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Evaluation of Toll-like-receptor gene family variants as prognostic biomarkers in rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) is a systemic autoimmune disease whose main feature is persistent joint inflammation. Toll-like receptors (TLRs) play critical roles in the activation of innate and adaptive immune responses, and influence the activity of NFκB, a key player in chronic inflammation. We aimed at investigating the association of TLR allelic variants with susceptibility and severity of RA through a systematic, high-throughput, analysis of TLR genes. All coding exons and flanking regions of nine members of the TLR family (TLR1-9) were analyzed in 66 patients with RA and 30 healthy controls by next generation sequencing. We focussed on three single allelic variants, N248S in TLR1, Q11L in TLR7 and M1V in TLR8 based on the allelic frequencies in both patient and control populations, the predicted impact on protein function and the novelty in RA research. Analysis of these selected variants in a larger cohort of 402 patients with RA and in 208 controls revealed no association with susceptibility. However, the M1V allele was associated with a lower need for disease-modifying antirheumatic drugs (DMARDs) ($p = 0.008$) and biologic treatments ($p = 0.021$). Functional studies showed that the M1V variant leads to a reduced production of inflammatory cytokines, IL-1β, IL-6 and TNFα, in response to TLR8 agonists. Thus, the presence of this variant confers a significant protective effect on disease severity. These results show for the first time the association between the M1V variant of TLR8 and reduced disease severity in RA, which could have prognostic value for these patients.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease caused by the chronic inflammation of the synovial lining. It is characterized by progressive joint destruction [1]. Although a number of genes have been identified as possible targets in this pathology, the genetic regulation that contributes to the development and progression of disease in RA patients remains unclear [2]. It has been suggested that viruses and bacteria may contribute to initiate or exacerbate RA by binding to Toll-like receptors (TLRs) [3,4]. TLRs constitute a family of transmembrane proteins whose activation has been implicated in the loss of self-tolerance leading to autoimmunity and chronic inflammation [5–7]. They play an essential role in the activation and regulation of innate and acquired immune responses through recognition of specific pathogen-associated molecular patterns and endogenous peptides [6,8]. The stimulation of the TLR pathway modulates NFκB activation and thus the production of proinflammatory cytokines and cell-adhesion molecules [9,10]. Activation of the NFκB pathway plays a key role in the pathogenesis of chronic inflammatory diseases, including RA and inflammatory bowel disease [11]. The most recently described TLRs involved in responding to viral stimulation are TLR7 and TLR8. They are located at the membranes of the endosomal compartment and recognize viral single-stranded RNA and short double-stranded RNA [12,13]. Human TLR8 is expressed in monocytes/macrophages and myeloid dendritic cells [14]. TLR8 signaling is mediated by the adaptor protein MyD88 which activates NFκB, IRF-7

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and p38 MAPK, resulting in the induction of proinflammatory cytokines and tissue-destructive enzymes [15]. TLR8 is located on the X chromosome and spans 15.5 kb (Xp22.3–p22.2). At the genomic level, two splice variants with alternative translation start sites, due to SNP rs3764880 (p.Met1Val), are encoded by TLR8 gene (TLR8v1 and TLR8v2) [16,17]. Although TLR8v2, that lacks the first three amino acids, is the most conserved isoform of TLR8 among primates, the long isoform (TLR8v1) plays a major role in the positive regulation of TLR8 function in differentiated monocytes [18].

Genetic variants in TLRs have been mainly associated with disease susceptibility in patients with RA with variable level of significance and even discordant results [5,19–22]. Although several polymorphisms of TLR8 have been studied in RA patients, only rs5741883 has shown a moderate association with rheumatoid factor (RF) positivity [5,23].

In this study, we analyzed the association of variants N248S in TLR1, Q11L in TLR7 and M1V in TLR8 in 402 patients with RA and showed that M1V variant is significantly associated with a reduced need for disease-modifying antirheumatic drugs (DMARDs) and biologic treatment. We also described that monocytes from M1V variant carriers had a reduced production of inflammatory cytokines, IL-1β, IL-6 and TNFα, in response to TLR8 agonists.

2. Materials and methods

2.1. Patient samples

A first cohort of 66 selected RA patients with high disease severity (RF and/or ACCP positivity, erosive disease and failure to at least one DMARD) and 30 healthy controls were enrolled for next-generation sequencing (NGS). Identified variants were analyzed in a second cohort of 402 unselected patients with RA (Table 1), diagnosed according to the 1987 American College of Rheumatology (ACR) classification criteria [24].

In this retrospective cohort study, anonimyzed clinical, laboratory and treatment data were registered, annotating RF or ACCP positivity and the number of DMARDs and biologics. As a control population, 208 sex and age matched individuals who had no known history of serious disease, including autoimmune or chronic inflammatory disorders, were also genotyped. All patients were followed at Hospital Universitario Marques de Valdecilla (HUMV) (Santander, Spain) or Hospital Universitario La Paz (Madrid, Spain). Clinical information, including demographic data, disease characteristics, and treatment, has been previously described [25]. The study was approved by the corresponding Research Ethics Committees and informed consent was obtained from all subjects.

2.2. Sequencing analyses

The coding exons and flanking regions of the TLR family (TLR1-9) gene were sequenced in 66 RA patients and 30 healthy controls by NGS.

Table 1
Main features of the patients.

Female sex, %	72.9
Mean age ± SD, years	65.99 ± 14.20
Mean duration of follow-up ± SD, months	124.76 ± 91.27
Extra-articular manifestations, %	23.1
Erosive disease, %	57.7
RF[a] positive, %	63.7
Patients (%) treated with:	
DMARDs	97.6
Corticoid therapy	59.9
Biologic therapy	37.0
Mean number of DMARDs ± SD	2.22 ± 1.48
Mean number of biologics ± SD	1.81 ± 1.21

[a] RF, rheumatoid factor.

DNA libraries were processed for hybrid enrichment using a custom SeqCap EZ design (Roche Nimblegen, Basel, Switzerland) containing the coding sequences of TLRs. Then, double barcoded libraries were sequenced by using a MiSeq NGS platform (Illumina, Madison, WI). Allelic variants were analyzed in other 402 patients with RA and 208 age-matched control individuals by NGS sequencing. DNA was extracted from whole blood by using the QIAamp DNA blood kit (Qiagen, Hamburg, Germany) and amplified with primers for human TLR8 5′-CTCTTCTCGGCCACCTCCTG-3′ and 5′-GCAAGCCGCTTTACCTGCAT-3′, TLR7 5′-GGGGTTGGGGATGCTGTTTA-3′ and 5′-TGCAGTCCACGATCACATGG-3′, and TLR1 5′-ATGCCAAACCAGCTGGAGGA-3′ and 5′-CCCTGAGGGCCTTCAAGACT-3′.

2.3. Expression analyses of NFκB target cytokines

TLR8 activity was assessed by measuring the production of intracellular cytokines in monocytes as previously described [26]. Briefly, blood cells were stimulated with the TLR8 agonist ssRNA40 (InvivoGen, San Diego, CA) for 18 h in the presence of brefeldin A (Sigma-Aldrich, St Louis, MO) to prevent cytokine release. Cells were then stained with FITC-conjugated anti-human CD14 (BD Biosciences, San Jose, CA) to identify the monocyte population. Erythrocytes were lysed with FACS lysing solution (BD Biosciences). Mononuclear cells were permeabilized and intracellularly stained with phycoerythrin (PE)-labelled monoclonal antibodies against IL-1β, TNFα or IL-6 (BD Biosciences). Cytokine expression was analyzed by flow cytometry using Cell Quest Pro Software (BD Biosciences).

2.4. Statistical analysis

All statistical analyses were performed using SPSS 20 program (IBM, Armonk, NY). Differences in quantitative variables between groups of patients were compared with the Mann-Whitney *U* test, and the chi-squared statistic was used for categorical variables. For functional studies, the statistical comparisons of data between genotypes were performed using the Mann-Whitney *U* test. The significance level was set at $p < 0.05$.

3. Results

Genetic variants within human TLR genes have been reported to be associated with susceptibility to RA with variable level of significance and even discordant results. We aimed to assess whether TLR variants contribute to modify the course of the disease in RA patients. To approach this goal, we sequenced the coding exons of nine members of the TLR family (TLR1-9) gene in 66 selected patients with RA and 30 healthy controls. A total of 71 variants were identified (Table 2).

Three variants, N248S in TLR1, Q11L in TLR7 and M1V in TLR8 genes were selected based on the allelic frequencies in both patient and control populations, the novelty in RA research and the predicted functional impact on the protein as assessed by using PolyPhen, SIFT and SNPs3D programs. We studied these variants in a larger cohort of 402 patients with RA and in 208 controls and showed that none of them was associated with disease susceptibility as the genotypes distribution was similar in both patient and control populations (Table 3).

Then we analyzed a number of clinical findings associated with disease severity. Interestingly, we found that the presence of two copies of the G allele of TLR8 gene tended to correlate with clinical remission, better prognosis (less surgical interventions and prostheses), lower need for pharmacological therapies and the absence of two well-known serological markers of disease severity, RF and ACCP (Fig. 1A). Furthermore, when we analyzed the need for disease-modifying antirheumatic drugs (DMARDs) and biologic therapy among GG (homozygotes for the V variant) and AG/AA genotype carriers, we showed that the GG genotype was significantly associated with lower number of DMARDs ($p = 0.008$) and biologics ($p = 0.021$) (Fig. 1B and C). Thus,