

While, the mean differences in age between groups were compared by Student's t test, otherwise, One-way ANOVA was applied for comparison among more than two independent group. When the p value from One-Way ANOVA is statistically significant the post hoc Tukey HSD test was used to know which group differ from which others. Categorical data were analyzed by Pearson's Chi-square or Fisher's exact test, where appropriate.

While, the effects of genotypes and allele frequency on the existence knee osteoarthritis were evaluated by Logistic regression analyses, otherwise, Multinominal Logistic regression analyses were applied for determining the effects of genotypes and allele frequency on the severity of knee osteoarthritis. The odds ratios and 95% confidence intervals which were crude and also after adjustment for age and gender were calculated.

Data analysis was performed by using IBM SPSS Statistics version 17.0 software (IBM Corporation, Armonk, NY). A p value less than 0.05 was considered as statistically significant.

RESULTS

The descriptive characteristics and clinical data of the study participants are summarized in Table 1.

The distribution of both allele and genotype frequencies of the case and control groups are shown in Table 2. Association analysis revealed that T-1486C was associated with OA. In the OA group, the frequencies of the TT, TC, and CC genotypes at the −1486T/C SNP were 34.9%, 44.1%, and 21.0%, respectively. The wild-type TT genotype was detected in 35.5% and the heterozygous TC genotype in 53.4%, while the homozygous CC genotype was detected in 11.1% of healthy individuals (Table 2). When the *TLR*-9 −1486TT genotype was used as the reference group, the −1486CC genotype appeared to have a higher risk for OA (crude OR = 1.909, 95% CI = 1.46–3.181, $p = 0.013$). However, after adjustment for age and gender no significant effect was observed at −1486CC genotype risk for OA (adjusted OR = 1.697, 95% CI = 0.992–2.904, $p = 0.053$) Table 2.

TLR-9 −1486TT and −1486CT genotypes have a protective effect against the development of OA for both crude and adjusted OR results whereas the −1486CC genotype may increase susceptibility to this disease (crude OR = 0.473, 95% CI = 0.297–0.754, $p = 0.002$, adjusted OR = 0.531, 95% CI = 0.326–0.864, $p = 0.011$) Table 2. The demographic features of OA case groups among grades are shown in Table 3. There were no differences in the genotypes and allele frequencies in Grade 2 OA case group and control groups ($p > 0.05$) (Table 4).

When the *TLR*-9 −1486TT genotype was used as the reference group, the −1486CC genotype appeared to have a higher risk for both crude and adjusted OR results for Grade 3 OA (crude OR = 2.758, 95% CI = 1.433–5.308, $p = 0.002$ adjusted OR = 3.217, 95%

Table 1. Demographic Characteristics of Control and Case Groups

Groups	Control (n = 296)	Case (n = 272)	p-Value
Age (year)	54.3 ± 7.4	57.0 ± 9.0	<0.001[a]
Gender			<0.001[b]
Female	178 (60.1%)	237 (87.1%)	
Male	118 (39.9%)	35 (12.9%)	
Knee grade			–
0	161 (54.4%)	–	
1	135 (45.6%)	–	
2	–	149 (54.8%)	
3	–	98 (36.0%)	
4	–	25 (9.2%)	

[a]Student's t test. [b]Pearson's Chi-square test.

Table 2. Distribution of Genotype and Allele Frequencies Acording to Control and Case Groups

	Control ($n = 296$) (%)	Case ($n = 272$) (%)	Crude OR (95%CI)	p-Value	Adjusted OR (95%CI)[a]	p-Value
Genotype						
TT	105 (35.5)	95 (34.9)	1.000 (reference)	–	1.000 (reference)	–
TC	158 (53.4)	120 (44.1)	0.839 (0.583–1.209)	0.348	0.835 (0.566–1.230)	0.361
CC	33 (11.1)	57 (21.0)	1.909 (1.146–3.181)	**0.013**	1.697 (0.992–2.904)	0.053
Genotype						
TT	105 (35.5)	95 (34.9)	1.000 (reference)	–	1.000 (reference)	–
CC + TC	191 (64.5)	177 (65.1)	1.024 (0.726–1.446)	0.892	0.995 (0.690–1.435)	0.979
Genotype						
CC	33 (11.1)	57 (21.0)	1.000 (reference)	–	1.000 (reference)	–
TT + TC	263 (88.9)	215 (79.0)	0.473 (0.297–0.754)	**0.002**	0.531 (0.326–0.864)	**0.011**
Allele						
T	368 (62.2)	310 (57.0)	1.000 (reference)	–		
C	224 (37.8)	234 (43.0)	1.240 (0.978–1.572)	0.076		

OR, odds ratio; CI, confidence interval. [a]After adjustment for age and gender.

CI = 1.550–6.676, $p = 0.002$) (Table 5). *TLR*-9 −1486TT and −1486CT genotypes have a protective effect against the development of Grade 3 OA for both crude and adjusted OR results whereas the −1486CC genotype may increase susceptibility to this disease (crude OR = 0.347 95% CI = 0.195–0.618, $p < 0.001$, adjusted OR = 0.289, 95% CI = 0.152–0.550, $p < 0.001$) and C allele frequency has significantly higher risk for Grade 3 OA (crude OR = 1.514 95% CI = 1.093–2.097, $p = 0.013$) (Table 5).

When the *TLR*-9 −1486TT genotype was used as the reference group, the −1486CC genotype appeared to have a higher risk for Grade 4 OA (adjusted OR = 4.383, 95% CI = 1.152–16.670, $p = 0.030$) (Table 6). *TLR*-9 −1486TT and −1486CT genotypes have a protective effect against the development of Grade 4 OA for both crude and adjusted OR results whereas the −1486CC genotype may increase susceptibility to this disease (crude OR = 0.323 95% CI = 0.125–0.830, $p = 0.019$, adjusted OR = 0.124, 95% CI = 0.035–0.434, $p < 0.001$) (Table 6).

DISCUSSION

In this study, the relationship was investigated between OA and (SNP) rs187084 Toll-like receptor 9 single-nucleotide polymorphisms in the Turkish population. The results demonstrated that the −1486CC genotype seemed to have a higher risk for Grade 3 and 4 OA and *TLR*-9 −1486TT and −1486CT genotypes have a protective effect against the development of Grade 3 and 4 OA. C allele frequency was determined to have a significantly higher risk for Grade 3 OA.

Although it is inevitable that OA affects joint cartilage, it includes all the tissues of the joint. Loss of joint cartilage is accompanied by remodelling of the subchondral bone with sclerosis, osteophyte formation in the joint margins, cyst development, relaxation and contracture in the ligaments, muscular atrophy, spasm, and inflammation of the synovial membrane. Cartilage repair is not sufficient because of the normal anabolic and catabolic imbalance within the cartilage. This destructive imbalance is created by inflammatory cytokines.[16,17] The pro-inflammatory cytokines, IL-1 and TNF-a are produced by inflammatory cells, articular chondrocytes, or activated synoviocytes and have a key role in the onset and progression of OA.[18]

There have been recent studies which have shown that *TLR*s in both chondrocytes and pioneer cells and *TLR* ligands constitute a potentially significant group of local chondrocyte stimulators of cartilage and at the same time, OA could be an autoinflammatory disease created in the chondrocytes as a primary inflammatory cell.[19,20] *TLR*s bind to various damage-associated molecular patterns (DAMP) which are endogenous danger signals. Just as this causes the formation of autoinflammatory conditions, it also causes the production of co-stimulatory signals which are necessary for adaptive immune reactions.[21]

Table 3. The Demographic Characteristics of the Patients Releted to Osteoarthritis Grade

	Control ($n = 296$)	Grade 2 ($n = 149$)	Grade 3 ($n = 98$)	Grade 4 ($n = 25$)	p-Value
Age (year)	$54.3 \pm 7.4^{a,b,c}$	$51.9 \pm 6.5^{a,d,e}$	$61.2 \pm 7.3^{b,d,f}$	$70.4 \pm 5.6^{c,e,f}$	$<0.001^{\dagger}$
Gender					$<0.001^{\ddagger}$
Female	178 (60.1%)[a,b,c]	130 (87.2%)[a]	85 (86.7%)[b]	22 (88.0%)[c]	
Male	118 (39.9%)[a,b,c]	19 (12.8%)[a]	13 (13.3%)[b]	3 (12.0%)[c]	

a, control versus grade 2 ($p < 0.01$); b, control versus grade 3 ($p < 0.001$); c, vontrol versus grade 4 ($p < 0.01$); d, grade 2 versus grade 3 ($p < 0.001$); e, grade 2 versus grade 4 ($p < 0.001$); f, grade 3 versus grade 4 ($p < 0.001$). [†]One-way ANOVA. [‡]Pearson's Chi-square test.

Table 4. An Examination of the Effect of Genotype and Allele Frequency in Respect of Developing Grade 2 Osteoarthritis

	Control ($n = 296$) (%)	Grade 2 ($n = 149$) (%)	Crude OR (95%CI)	p-Value	Adjusted OR (95% CI)[a]	p-Value
Genotype						
TT	105 (35.5)	53 (35.6)	1.000 (reference)	–	1.000 (reference)	–
TC	158 (53.4)	72 (48.3)	0.903 (0.586–1.391)	0.643	0.954 (0.605–1.504)	0.838
CC	33 (11.1)	24 (16.1)	1.441 (0.774–2.681)	0.249	1.184 (0.620–2.258)	0.609
Genotype						
TT	105 (35.5)	53 (35.6)	1.000 (reference)	–	1.000 (reference)	–
CC + TC	191 (64.5)	96 (64.4)	0.996 (0.660–1.502)	0.984	0.993 (0.644–1.531)	0.975
Genotype						
CC	33 (11.1)	24 (16.1)	1.000 (reference)	–	1.000 (reference)	–
TT + TC	263 (88.9)	125 (83.9)	0.654 (0.371–1.152)	0.142	0.821 (0.455–1.480)	0.511
Allele						
T	368 (62.2)	178 (59.7)	1.000 (reference)	–		
C	224 (37.8)	120 (40.3)	1.108 (0.833–1.473)	0.482		

OR, odds ratio; CI, confidence interval. [a]After adjustment for age and gender.

Potential ligands *TLR* have been identified as chondrocyte-derived heat-shock proteins, high-mobility group box 1, and several components of the cartilage extracellular matrix (ECM)—for example, low-molecular-weight hyaluronan, heparan sulphate, and fibronectin fragments.[22,23] It has been suggested that *TLR*s have a role in the exacerbation of the inflammatory response and arthritic joint destruction because stimulation of *TLR-9* by ligands trigger a common signaling pathway for all *TLR*, which results in the activation of NF-kB transcription factor and the production of proinflammatory cytokines, including TNF-a, IL-1b, and IL-6.[24–27] In a recent study, *TLR*1, *TLR*2, *TLR*4, and *TLR-9* were observed to increase with the progression of OA. It was reported that *TLR*1 and *TLR*2 are expressed during chondrogenesis, in mature primary chondrocytes, and during the course of OA, in degenerated chondrocytes.[20]

This study provides the first demonstration that the −1486T/C genotype might be an important marker in Turkish osteoarthritic patients compared to a healthy control group. There are few studies in literature related to how −1486T/C polymorphism has a functional effect on the formation of OA or its progression. Problems have been experienced in the definition of factors affecting the pathogenesis of cartilage degradation in articular chondrocytes in several forms of arthritis. It has been shown that there is *TLR*2 expression in normal articular chondrocytes.[28] In an experimental model by Kim et al., ligands for *TLR*2, 3, 4, and 9 were shown to have stimulated arthritis.[29] *TLR* ligands have an important role in the catabolic stimulation of articular chondrocytes and the upregulation of cartilagization by *TLR*2 and *TLR*4 has been shown in advanced OA patients.[30] However, there are few studies related to the expression of *TLR*s in

Table 5. An Examination of the Effect of Genotype and Allele Frequency in Respect of Developing Grade 3 Osteoarthritis

	Control ($n = 296$) (%)	Grade 3 ($n = 98$) (%)	Crude OR (95%CI)	p-Value	Adjusted OR (95%CI)[a]	p-Value
Genotype						
TT	105 (35.5)	30 (30.6)	1.000 (reference)	–	1.000 (reference)	–
TC	158 (53.4)	42 (42.9)	0.930 (0.548–1.580)	0.789	0.887 (0.495–1.591)	0.688
CC	33 (11.1)	26 (26.5)	2.758 (1.433–5.308)	**0.002**	3.217 (1.550–6.676)	**0.002**
Genotype						
TT	105 (35.5)	30 (30.6)	1.000 (reference)	–	1.000 (reference)	–
CC + TC	191 (64.5)	68 (69.4)	1.246 (0.762–2.036)	0.380	1.265 (0.737–2.171)	0.394
Genotype						
CC	33 (11.1)	26 (26.5)	1.000 (reference)	–	1.000 (reference)	–
TT + TC	263 (88.9)	72 (73.5)	0.347 (0.195–0.618)	**<0.001**	0.289 (0.152–0.550)	**<0.001**
Allele						
T	368 (62.2)	102 (52.0)	1.000 (reference)	–		
C	224 (37.8)	94 (48.0)	1.514 (1.093–2.097)	**0.013**		

OR, odds ratio; CI, confidence interval, [a]After adjustment for age and gender.

Table 6. An Examination of the Effect of Genotype and Allele Frequency in Respect of Developing Grade 4 Osteoarthritis

	Control ($n = 296$) (%)	Grade 4 ($n = 25$) (%)	Crude OR (95%CI)	p-Value	Adjusted OR (95%CI)[a]	p-Value
Genotype						
TT	105 (35.5)	12 (48.0)	1.000 (reference)	–	1.000 (reference)	–
TC	158 (53.4%)	6 (24.0)	0.332 (0.121–0.913)	**0.033**	0.248 (0.068–0.899)	**0.034**
CC	33 (11.1)	7 (28.0)	1.856 (0.675–5.101)	0.230	4.383 (1.152–16.670)	**0.030**
Genotype						
TT	105 (35.5)	12 (48.0)	1.000 (reference)	–	1.000 (reference)	–
CC + TC	191 (64.5)	13 (52.0)	0.596 (0.262–1.352)	0.215	0.655 (0.235–1.825)	0.418
Genotype						
CC	33 (11.1)	7 (28.0)	1.000 (reference)	–	1.000 (reference)	–
TT + TC	263 (88.9)	18 (72.0)	0.323 (0.125–0.830)	**0.019**	0.124 (0.035–0.434)	<**0.001**
Allele						
T	368 (62.2)	30 (60.0)	1.000 (reference)	–		
C	224 (37.8)	20 (40.0)	1.095 (0.607–1.975)	0.762		

OR, odds ratio; CI, confidence interval. [a]After adjustment for age and gender.

chondrocytes, some of which are debatable and the role of *TLR-9* in cartilage has not yet been clarified. We studied the −1486T/C SNPs, which is located within the putative promoter region of *TLR-9* gene. An analysis of *TLR-9* promoter −1486T/C polymorphism revealed that the −1486CC genotype appeared to have a higher risk for OA and −1486TT and −1486CT genotypes have a protective effect against the development of OA (crude OR = 0.473, 95% CI = 0.297–0.754, $p = 0.002$, adjusted OR = 0.531, 95% CI = 0.326–0.864, $p = 0.011$). While Grade 3 OA was seen at a statistically significant level in individuals carrying C allele, there was also an increased risk of OA in individuals carrying C allele at Grade 4 OA, although not at a significant level.

Although there is no available data for *TLR-9* −1486T/C polymorphism, there is a strong possibility that it changes the function of the promoter. In literature there is only one report of this polymorphism studied in OA patients. In contrast to the current study, in a study of a Chinese population, the −1486TT genotype seemed to have a higher risk for OA.[13] There could be many reasons for these regional differences. Different populations have a different genetic background, which can cause ethnic heterogenity. The size of the samples in the studies could also have affected the results.

In recent years, *TLR-9* gene polymorphism has been associated with several diseases such as rheumatoid arthritis,[31] systemic lupus erythematosus,[32] symptomatic malaria,[33] Graves' ophthalmopathy,[34] and cervical cancer.[35] Holla et al. reported that there may be a correlation of *TLR-9* haplotypes with a susceptibility to chronic periodontitis (CP).[36] Paradowska et al. suggested that the *TLR-9* −1486T/C and 2848C/T polymorphisms may be a genetic risk factor in the development of human cytomegalovirus (HCMV) disease.[37] However, studies of Spanish[38] and French[39] populations with RA have demonstrated no evidence of any major effects of *TLR*-1, 2, 4, 6, 9, gene polymorphisms. In the Turkish population, *TLR*-9 T-1237C SNP, but not *TLR*-3 and genotypes, have been associated with RA risk.[6]

Genetic studies related to OA have increased greatly in recent years and these studies are important in respect of understanding the etiology of the disease and finding early resolutions. In conclusion, from the results of this study it can be considered that there is a correlation of *TLR*-9 T-1486C gene polymorphism with advanced knee OA in a Turkish population. Our finding indicate that changed in *TLR* expression due to different allelles may cause osteoarthrith development outcome cartilage degeneration.

AUTHORS' CONTRIBUTIONS

Conception and design, acquisition of data, or analysis and interpretation of data: Ozlem Balbaloglu, Seda Sabah Özcan. Drafting the article or revising it critically for important intellectual content: Ozlem Balbaloglu, Seda Sabah Özcan, Murat Korkmaz, Neziha Yılmaz. Final approval of the version to be published: Ozlem Balbaloglu, Seda Sabah Özcan, Murat Korkmaz, Neziha Yilmaz. All authors have read and approved the final submitted manuscript.

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