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.

Keywords: Toll-like receptor; rheumatoid arthritis; gene variant; prognosis

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 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].

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.

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. 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).

Table 2. Allelic variants of TLR genes in control and RA populations.

Gene	ID number	MAF[a] controls	MAF cases	P value	AA[b] change	Impact
TLR1	rs113706342	0	0,007576	0,4991	H720P	MODERATE[c]
TLR1	rs41311400	0	0,007576	0,4991	G676G	LOW[d]
TLR1	rs151036585	0	0,007576	0,4991	D605Y	MODERATE
TLR1	rs5743618	0,4333	0,3333	0,1821	S602I	MODERATE
TLR1	rs5743614	0,3929	0,254	0,05834	S506S	LOW
TLR1	rs201398822	0,01667	0	0,137	L371F	MODERATE
TLR1	rs184548723	0	0,007576	0,4991	T363M	MODERATE
TLR1	rs3923647	0,01667	0,01515	0,9375	H305L	MODERATE
TLR1	rs4833095	0,4333	0,303	0,07807	N248S	MODERATE
TLR1	rs5743611	0,05	0,09091	0,3276	R80T	MODERATE
TLR1	rs145135062	0	0,007576	0,4991	I57M	MODERATE
TLR1	rs5743610	0,01667	0,01515	0,9375	H38H	LOW
TLR2	rs5743697	0	0,007576	0,4991	G38G	LOW
TLR2	rs200686000	0	0,007576	0,4991	T138I	MODERATE
TLR2	rs3804099	0,5167	0,4621	0,4831	N199N	LOW
TLR2	rs5743698	0	0,007576	0,4991	L213L	LOW
TLR2	rs3804100	0,06667	0,08333	0,69	S450S	LOW
TLR2	rs5743700	0,05	0,02273	0,3141	F541F	LOW

TLR2	rs5743704	0,01667	0,02273	0,7852	P631H	MODERATE
TLR2	rs5743708	0	0,007576	0,4991	R753Q	MODERATE
TLR3	rs3775291	0,4333	0,3409	0,2188	L135F	MODERATE
TLR3	rs3775290	0,3333	0,3258	0,9175	F182F	LOW
TLR3	rs73873710	0,06667	0,02273	0,1321	F574F	LOW
TLR4	rs78848399	0,01667	0	0,137	Y46C	MODERATE
TLR4	rs137853920	0,05	0,01515	0,1599	C241Y	MODERATE
TLR4	rs4986790	0,1	0,06061	0,3305	D259G	MODERATE
TLR4	rs56070048	0	0,007576	0,4991	K314K	LOW
TLR4	rs4986791	0,1	0,06061	0,3305	T359I	MODERATE
TLR4	rs5030721	0,01667	0,0303	0,5824	K613K	LOW
TLR4	rs5030724	0,01667	0	0,137	Q794K	MODERATE
TLR5	rs1053954	0,08333	0,07576	0,8561	K841K	LOW
TLR5	rs7512943	0	0	NA	F822L	MODERATE
TLR5	rs5744174	0,3333	0,3333	1	F616L	MODERATE
TLR5	rs2072493	0,1667	0,2652	0,1354	N592S	MODERATE
TLR5	rs5744169	0	0,007576	0,4991	L444L	LOW
TLR5	rs5744168	0,01667	0,007576	0,5652	R392*	HIGH[e]
TLR5	rs45528236	0,01667	0,007576	0,5652	Q181K	MODERATE
TLR5	rs764535	0,01667	0	0,137	T82I	MODERATE
TLR5	rs5744165	0,03333	0,03788	0,8762	V61V	LOW
TLR5	rs187499609	0	0,007576	0,4991	R34H	MODERATE
TLR6	rs138342666	0,01667	0,02273	0,7852	I461V	MODERATE
TLR6	rs5743820	0,03333	0,007576	0,1822	T440M	MODERATE
TLR6	rs5743818	0,2167	0,2424	0,6961	A644A	LOW
TLR6	rs5743816	0,01667	0	0,137	V465I	MODERATE
TLR6	rs5743815	0,01667	0,0303	0,5824	V427A	MODERATE
TLR6	rs3775073	0,3	0,3182	0,8011	K421K	LOW
TLR6	rs3821985	0,3167	0,303	0,8494	T361T	LOW
TLR6	rs5743810	0,35	0,3712	0,7771	S249P	MODERATE

TLR6	rs189784331	0	0,01515	0,3378	V213V	LOW
TLR7	rs179008	0,3167	0,197	0,06955	Q11L	MODERATE
TLR7	rs55907843	0	0,007576	0,4991	V222D	MODERATE
TLR7	rs5743780	0	0,02273	0,2392	L345L	LOW
TLR7	rs5743781	0	0,0303	0,173	A448V	MODERATE
TLR7	rs864058	0,15	0,08333	0,161	T801T	LOW
TLR7	rs141270925	0	0,01515	0,3378	Y897Y	LOW
TLR7	rs189681811	0	0,007576	0,4991	R920K	MODERATE
TLR8	rs3764880	0,3667	0,2424	0,07593	M1V	HIGH
TLR8	rs56194919	0,01667	0,0303	0,5824	C25C	LOW
TLR8	rs2159377	0,2	0,1894	0,8629	D118D	LOW
TLR8	rs5744080	0,35	0,3636	0,8552	H215H	LOW
TLR8	rs2407992	0,4	0,3636	0,6295	L651L	LOW
TLR8	rs3747414	0,3	0,2803	0,7796	I751I	LOW
TLR8	rs2109135	0	0	NA	D923D	LOW
TLR9	rs445676	0,03333	0,02308	0,6814	Y980Y	LOW
TLR9	rs5743845	0	0,007576	0,4991	R863Q	MODERATE
TLR9	rs352140	0,4833	0,4848	0,9845	P545P	LOW
TLR9	rs141799890	0	0,007576	0,4991	H539Q	MODERATE
TLR9	rs35342983	0	0,007576	0,4991	S509S	LOW
TLR9	rs35654187	0	0,007576	0,4991	T383T	LOW
TLR9	rs150459369	0,01667	0,007576	0,5652	A7T	MODERATE
TLR9	rs5743842	0,01667	0	0,137	R5C	MODERATE

[a]MAF, minor allele frequency; [b]AA, amino acid; [c]non-disruptive variant that might change protein effectiveness, [d]mostly harmless or unlikely to change protein behaviour, [e]assumed to have high or disruptive impact in the protein, probably causing protein truncation or loss of function.

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).

Table 3. Genotype frequencies of selected TLR variants in controls and RA patients.

Variant	Genotype	Controls %	Cases %	P value	OR[a] (95% CI[b])
TLR1-N248S	TT	0.35	0.36		
TLR1-N248S	TC	0.46	0.48	0.900	1.032 (0.636-1.672)
TLR1-N248S	CC	0.18	0.14	0.461	0.788 (0.417-1.487)
TLR7-Q11L	AA	0.64	0.69		
TLR7-Q11L	AT	0.29	0.20	0.906	0.660 (0.399-1.095)
TLR7-Q11L	TT	0.05	0.09	0.350	1.539 (0.620-3.822)
TLR8-M1V	AA	55.7	55.7		
TLR8-M1V	AG	30.6	30.1	0.941	0.982 (0.608-1.585)
TLR8-M1V	GG	13.9	14.2	0.957	1.018 (0.539-1.923)

[a]OR, odds ratio; [b]CI, confidence interval

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 1C). Thus, this variant

appears to select a group of patients with less severe and refractory disease. In order to correlate these data with the level of inflammatory mediators, we stimulated patient blood cells with a TLR8 agonist and analyzed the production of inflammatory cytokines by flow cytometry. As shown in Fig. 2, IL-1β, TNF-α and IL-6 levels in circulating monocytes were lower in GG genotype carriers compared with those in patients carrying the AA genotype. Although these differences did not reach statistical significance, most likely because of the low frequency of the rare allele, the results are consistent with our previous data and strengthen the association of the GG genotype with a reduced inflammatory response following activation of TLR8.

4. Discussion

TLR8 variants have been associated with susceptibility to infectious and autoimmune diseases including tuberculosis and systemic lupus erythematosus [27-29]. In this study, we show that M1V variant of TLR8 is associated with disease severity in RA, and selects a group of patients with a lower need for therapeutic interventions. A meta-analysis that included 14 studies worldwide has been recently performed to determine whether TLR polymorphisms confer susceptibility to RA and/or influence the clinical features. This study, that included a TLR8 polymorphism (rs5741883) in the non-coding upstream region of the gene, did not find a clear association with any of the variants analyzed [5]. However, the lack of a statistically significant amount of data precluded the possibility of reaching conclusive results. Another study that genotyped RA patients for 22 polymorphisms in 7 TLR genes concluded that only rs5741883 showed a moderate association with rheumatoid factor positivity [23]. To avoid SNP preselection bias, we conducted a systematic and high-throughput study by sequencing all coding and flanking

219 regions of nine TLR genes in 66 patients. The selected TLR8 polymorphism, M1V, was
220 further studied in a larger cohort of 402 patients to validate our data.

221 The SNP that leads to M1V variant (rs3764880, A>G) has been previously studied in
222 patients with tuberculosis, showing that the major allele A was associated with
223 susceptibility to this infectious disease [30]. In line with this, another report showed that
224 the G allele conferred a significant protective effect to HIV-positive adults regarding
225 progression of the disease [31]. Furthermore, TLR8 signaling results in the induction of
226 proinflammatory cytokines [15]. Consistently, we found that GG genotype carriers
227 produced significantly lower levels of inflammatory cytokines, IL1β, IL6 and TNFα,
228 when blood mononuclear cells were stimulated with TLR8 agonists. A>G change
229 eliminates the start codon and provokes the use of the next in-frame ATG, which
230 generates a deletion of the first three amino acids of TLR8, giving rise to TLR8v2
231 isoform. We showed that G allele carriers develop a less severe disease and reduce the
232 need for therapy. A likely mechanistic explanation might be in the stability of TLR8
233 mRNA. Transfection experiments with both A and G allelic variants of TLR8 showed
234 that the amount of the G variant mRNA was significantly lower, suggesting that this
235 mRNA may be less stable [32]. A similar polymorphism has been described in the vitamin
236 D receptor gene [33]. A T>C change at the initiation codon gives rise to a shorter isofom
237 lacking the first three amino acids, which has functional consequences due to differences
238 in the transcriptional capacity of both alleles. Although a scenario where the G allele
239 correlates with a lower inflammatory activity is consistent with our data, it has also been
240 described that the same allele is more efficient than the A allele in promoting activation
241 of the NFκB proinflammatory pathway [32]. This result was obtained by cotransfecting
242 cells with a luciferase reporter construct and the A/G allelic variants of TLR8. Our results
243 are based on in vivo data in a large cohort of RA patients.

In conclusion, our study suggests that the presence of the TLR8 allelic variant M1V, may be associated with a reduced severity of rheumatoid arthritis in a Caucasian Spanish population. Replication of these results in other cohorts of patients, including populations with different genetic background, would strengthen the prognostic value of M1V variant in patients with RA.

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346 **Figure Legends**

347 **Fig 1. Association of the M1V variant of TLR8 with disease severity in a cohort of**
348 **402 patients with RA.** (A) Carriers of the indicated genotypes were distributed in regards
349 to presence of serum ACCP and RF, need of surgery, and clinical remission. ACCP, anti-
350 cyclic citrullinated protein antibodies; RF, rheumatoid factor. Box plot showing the
351 association between the total number of disease-modifying antirheumatic drugs
352 (DMARDs) (B), or biologics (C) that each patient has received during the course of the
353 disease, and TLR8 genotypes in RA patients.

354 **Fig 2. TLR 8 activity according to TLR8 genotypes in patients with RA**. (A-C) TLR8
355 activity was assessed by measuring intracellular proinflammatory cytokine production
356 following treatment of circulating monocytes with ssRNA40, a TLR8 specific agonist.
357 Cytokine and CD14 co-staining with specific antibodies was used to reveal the expression
358 of cytokines in monocytes by using flow cytometry. Values represent the difference
359 between the percentage of cells expressing the cytokine with and without treatment with
360 the TLR8 agonist (delta).

361

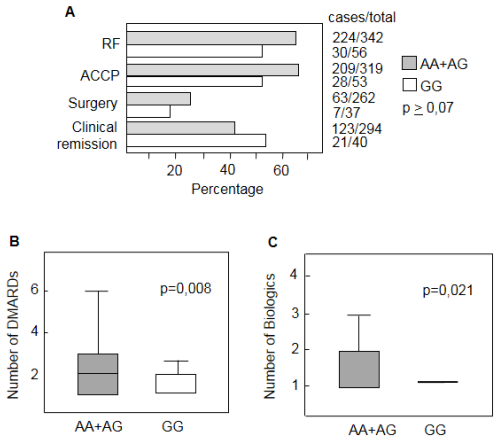
A
cases/total
RF
224/342
30/56
ACCP
209/319
28/53
Surgery
63/262
7/37
Clinical remission
123/294
21/40
AA+AG
GG
p ≥ 0,07
20
40
60
Percentage
B
Number of DMARDs
p=0,008
AA+AG
GG
C
Number of Biologics
p=0,021
AA+AG
GG

Fig 1

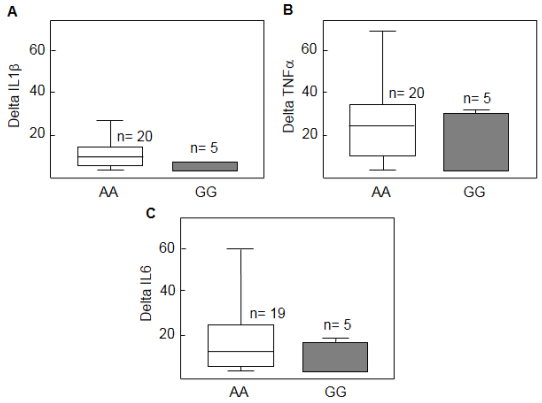
A
Delta IL1β
n= 20
n= 5
AA
GG
B
Delta TNFα
n= 20
n= 5
AA
GG
C
Delta IL6
n= 19
n= 5
AA
GG

Fig 2