Lack of Association Between TLR4 rs4986790 Polymorphism and Risk of Cardiovascular Disease in Patients with Rheumatoid Arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory disease associated with increased cardiovascular (CV) mortality. Toll-like receptor-4 (TLR4) activates the innate immune response via NF-κB pathway and mitogen-activated protein kinase signaling, leading to expression of proinflammatory cytokines and chemokines. The G allele of TLR4 rs4986790 (+896A>G, Asp299Gly) gene polymorphism has been implicated in reduction of risk of atherosclerosis. In this study, 1481 RA patients fulfilling the 1987 American College of Rheumatology (ACR) criteria were genotyped for the rs4986790 TLR4 variant to determine the influence of this variant in the risk of CV events in these patients. Also, HLA-DRB1 status was determined using molecular based methods. Moreover, potential influence of rs4986790 variant in the development of subclinical atherosclerosis was assessed in a subgroup of RA patients with no history of CV events by the measurement of surrogate markers of subclinical atherosclerosis. No statistically significant differences in allele or genotype frequencies for the rs4986790 variant between RA patients who experienced CV events or not were found. Likewise, no significant association between this gene variant and any of the surrogate markers of subclinical atherosclerosis was found. In summary, results in our study do not support the hypothesis that the rs4986790 (+896A>G, Asp299Gly) TLR4 variant may influence predisposition for subclinical atherosclerosis and clinically evident CV disease in RA patients.

Introduction

RHEUMATOID ARTHRITIS (RA) is a complex autoimmune disease associated with accelerated atherosclerosis (Satir et al., 2003; González-Gay et al., 2005). Besides traditional cardiovascular (CV) risk factors, chronic inflammation (González-Gay et al., 2007) and genetic factors (Farragher et al., 2008; González-Gay et al., 2009; Palomino-Morales et al., 2009a, 2010a; Panoulas et al., 2009; Rodriguez-Rodriguez et al., 2011c) have been implicated in the increased CV mortality observed in RA patients.

Human toll-like receptors (TLRs) participate in the innate response and signal the activation of adaptive immunity. The toll-like receptor-4 (TLR4) plays an important role in the recognition of microbial components, particularly lipopolysaccharides. In addition, TLR4 also interacts with endogenous proteins present during inflammation such as oxidized low-density lipoprotein, heat shock proteins 60 and 70, fibrinogen, and fibronectin (Akira et al., 2001; Xu et al., 2001). TLR4 activates the innate immune response via the NF-κB pathway and mitogen-activated protein kinase signaling, through IRAK-1 and -4 and TRAF6 (Huang and Pope, 2010) leading to the expression of proinflammatory cytokines and chemokines. TLR4 downregulates disease severity in experimental autoimmune encephalomyelitis and Th17 cell responses, but promotes Th1 cell responses that may inhibit...
the differentiation of Th17 cells (Marta et al., 2009). A single-nucleotide polymorphism (SNP) in the coding region of the human TLR4 gene (rs4986790, +896A>G) at exon 3 results in amino acid exchange at position 299 (Asp299Gly). The presence of Asp299Gly in Caucasian individuals alters the structure of the extracellular domain of TLR4 (Akira et al., 2001; Kiechl et al., 2002) because it is a change in the ligand-binding site of the receptor (Rallabandi et al., 2006).

TLR4 has been shown to be expressed by macrophages and endothelial cells in human atherosclerotic lesions (Xu et al., 2001; Edfeldt et al., 2002). Kiechl et al. reported that carriers of the +896G allele had lower levels of proinflammatory cytokines, acute-phase reactants, and soluble adhesion molecules. They also found that these +896G allele carriers were more susceptible to severe bacterial infections but had a lower risk of atherosclerosis manifested by a smaller intima-media thickness in the common carotid (Kiechl et al., 2002). Further, individuals with angiographically documented coronary atherosclerosis treated with pravastatin that carried the 299Gly were found to have lower risk of CV events during follow-up than noncarriers (Boekholt et al., 2003).

Some studies have reported association between TLR4 polymorphisms and susceptibility to RA (Radstake et al., 2004). Although association of TLR4 polymorphisms with other autoimmune diseases has also been described (Marshall-Rothstein, 2006), in keeping with data from a recent meta-analysis on association of TLR4 (+896A/G) gene polymorphism with susceptibility to giant cell arteritis (Alvarez-Rodriguez et al., 2011), a disease that like RA is associated with HLA-DRB1*04 alleles (Gonzalez-Gay et al., 2003; Sanchez et al., 2004) did not confirm association of TLR4 polymorphisms with RA in the Spanish population. Also, no association of the TLR4 rs4986790 gene polymorphism with RA was found in Dutch (Emonts et al., 2011) and French populations (Jaen et al., 2009).

Despite those negative results, in the present study we aimed to determine whether the common SNP of the TLR4 gene rs4986790 (+896A>G, Asp299Gly) may be associated with the risk to develop CV disease in patients with RA, given the crucial role of TLR4 in mediating inflammatory signaling.

Materials and Methods

Patients

Between March 1996 and September 2008, 1481 consecutive patients who fulfilled the 1987 American College of Rheumatology classification criteria for RA (Arnett et al., 1988) were recruited from the Rheumatology Outpatient Clinics of Hospital Xeral-Calde (Lugo), Hospital Clinico San Carlos (Madrid), Hospital Universitario La Paz (Madrid), Hospital Universitario La Princesa (Madrid), Hospital Universitario Bellvitge (Barcelona), and Hospital Universitario Marques de Valdecilla (Santander), Spain. Patients were assessed for differences in the TLR4 rs4986790 gene variant.

Study protocol

Between December 2009 and January 2010, patient’s clinical records were examined until patient’s death, loss of follow-up, or December 1st, 2009. Information on the main demographic data, clinical characteristics of the patients enrolled in the study, CV risk factors, and CV events of patients are shown in Table 1. Two hundred and sixty-one (17.62%) of these 1481 patients with RA experienced CV events. Clinical definitions for CV events (ischemic heart disease, heart failure, cerebrovascular accident, or peripheral arteriopathy) and classic CV risk factors were established as previously described (Gonzalez-Gay et al., 2007; Gonzalez-Juanatey et al., 2009).

To determine the potential association between the TLR4 rs4986790 polymorphism and the presence of subclinical atherosclerosis, between March 2007 and September 2009 a random subgroup of patients from the Lugo cohort with no previous history of CV events was assessed. Presence of endothelial dysfunction was established by a brachial artery reactivity study in 134 patients. Flow-mediated endothelium-dependent vasodilatation-FMD (post-ischemia) and endothelium-independent-NTG (post-nitroglycerin) vasodilatation were measured by brachial ultrasonography as previously reported (Gonzalez-Juanatey et al., 2003b; Gonzalez-Gay et al., 2008). Also, carotid ultrasonography studies were performed in 112 patients to determine the carotid artery intima-media wall thickness (IMT). It was assessed in the right common carotid artery as earlier described (Gonzalez-Juanatey et al., 2006; Gonzalez-Gay et al., 2008).

A subject’s written consent was obtained according to the declaration of Helsinki, and the design of the work was approved by the Ethics Committee of Galicia (Spain). The Ethics Committees of the Hospital Clinico San Carlos (Madrid), Hospital La Paz (Madrid), Hospital de la Princesa (Madrid), Hospital Universitario Bellvitge (Barcelona), and Hospital Universitario Marques de Valdecilla (Santander) also approved the study.

Genotyping

TLR4 genotyping. DNA was obtained from peripheral blood using standard methods. Subjects were genotyped to

<table>
<thead>
<tr>
<th>Variables</th>
<th>n=1481</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td>1100 (74.27)</td>
</tr>
<tr>
<td>Age of patients at the time of disease diagnosis, years, median (IQR)</td>
<td>54.2 (43–64)</td>
</tr>
<tr>
<td>Time follow-up, years, median (IQR)</td>
<td>10.6 (5.5–17)</td>
</tr>
<tr>
<td>anti-CCP positive (n=1154)</td>
<td>672 (58.23)</td>
</tr>
<tr>
<td>Rheumatoid Factor positive (n=1449)</td>
<td>1012 (69.84)</td>
</tr>
<tr>
<td>Shared epitope, presence (n=753)</td>
<td>471 (62.55)</td>
</tr>
<tr>
<td>Rheumatoid arthritis subjects with cardiovascular disease</td>
<td>261 (17.62)</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>141 (9.36)</td>
</tr>
<tr>
<td>Cerebrovascular accident</td>
<td>73 (4.85)</td>
</tr>
<tr>
<td>Heart failure</td>
<td>80 (5.32)</td>
</tr>
<tr>
<td>Peripheral arteriopathy</td>
<td>26 (1.73)</td>
</tr>
<tr>
<td>Hypertension (n=1476)</td>
<td>579 (39.23)</td>
</tr>
<tr>
<td>Diabetes mellitus (n=1470)</td>
<td>189 (12.86)</td>
</tr>
<tr>
<td>Dyslipidemia (n=1445)</td>
<td>579 (40.07)</td>
</tr>
<tr>
<td>Obesity (n=1401)</td>
<td>264 (18.84)</td>
</tr>
<tr>
<td>Smoking habit (n=1427)</td>
<td>356 (24.95)</td>
</tr>
</tbody>
</table>

Except where indicated otherwise, values are n (%). IQR, interquartile range; anti-CCP, anti-cyclic citrullinated peptide antibodies.
determine the TLR4 rs4986790 (+896A>G) polymorphism located in exon 3, which leads to an aminoacid change Asp299Gly (Arbour et al., 2000), using TaqMan Assays-on-Demand and analyzed using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), following manufacturer’s instructions. Negative controls and duplicate samples were included to check the accuracy of genotyping.

### Shared epitope determination

Several HLA-DRB1 alleles (HLA-DRB1*0401, *0404, *0405, *0408, *0101, *0102, *1001, *1402) are associated with susceptibility to RA. These alleles encode a conserved amino acid sequence (QKRAA, QRRAA, or RRRRAA), called the shared epitope, at position 70–74 in the third hypervariable region of the HLA-DRB1 molecule (Gregersen et al., 1987). HLA-DRB1-shared epitope alleles are also implicated in the severity of the disease (González-Cay et al., 2002). HLA-DRB1 typing was carried out using a reverse dot-blot kit with sequence-specific oligonucleotide (SSO) probes (Dynal RELITM SSO HLA-DRB1 typing kit; Dynal Biotech, Bromborough, United Kingdom).

### Statistical analysis

All genotype data were checked for deviation from Hardy–Weinberg equilibrium (HWE) using http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. Comparison of proportions was carried out using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), following manufacturer’s instructions. Negative controls and duplicate samples were included to check the accuracy of genotyping.

**Table 2. Differences in Genotype and Allele Frequencies of TLR4 rs4986790 Polymorphism Between Rheumatoid Arthritis Patients with Cardiovascular Events or Without Cardiovascular Events**

<table>
<thead>
<tr>
<th>TLR4 rs4986790</th>
<th>With CV events</th>
<th>Without CV events</th>
<th>p-Value</th>
<th>OR [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>219 (87.95)</td>
<td>1039 (88.58)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AG</td>
<td>29 (11.65)</td>
<td>127 (10.82)</td>
<td>0.71</td>
<td>1.08 (0.69–1.70)</td>
</tr>
<tr>
<td>GG</td>
<td>1 (0.40)</td>
<td>0 (0.00)</td>
<td>0.71</td>
<td>0.68 (0.03–5.50)</td>
</tr>
<tr>
<td>AG+GG</td>
<td>30 (12.05)</td>
<td>134 (11.42)</td>
<td>0.78</td>
<td>1.06 (0.68–1.65)</td>
</tr>
<tr>
<td>A</td>
<td>467 (93.78)</td>
<td>2205 (93.99)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>G</td>
<td>31 (6.22)</td>
<td>141 (6.01)</td>
<td>0.85</td>
<td>1.04 (0.68–1.58)</td>
</tr>
</tbody>
</table>

CV, cardiovascular; OR (95% CI), odds ratio with 95% confidence interval.

### Results

**Frequencies of the TLR4 rs4986790 variant and CV events in RA patients**

The study had 74% statistical power to detect allelic ORs greater than 1.65, based on calculation done from the association study in RA (Radstake et al., 2004) and reported in biopsy-proven giant cell arteritis association study (Palomino-Moraes et al., 2009b), at the stated significance level (z=0.05), with a minor allele frequency of 0.06 and a prevalence of the disease in Spanish population 0.005 (Carmona et al., 2002).

The study reached a genotyping success >95%. Genotype frequencies of the TLR4 rs4986790 variant studied were in HWE equilibrium in the population under study. Minor allele frequency of rs4986790 SNP was in keeping with other studies previously reported (Radstake et al., 2004; Sánchez et al., 2004; Palomino-Moraes et al., 2009b), around 6% in most European populations.

Table 2 shows the genotype frequencies of the TLR4 rs4689790 gene polymorphism assessed in this cohort of RA patients stratified according to the presence or absence of CV events. No statistically significant differences in the genotype or allele frequency of the TLR4 rs4986790 (+896G>A) gene polymorphism between RA patients who experienced CV events or not were seen.

Also, no significant differences in the age at the onset of the disease, rheumatoid factor, anti-cyclic citrullinated peptide antibodies, shared epitope, and age at the time of disease diagnosis were observed according to the different TLR4 rs4986790 genotypes in this series of RA patients (data not shown).

To investigate a potential role of the TLR4 rs4986790 SNP in the development of CV events, we assembled a logistic regression model to explain the presence of CV disease in patients with RA according to TLR4 rs4986790 allele distribution adjusted for gender, age at the time of RA diagnosis, follow-up time, presence or absence of shared epitope, and classic CV risk factors, which did not disclose statistically significant differences: $p=0.86$, adjusted $p=0.68$. Moreover, no association with the TLR4 rs4986790 was found when we...
specifically assessed the subtypes of CV events studied (ischemic heart disease, cerebrovascular accidents, peripheral arteriopathy, or heart failure) (data not shown).

Since some studies suggested a potential role of TLR4 gene in the risk of metabolic syndrome (Cuda et al., 2011) or type 1 diabetes mellitus (Kolek et al., 2004), we also sought for potential association of the TLR4 rs4986790 polymorphism with diabetes mellitus or dyslipidemia in our series. However, no association was disclosed (data not shown).

**TLR4 rs4986790 gene polymorphism and subclinical atherosclerosis markers**

Previous studies have shown an increased frequency of subclinical atherosclerosis in RA patients without clinically evident CV disease (González-Juanatey et al., 2003a, 2003b). Because of that, we also aimed to establish the possible influence of this TLR4 polymorphism in the development of subclinical atherosclerosis using two well-defined surrogate markers of atherosclerosis, endothelial function, and the carotid IMT (González-Juanatey et al., 2006; González-Gay et al., 2008), which have been proved to be predictors of future CV events in asymptomatic stages of the atherosclerotic disease (González-Gay et al., 2006; Full et al., 2009; Emonts et al., 2011).

Although the results described in the present study confirmed the presence of endothelial dysfunction in patients with long-standing RA (González-Gay et al., 2008), no association between TLR4 rs4986790 polymorphism and markers of subclinical atherosclerosis was found in this series of RA patients. Data from this series of patients with RA without clinically evident CV disease stratified according to the genotype distribution were the following:

- Carotid IMT—mean (standard deviation [SD]): AA (n = 104) 0.74 mm (0.18), AG (n = 8) 0.65 mm (0.12), GG (n = 0); p = 0.20.
- FMD%—mean (SD): AA (n = 124) 5.60 (4.93), AG (n = 10) 7.05 (4.72), GG (n = 0); p = 0.38.

No significant association in genotype distribution was found for NTG% (p = 0.34).

This study had >90% statistical power to detect a difference in carotid IMT of 0.15 mm or higher. Statistical power was >60% to detect a variation of 3.5% or higher in FMD-endothelium-dependent vasodilatation.

In the ANCOVA model adjusted for gender, age at the time of the ultrasonography assessment, follow-up time, absence or presence of shared epitope, and traditional CV risk factors, no significant differences were found according to TLR4 rs4986790 alleles (TLR4 rs4986790 carotid IMT: p = 0.93; FMD: p = 0.71; NTG: p = 0.94).

**Discussion**

Previous studies have described expression of TLR4 by macrophages and endothelial cells within human atherosclerotic lesions (Xu et al., 2001; Edfeldt et al., 2002).

Association of TLR4 rs4986790 allele G polymorphism with reduction of CRP levels and with decreased risk of angiographic coronary artery disease and clinical diabetes was observed in 1894 patients without acute myocardial infarction (MI) undergoing coronary angiography (Kolek et al., 2004). In keeping with this observation, a case-control study on 183 patients with acute coronary syndromes and 216 controls disclosed that the TLR4 rs4986790 allele G was associated with a decreased risk of coronary events independent of the presence of classic CV risk factors (Ameziane et al., 2003). Also, association of this variant with MI has been described (Balistreri et al., 2004) in older subjects.

Taking into account these results that suggested a potential influence of the TLR4 rs4986790 gene polymorphism in the risk of atherosclerosis, we conducted a study in a large series of patients with RA, a disease associated with accelerated atherogenesis and increased incidence of CV events (González-Gay et al., 2005, 2006), to determine whether the TLR4 rs4986790 gene variant might also account for a decreased incidence of CV events in white individuals with RA. However, our data did not confirm any association of the TLR4 rs4986790 gene polymorphism with the risk of CV events in RA patients. It was also the case when we stratified RA patients according to the presence of specific CV events such as coronary heart disease or cerebrovascular accidents. Interestingly, our results in RA are in accordance with a study on 3657 patients with MI and 1211 controls with angiographically normal coronary arteries and without signs or symptoms of MI that did not show influence of the TLR4 gene polymorphism in the risk of MI in Caucasian individuals (Koch et al., 2006). Likewise, another study did not disclose differences in the prevalence of the TLR4 rs4986790 genotypes in patients with cerebral ischemia and control subjects (Reismann et al., 2004). Moreover, when the TLR4 rs4986790 gene polymorphism was assessed in 1400 patients from the Southampton Atherosclerosis Study that were stratified according to the presence of 0, 1, 2, or 3 coronary arteries with >50% stenosis, no significant differences in the genotype distribution were observed (Yang et al., 2003). Further, to the best of our knowledge, genome-wide association studies (GWAS) have not shown associations between TLR4 gene and RA disease susceptibility or CV traits (www.genome.gov/gwastudies, accessed November 30th, 2011).

With regard to surrogate markers of atherosclerosis, a study performed in 287 Dominicans from the Northern Manhattan Study showed an association of carotid plaques with genes involved in inflammation, including the TLR4 gene variant (Gardener et al., 2011). Also, the G allele of the TLR4 gene—Asp299Gly polymorphism was found to be associated with increased carotid artery compliance in young adults (Hernesniemi et al., 2008). Regrettably, in accordance with these negative results in terms of CV events, no associations of surrogate markers of subclinical atherosclerosis in RA patients without clinically evident CV disease (carotid IMT or endothelial function) with the TLR4 rs4986790 (A/G) gene polymorphism were found in our study.

Our negative results are in line with previous reports of our group on several gene polymorphisms implicated in the inflammatory response that failed to establish an association of gene polymorphisms with clinically evident CV disease or subclinical atherosclerosis in Spanish RA patients: other gene variants located outside the major histocompatibility complex region (MHC) (PTPN22, STAT4, and TRAF1/C5), which are also associated with increased disease susceptibility to RA (Palomo-Morales et al., 2010b); polymorphisms implicated in inflammation such as MIF-173 (Palomo-Morales et al., 2010c) or vascular endothelial growth factor A (VEG-FA) polymorphisms (Rodríguez-Rodríguez et al., 2011a), as well. Neither association with CV disease was observed.
when distinct adipokines polymorphisms implicated in the development of metabolic syndrome nor association with the degree of inflammation in RA patients with severe disease was assessed (García-Bermúdez et al., 2011; Rodríguez-Rodríguez et al., 2011b).

In conclusion, shown in our study do not support the hypothesis that TLR4 rs4986790 (+896A>G, Asp299Gly) polymorphism would be a risk factor for CV disease in patients with RA. However, since TLR4 is a mediator of inflammatory reactions (Akira et al., 2001) and atherosclerosis is also an inflammatory disease, further studies in diverse populations with different genetic backgrounds are needed to completely exclude the role of the TLR4 rs4986790 gene polymorphism in the development of the accelerated atherosclerosis found in patients with RA.

Acknowledgments

This study was supported by two grants from Fondo de Investigaciones Sanitarias PI06-0024 and PS09/00748 (Spain). This work was partially supported by RETICS Program, RD08/0075 (RIER) from Instituto de Salud Carlos III (ISCIII), within the VI PN de I+D+i 2008–2011 (FEDER). M.G.B. is supported by a grant from Fundación Española de Reumatología (FER). R.L.M. is supported by a grant by IFIMAV, Santander (Spain). We sincerely thank all the patients for their fundamental collaboration. We thank Sofia Vargas, Sonia García, and Gema Robledo for their outstanding technical assistance. We also thank Mr. Rodrigo Ochoa for his important contribution to the recruitment of patients.

Disclosure Statement

The authors declare that no competing financial interests exist.

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Received for publication December 16, 2011; received in revised form January 25, 2012; accepted January 26, 2012.