

A biologically important single nucleotide polymorphism within the toll-like receptor-4 gene is not associated with rheumatoid arthritis

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ABSTRACT

Background. Rheumatoid arthritis (RA) is a heterogeneous condition affecting 1-2% of the population. Genetics account for 30% of disease susceptibility, with one third arising from the Major Histocompatibility Complex. The toll-like receptor 4 (TLR-4) gene which has been mapped to chromosome 9 (9q32-q33) is involved in innate immune recognition with subsequent proinflammatory cytokine release including TNF. A single nucleotide polymorphism (+896A → G) resulting in the amino acid substitution (Asp299Gly) has been shown to interrupt TLR-4 mediated signalling.

Objective. We sought to determine if this TLR-4 polymorphism influences susceptibility to rheumatoid arthritis.

Methods. DNA was extracted from 879 healthy controls and 212 rheumatoid arthritis patients recruited from the north of England. Genotyping was performed using a 5' nuclease Taqman allelic discrimination assay. Allele frequencies were compared between the two groups. We also examined whether an association existed in non-carriers of the DRB1 shared epitope alleles.

Results. The frequency of the rare allele was 5.9% in the controls and 7% in the patients. Comparison of rare allele carriage between controls and patients revealed no significant difference $p = 0.13$. This was also the case in shared epitope negative individuals $p = 0.92$.

Conclusion. The TLR-4 +896 polymorphism does not appear to influence susceptibility to rheumatoid arthritis.

Introduction

Rheumatoid arthritis is a complex heterogeneous inflammatory autoimmune condition, in which proinflammatory cytokines including tumour necrosis factor (TNF) and interleukin-1 (IL-1) play a pivotal role in disease pathogenesis. Susceptibility to RA is multifactorial with both environmental and genetic factors being important, the latter is believed to contribute around 30% of susceptibility (1). Approximately one third of the total genetic susceptibility arises from the Major Histocompatibility Complex (MHC) with the shared epitope alleles of HLA-DRB1 being

the most convincingly implicated (2). The shared epitope sequence encodes amino acids 69-73 of the mature peptide and is important in determining the repertoire of peptides which are bound by HLA class II molecules and presented to T cells (3).

The human Toll like receptor-4 (TLR4) is a type I transmembrane protein with an extracellular domain consisting of a leucine rich repeat region and an intracellular domain homologous to that of the human interleukin-1 receptor (IL-1R). TLR4 is an innate immune recognition receptor activated by chemically diverse microbial products including lipopolysaccharide (LPS) (4). LPS is derived from gram-negative bacterial cell walls and results in production of TNF, IL-1 and IL-6 from macrophages, recognition of LPS by cells of the innate immune system enables prompt clearance of pathogens before they become widely disseminated. Exposure to bacterial products, such as (LPS) or proinflammatory cytokines increases TLR4 expression in monocytes and PMN, an effect which is blocked by the anti-inflammatory cytokine IL-10 (5). Over-expression of TLR4 has been shown to drive NFκB activation and cytokine production including TNF, IL-1, IL-6 and IL-8 (6). Two TLR4 polymorphisms have been described, an adenine for guanine substitution at 896 nucleotides from the transcription start site of TLR4 cDNA (+896) leads to an amino acid change at 299 from aspartic acid to glycine (Asp299Gly). This missense mutation within the fourth exon alters the extracellular domain of the TLR4 receptor interrupting TLR4 mediated signalling resulting in endotoxin hyporesponsiveness. An additional missense mutation replacing a non-conserved threonine with an isoleucine at amino acid 399 (Thr399Ile) in the extracellular domain of the TLR4 receptor has also been described but does not interrupt TLR4 mediated LPS signalling (7).

In view of the ability of TLR4 activation to stimulate inflammatory cytokine release including TNF, we were interested to determine whether this functional TLR4 (+896) polymorphism influenced susceptibility to RA.

Materials and methods

Controls and patient populations

DNA was extracted from EDTA anti-coagulated blood by standard methods from 879 ethnically matched healthy controls and 212 patients with rheumatoid arthritis. All patients satisfied the 1987 revised American College of Rheumatology criteria for the diagnosis of RA (8).

Genotyping of the Asp299Gly polymorphism of TLR4 was performed using a 5' nuclease assay (Taqman allelic discrimination test; Perkin Elmer Biosystems, Foster City, USA). Each amplification used 20ng of genomic DNA as template, 900nM of each primer (Forward 5'CCATTGAAGAA-TTCCGATTAGCAT-3'; Reverse 5'GGGAAAATGAAGAAACATTTGTCAA-3'), 50nM of Allele 1 probe (5'FAM-TAGACTACTACCCTCGATGATATTATTGACTTATTTAATTTC-3') and 200nM of allele 2 probe (5'TETACTACTACTCTCGATGGTATTATTGACTTATTTAATTGT-3'). Thermal cycling conditions were as follows: an initial cycle of 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 62°C for 1 minute with a final holding cycle of 15°C. Genotype was ascertained using an ABI Prism®7200 Sequence Detection System Machine (Perkin Elmer Biosystems, Foster City, USA).

The validity of the TaqMan genotyping technique was checked by dideoxy sequencing using an ABI3700 (Perkin Elmer Biosystems, Foster City, USA). A 79 base-pair segment including the polymorphic site was sequenced in 25 individuals and no discrepancies with genotyping were detected.

Genotyping of HLA-DRB1 was performed in 164 controls and all of the RA patients by polymerase chain reaction sequence specific primer reactions (PCR-SSP) amplification followed by agarose gel electrophoresis (9). The shared epitope (SE) alleles were defined as DRB1*01, *0401, *0404, *0405, *0408, *10.

Statistical analysis

Allele frequencies between the two groups were compared, p-values were

calculated using the χ^2 test, with a value of < 0.05 considered statistically significant.

The power to detect a relative risk of greater than or equal to 2 for those carrying the G-allele compared to non carriers was 91% found by simulation.

Results

The results of genotyping of the TLR4 +896 polymorphism are shown in Table I. No significant difference was found between the controls and patients with 90% of the controls and 86% of the RA patients being homozygote for the TLR4 +896 A allele. The frequency of the rare allele was 5.9% in controls and 7% in patients.

Comparison of carriage of the rare allele between controls and patients revealed no significant difference ($p = 0.13$) with an estimated relative risk of 1.3 for carriers of allele G compared to non-carriers. We then analysed allele frequency in those individuals who did not carry the shared epitope, 91 of the 164 controls typed for HLA-DRB1 and 46 of the 212 rheumatoid patients were shared epitope negative. The rare allele frequency was 4.4% in the shared epitope negative controls and 5.4% in the rheumatoid arthritis patients. There was no significant difference in G allele frequency between cases and controls $p = 0.69$. The relative risk of carriers of allele G compared with non-carriers was 1.2.

Discussion

We did not detect a significant correlation between the TLR4 (+896) polymorphism and susceptibility to rheumatoid arthritis despite a large study sample and a power set of 99% to de-

tect a GRR of 2 or more.

The role of the MHC in the susceptibility to rheumatoid arthritis has been well documented, but the location of non-MHC susceptibility genes, which contribute two-thirds of the genetic risk for RA, are largely unknown. In support of our results TLR4 has been mapped to chromosome 9 (9q32-q33) (10), within a region that has not previously been genetically linked with RA.

The innate immune system provides the initial defence mechanism to foreign pathogens and is a non-antigen specific response. The major cells and molecules of the innate immune system have been found in rheumatoid synovium and may be involved in the initiation of inflammation (11). The Toll-like receptors are vital pattern recognition receptors (PRRs) in the innate immune system (12). Activation of a Toll receptor results in a common signal transduction pathway involving MyD88, IL-1R associated kinase (IRAK) and TNF receptor associated factor 6 (TRAF6) (13). The TLR signalling pathway is highly homologous to that of IL-1 receptor, the receptors share sequence similarities in their cytoplasmic domain Toll-IL-1-R (TIR) and signal via common components including MyD88 and IRAK (14). The role of IL-1 has been well documented in the pathogenesis of RA (15) and polymorphisms within the IL-1 cluster have been shown to be associated with rheumatoid arthritis (16,17). Evidence exists that TLR4 is expressed in synovial cells from patients with RA (18). The homology between the TLR4 and IL-1 signalling pathways supports a potential candidate role for TLR4.

The TLR4 +896 polymorphism des-

Table I. Genotype distribution of the TLR4+896 polymorphism in the controls and rheumatoid arthritis patients. The genotype relative risk is shown for both shared epitope positive and negative individuals.

	Shared epitope positive			Shared epitope negative		
	Genotype		Relative	Genotype		Relative
	AA	AG+GG	risk	AA	AG+GG	risk
Controls	787	92		83	8	
Patients	182	30	1.3	41	5	1.2

cribed by Arbor et al has been demonstrated to interrupt TLR4 mediated LPS-signalling and reduce production of TNF by inflammatory cells (7). To date, work has focussed on the potential role of this polymorphism in infections, but clearly there are potential implications in systemic inflammatory diseases. To our knowledge this is the first study to investigate the relationship between the TLR4 + 896 polymorphism and rheumatoid arthritis.

In conclusion the TLR-4 +896 polymorphism does not appear to influence susceptibility to rheumatoid arthritis.

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