Association of Toll-like Receptor 4 Variants and Ankylosing Spondylitis: A Case-Control Study

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ABSTRACT. ***Objective.*** Functional single nucleotide polymorphisms within the ectoplasmic domain of the Toll-like receptor 4 (TLR4) gene have been shown to result in an endotoxin-hyporesponsive phenotype and aberrant signal transduction for bacterial agonists. TLR4 is in proximity to a genome-wide linkage peak in 9q32-33. Given the proposed function and location of TLR4, we examined the association of 2 functional variants of TLR4 in patients with ankylosing spondylitis (AS) in Newfoundland.

Methods. In total, 101 AS patients and 100 ethnically matched controls were genotyped, using the Sequenom MassArray platform, for 2 functional variants in the TLR4 gene: Asp299Gly (A/G polymorphism) and Thr399Ile (C/T polymorphism).

Results. The minor allele frequency for the 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% (OR 2.59, p = 0.071). Haplotype analysis using Haploview noted a higher proportion of GT in the cases (for GT, chi-squared p = 0.023).

Conclusion. Given the functional role of TLR4 variants in the innate immune system, larger studies are now warranted to elucidate the association of TLR4 variants in AS. (First Release Dec 1 2006; J Rheumatol 2007;34:368–70)

Key Indexing Terms:
ANKYLOSING SPONDYLITIS ARTHRITIS FOUNDER POPULATION
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Ankylosing spondylitis (AS) is an inflammatory joint disease predominantly affecting the axial spine. Although the etiology of AS is likely multifactorial, there is a substantive role for genetic factors in AS susceptibility and expression. Twin studies in AS estimate the heritability to be over 90%, while the sibling risk (λs) for AS has been reported to be as high as 82[1]. Strong association of AS and HLA-B27 has been consistently demonstrated in multiple ethnic AS populations for over 3 decades[2]. Despite the universality of this association, it is estimated that the presence of the HLA-B27 allele, at most, accounts for one-third of the entire genetic contribution of AS[1,2]. Thus, non-HLA genes are being actively investigated, with recent associations noted within the IL1 gene cluster, CYP450, and the ANKH gene[3-5].

We investigated the Toll-like receptor 4 (TLR4) gene as a possible candidate gene in AS. TLR4 variants were selected based on their proposed function, their association with a related phenotype (Crohn's disease, CD), and their location within the genome. TLR mediate intracellular signaling and recognize a wide spectrum of exogenous and endogenous agonists. TLR4 is the most extensively studied and versatile TLR, as functional single nucleotide polymorphisms (SNP) within the ectoplasmic domain of TLR4 gene have resulted in an endotoxin-hyporesponsive phenotype[6]. This is associated with aberrant signal transduction for bacterial agonists. Second, TLR4 variants have been associated with CD in multiple independent populations[7-9]. This is relevant in AS, since bowel inflammation often coexists in AS. Finally, TLR4 is in proximity to a linkage peak in 9q32-33 that has been noted in a previous linkage study in AS[10]. Thus we examined the association of 2 functional variants of TLR4 gene in the AS population of Newfoundland.

MATERIALS AND METHODS

Clinical data. This study was approved by the ethics committee of Memorial University. Informed consent was obtained from all patients. All AS probands were Caucasians and satisfied the Modified New York criteria for the classification of AS[11]. Information was collected systematically and included standardized measures of disease activity and function for AS [Bath AS Disease Activity Index (BASDAI) and Bath AS Functional Index (BASFI)]. Control

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subjects were healthy volunteers from Newfoundland who participated after a local campaign seeking controls for genetic studies.

Laboratory data. Whole-blood samples were obtained from AS probands and controls. DNA was extracted using the Promega Wizard Genomic DNA purification kit. The TLR4 polymorphisms Asp299Gly (A/G polymorphism) and Thr399Ile (C/T polymorphism) were typed using the Sequenom chip-based MALDI-TOF mass spectrometry platform (Sequenom, San Diego, CA, USA). In brief, polymerase chain reaction (PCR) and extension reactions were designed using MassArray software (Sequenom). Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). The PCR primers were used to amplify 2.5 ng of genomic DNA using standard conditions for MassArray genotyping. Unincorporated nucleotides in the PCR product were deactivated using shrimp alkaline phosphatase. The amplification of the SNP site was carried out using the MassExtend primer and involved the use of specific d/ddNTP termination mixes, which were also determined using MassArray assay design software. The primer extension products were then cleaned and spotted onto a SpectroChip. The chip was scanned using a mass spectrometry workstation (Bruker) and the resulting spectra were analyzed using the Sequenom SpectroTyper-RT software.

Statistical methods. A simple chi-square test for departure from the Hardy-Weinberg equilibrium was performed for both TLR4 SNP among the controls. Single-marker case-control differences were evaluated for both TLR4 variants using Fisher's exact test, and odds ratios for the 2 markers in the contingency tables were calculated. Haplotype analysis was done using Haploview; this program takes one haplotype at a time and compares its frequency between cases and controls. QTL regression analyses with respect to genotypes and haplotypes were then performed among AS cases to assess genotype/haplotype associations with age of onset, BASDAI, and BASFI.

RESULTS

One hundred one AS probands and 100 ethnically matched controls were assessed. The mean age of patients with AS was 45.6 years (SD 12.4) and mean age at diagnosis was 25.0 years (SD 11.8). Seventy percent of AS patients were male and 87% were HLA-B27-positive. The mean age of controls was 44.0 years (SD 13.7), and 39% of controls were men. The mean BASDAI score for AS patients was 5.5 (SD 2.0) and the mean BASFI was 5.4 (SD 2.3).

Both control genotypes satisfied Hardy-Weinberg equilibrium. The minor allele frequency for the Asp299Gly variant (G) was 7.5% as compared to 2.5% for the controls (Fisher's exact p = 0.037, OR 3.10, 95% CI 1.10, 8.69). The minor allele frequency for the Thr399Ile variant (T) was 7.4% in the cases and 3.0% in controls (Fisher's exact p = 0.071, OR 2.59, 95% CI 0.99, 6.83). The GT haplotype was noted in 7.5% of the cases compared to 2.5% of controls. This difference was statistically significant when the haplotypes were analyzed using Haploview (chi-square p = 0.023). Genotypes of TLR4 variants in AS cases and controls are shown in Table 1. With respect to the age of onset, BASDAI, and BASFI, regression analysis among AS cases showed no statistical association with the genotype/haplotype indicator variables (all $p > 0.10$). With respect to the power of the study, assuming 100 cases and 100 controls and a minor allele frequency of 0.0255, there was 81% power to detect an odds ratio of 4.0. As the odds ratio in our study was close to 3.0, the magnitude of power was only about 57%. Thus we acknowledge that the study is underpowered and requires validation.

Table 1. Genotypes of TLR4 variants in AS cases and controls.

TLR4 Variants	Genotypes	Cases, n	Controls, n
Asp299Gly	AA	86	93
	AG	13	5
	GG	1	0
Thr399Ile	CC	87	94
	CT	13	6
	TT	1	0

DISCUSSION

Seronegative spondyloarthropathies (SpA) are postulated to be triggered by an enviromental agent, likely of infectious etiology, in a genetically susceptible host. This model is best demonstrated in reactive arthritis, which is often triggered by infectious diarrhea. Innate immunity has been implicated in this pathogenetic pathway, since this represents the first line of host defense against pathogens[12]. Toll-like receptors are key regulators of innate immunity, sensing and responding to invading microorganisms, and the potential importance of TLR is supported by both experimental and clinical evidence. In experimental reactive arthritis, TLR4 has been shown to play a key role in defining the severity and timing of joint inflammation[13]. In clinical SpA, active disease is associated with upregulation of TLR4 in both peripheral blood mononuclear cells and synovial tissue, and effective treatment with biologic therapy is accompanied by downregulation of TLR4 expression[14].

Arbour, *et al*[15] identified 2 mutations (Asp299Gly and Thr399Ile) within the extracellular domain of TLR4 that is associated with diminished airway responsiveness to inhaled lipopolysaccharide in humans. Since their identification, these mutations have been associated with increased susceptibility to gram-negative bacteremia and septic shock with gram-negative organisms.

Our study suggests that TLR4 variants appear to be a minor risk factor for AS, as a modest association was noted between the GT haplotype (for Asp299Gly/Thr399Ile variants) and AS. Our results differ from Dutch and Hungarian AS studies that found no association with the Asp299Gly variant[16,17]. In the Dutch study[16] 113 Dutch patients with AS and 170 ethnically matched controls were typed for one variant of TLR4 (Asp299Gly). Since only one variant was tested, they were unable to generate haplotypes. The Hungarian study[17] generated haplotypes of the 2 variants, but found no association to AS using 138 patients and 140 controls. The difference between our study and these 2 studies may be a result of different ethnic backgrounds or disease heterogeneity. This racial background effect also influences interpretation of our recent studies of AS in Korea, a population that is homozygous at the 299 and 399 loci[18]. As Newfoundland is known to have a relatively homogenous Caucasian population[19], an enhanced signal-to-noise ratio in the population may have facilitated the detection of TLR4 variants of modest effect.

The inconsistencies noted between the Newfoundland and Dutch AS cohorts are paralleled in CD, where conflicting results have been noted between TLR4 associations and CD. Brand, *et al*[7] recently noted an almost 2-fold difference in the frequency of TLR4 Asp299Gly phenotype in CD cases (14.2%) compared to controls (7.5%), and this association has also been reported in other independent populations[8,9]. However, in a large Dutch population, the Asp299Gly and Thr399Ile variants were found to have no association with inflammatory bowel disease, and the investigators proposed that these 2 polymorphisms are in linkage disequilibrium with disease susceptibility variants located elsewhere on TLR4[20].

We observed a modest association between 2 functional variants of TLR4 and AS in the Newfoundland population. Given the functional role of TLR4 variants in the innate immune system, larger studies are now warranted to delineate the association of TLR4 variants in AS.

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