Toll-like receptor 4 gene polymorphisms and myocardial infarction: no association in a Caucasian population

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KEYWORDS
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Introduction

The toll-like receptor 4 (TLR4) belongs to a family of pattern recognition molecules and is predominantly known for its role as an important mediator of innate and adaptive immunity.1–3 TLR4-dependent proinflammatory signalling has been implicated in the initiation, progression, and plaque destabilization stages of atherosclerosis.4–11 In agreement with detrimental properties attributed to TLR4, deficiency of TLR4 was reported to confer protection against neointima formation and arterial remodelling.6,10,12

With the use of single nucleotide polymorphisms (SNPs) as molecular markers, efforts were undertaken to demonstrate associations of the human TLR4 gene (TLR4; gene map locus 9q32-q33) with atherosclerosis and its clinical complications, including myocardial infarction (MI) and stroke.13–24 Attention has concentrated on co-segregating SNPs located in exon 3 of TLR4, an A to G substitution at position +896, relative to the first nucleotide of the start codon, and a C to T transition at position +1196.25 The 896A/G (rs4986790) and 1196C/T (rs4986791) SNPs cause amino acid exchanges at positions 299 (Asp299Gly) and 399 (Thr399Ile) which are located in the extracellular domain of the receptor.25 The 896G allele was found,14,20,25 but not always,16,19,23 to be associated with lower levels of proinflammatory serum markers, many of which have been implicated in atherogenesis. Results on the relationship of the 896A/G SNP (alone or in combination with the 1196C/T SNP) with MI in Caucasians were inconsistent between studies: for example, the 896G allele was linked to a reduced risk or increased risk or was reported not to be associated with the risk of MI.13–19 We investigated whether genotypes and haplotypes of the 896A/G and 1196C/T polymorphisms of the TLR4 gene or haplotypes based on these polymorphisms are not associated with MI.

Aims

The toll-like receptor 4 (TLR4) is predominantly known for its role as an important mediator of immune reactions and has been implicated in the initiation, progression, and plaque destabilization stages of atherosclerosis. We investigated whether genotypes and haplotypes of the 896A/G (Asp299Gly; rs4986790) and 1196C/T (Thr399Ile; rs4986791) single nucleotide polymorphisms of the gene encoding the TLR4 were associated with myocardial infarction (MI) in a large Caucasian sample.

Methods and results

The case group included 3657 patients with MI and the control group comprised 1211 individuals with angiographically normal coronary arteries and without signs or symptoms of MI. Genotypes were determined with the use of TaqMan assays. Genotype distributions of the 896A/G and 1196C/T polymorphisms were not significantly different between the control and patient groups (P > 0.30). The frequencies of haplotypes defined by the 896A/G and 1196C/T polymorphisms were similar between the control group and the patient group (P > 0.16). In addition, the distributions of haplotype-defined genotypes (diplotypes) were not significantly different between the control group and the patient group (P > 0.12). Separate analyses in women and men did not reveal sex-related associations of specific genotypes or haplotypes of the polymorphisms with MI (P > 0.11).

Conclusion

The results indicate that the 896A/G and 1196C/T polymorphisms of the TLR4 gene or haplotypes based on these polymorphisms are not associated with MI.

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95.2% (n = 4868) of the individuals who agreed to participate in the study. These individuals, 3657 patients with MI and 1211 controls, constituted the study population. Entry of data into a common database containing the participating clinics was done manually (anamnestic, clinical, angiographic, and genetic data) or by electronic transfer (clinical laboratory data). Source data check was done with 20% of the data. The study protocol was approved by the Institutional Ethics Committee and the reported investigations were in accordance with the principles of the current version of the Declaration of Helsinki.

Definitions
The diagnosis of MI was established in the presence of chest pain lasting ≥20 min combined with ST-segment elevation or pathologi-cal Q waves on a surface electrocardiogram. Patients with MI had to show either an angiographically occluded infarct-related artery or regional wall motion abnormalities corresponding to the electro-cardiographic infarct localization or both. Individuals were considered disease-free and therefore eligible as controls when their coronary arteries were angiographically normal and when they had no history of MI, no symptoms suggestive of MI, no electrocardio-graphic signs of MI, no regional wall motion abnormalities, and no relevant valvular abnormalities in echocardiograms. Coronary angiography in the control individuals was performed for the evaluation of chest pain. Systemic arterial hypertension was defined as a systolic blood pressure of ≥140 mmHg and/or a diastolic blood pressure of ≥90 mmHg.26 on at least two separate occasions, or anti-hypertensive treatment. Hypercholesterolaemia was defined as a documented total cholesterol value ≥240 mg/dL (≥6.2 mmol/L) or current treatment with cholesterol-lowering medication. Persons reporting regular smoking in the previous 6 months were considered as current smokers. Diabetes mellitus was defined as the presence of an active treatment with insulin or an oral anti-diabetic agent; for patients on dietary treatment, documentation of an abnormal fasting blood glucose, or glucose tolerance test based on the World Health Organization criteria27 was required for establishing this diagnosis.

Determination of TLR4 genotypes
Genomic DNA was extracted from peripheral blood leukocytes with the QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) or the High Pure PCR Template Preparation Kit (Roche Applied Science, Mannheim, Germany). Genotype analysis was performed with allele-specific fluorogenic oligonucleotide probes in an assay combining the polymerase chain reaction (PCR) and the 5′ nuclease reaction (TaqMan technique; Applied Biosystems, Darmstadt, Germany). Primers and probes were established with the Primer Express software (version 2.0.0; Applied Biosystems) after import of a DNA sequence (Homo sapiens TLR4 gene, TLR4A allele) deposited under accession number AF177765 in GenBank ([http://www.ncbi.nlm.nih.gov/; last access June 28, 2006]). The primers used for amplification of the 896A/G SNP (Asp299Gly; rs4986790) were 5′-GAAGAAACAT TTGAAGAATTCCGATTAGCATACTTAGAC 3′ and the primers used for amplification of the 1196C/T SNP (1199 (1199G/A SNP; rs4987233)) were 5′-TTGGGACAA CAGCT 3′-MGB (for 1196T). Oligonucleotides were synthesized by Applied Biosystems. The two-step thermocycling procedure consisted of 35 cycles of denaturation at 92°C for 15 s and primer annealing and extension at 60°C for 1 min. After cycling on a GeneAmp PCR System 9600 or 9700 (Applied Biosystems), endpoint fluorescent data acquisition and genotype calling was achieved on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). As a control, genotyping was repeated for 20% of the samples with the use of DNA prepared separately from the original blood sample. The ability of the TaqMan systems to provide correct genotype data was verified in separate analyses of 200 different PCR products with the allele-discriminating restriction enzyme Bcli (New England Biolabs, Frankfurt am Main, Germany) (896A/G SNP) and sequence analysis of 1196C/T SNP. This test included the samples (n = 59) with the relatively rare genotype combinations 896AA/1196CT (AA/CT), AG/CC, AG/TT, and GG/TT. The results obtained with the different methods were fully corresponding, which demonstrated the reliability of the TaqMan systems that were used for genotyping of the 896A/G and 1196C/T SNPs in the entire study population. Genotype determination was done by workers who had no knowledge of clinical, laboratory, or angiographic data of the individuals enrolled in the study.

A rare A variant at position +1199 (1199G/A SNP; rs4987233) located close to the 1196C/T SNP may interfere with correct 1196C/T genotype assignment. Presence of the 1199A allele was not observed in 200 different DNA samples examined with sequence analysis. For this reason and because of its reported extreme rarity ([http://www.ncbi.nlm.nih.gov/entrez/; last access June 28, 2006]), we considered it unlikely that the existence of the 1199A allele had a significant influence on the results of this study.

Statistical analysis
The analysis consisted of comparing separately genotype distributions, haplotype frequencies, and haplotype-based diplotype distributions between the control group and the group of patients with MI. In addition, separate analyses were performed in the groups of men and women because a sex-specific association of the 896G/1196T haplotype with MI was observed in a Swedish population.19 Discrete variables are expressed as counts (percentage) and compared with the use of the χ2 test or Fisher’s exact test, as appropriate. Continuous variables are expressed as mean ± SD and compared by means of the unpaired, two-sided t test. Genotype frequencies in the control and case groups were compared with values predicted by Hardy-Weinberg equilibrium with the use of the χ2 test. The measures of linkage disequilibrium (r2 and D′) between the 896A/G and 1196C/T SNPs were calculated from primary genotype data with the use of the software package Haplovie.28 A partition-igation-expectation-maximization algorithm was used to infer haplotype patterns from the genotype data of the SNPs.29 Statistical significance was accepted for P-values <0.05.

Results
The main baseline characteristics of the control group and the group of patients with MI are shown in Table 1. Mean age of the patients was higher than that of the control group, the proportion of women was lower in the patient group than in the control group, and history of arterial hypertension and hypercholesterolaemia, current cigarette smoking, and diabetes mellitus were more prevalent in the patient group than in the control group (P < 0.0001 for all comparisons; Table 1). The genotype distributions of the polymorphisms were not significantly different between the control group and the patient group (P > 0.30; [Table 2]). Genotype distributions of the SNPs in the control and patient groups were consistent with those expected
for samples in Hardy–Weinberg equilibrium (P ≥ 0.22). Calculated frequencies of the major and minor alleles of either SNP were similar in the control and patient groups (P ≥ 0.18; Table 2). ORs were 0.86 (95% CI 0.70–1.05) for the comparison of MI risk between the 896G allele carriers and the 896AA genotype carriers and 0.88 (95% CI, 0.72–1.0%) for the comparison between the 896A/G and 1196C/T SNPs. The possibility existed that a specific combination of the alleles in the 896A/G and 1196C/T SNPs was associated with MI. We observed a high degree of linkage disequilibrium between the 896A/G and 1196C/T SNPs (D′ = 0.98; 95% CI 0.96–1.00; r² = 0.92). Haplotype phases were assigned unambiguously in the individuals who were homozygous at least at one of the two polymorphic sites (n = 4382). Among these individuals, the proportions of the 896A/1196C (AC), GT, AT, and GC haplotypes were 99.1, 0.4, 0.4 and 0.1%, respectively. Because of the predominance of the AC haplotype and the relative rarity of the AT and GC haplotypes among the unambiguously typed individuals, the haplotypes AC and GT were assigned to the 486 carriers of the double heterozygous genotype combination 896AG/1196CT. The probability is 0.99994 that this inference is correct, indicating that the assignment was incorrect theoretically in less than one of the 486 double heterozygous individuals. The calculated frequencies of each of the four haplotypes were not significantly different between the control and patient groups (P ≥ 0.12; Table 4). ORs were 1.13 (95% CI 0.92–1.38) for the comparison between the AC/AC diplotype and the other diplotypes and 0.85 (95% CI 0.69–1.04) for the comparison between the AC/AC diplotype and the other diplotypes and 0.85 (95% CI 0.69–1.04) for the comparison between the AC/AC diplotype and the other diplotypes.

Figure 1 shows the structure of the TLR4 genomic region on chromosome 9 and the positions of SNPs in this region as obtained from the HapMap database of common variation in the human genome. Pair-wise correlations between SNPs indicated a high degree of linkage disequilibrium throughout the TLR4 region (Figure 1). The haplotypes of the 896A/G and 1196C/T SNPs yielded a marker system with 6.4% heterozygosity according to HapMap data on the CEU (Centre d’Etude du Polymorphisme Humain) sample and 11.0% heterozygosity according to the results obtained with the present sample. For comparison, typing all tag SNPs suggested from HapMap data (n = 9), a 6-haplotype marker system with a heterozygosity of 73.1% can be inferred. Thus, the present haplotype results captured

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**Table 1** Baseline characteristics of the control group and the group of patients with MI

<table>
<thead>
<tr>
<th></th>
<th>Control group (n = 1211)</th>
<th>MI group (n = 3657)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>60.3 ± 11.9</td>
<td>64.0 ± 12.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Women</td>
<td>598 (49.4)</td>
<td>885 (24.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Arterial hypertension</td>
<td>589 (48.6)</td>
<td>2246 (61.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>602 (49.7)</td>
<td>2067 (56.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Current cigarette smoking</td>
<td>134 (12.5)</td>
<td>1849 (50.6)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>65 (5.4)</td>
<td>754 (20.6)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Age is mean ± SD; other variables are presented as number (%) of control individuals and MI patients.

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**Table 2** Genotype distributions and minor allele frequencies of the TLR4 896A/G (Asp299Gly) and 1196C/T (Thr399Ile) polymorphisms in the control and MI groups

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control group</th>
<th>MI group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>896A/G (Asp299Gly)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA (AspAsp)</td>
<td>1069 (88.3)</td>
<td>3283 (89.8)</td>
<td>0.30</td>
</tr>
<tr>
<td>AG (AspGly)</td>
<td>138 (11.4)</td>
<td>360 (9.8)</td>
<td></td>
</tr>
<tr>
<td>GG (GlyGly)</td>
<td>4 (0.3)</td>
<td>14 (0.4)</td>
<td></td>
</tr>
<tr>
<td>1196C/T (Thr399Ile)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC (ThrThr)</td>
<td>1066 (88.0)</td>
<td>3267 (89.3)</td>
<td>0.44</td>
</tr>
<tr>
<td>CT (ThrIle)</td>
<td>140 (11.6)</td>
<td>375 (10.3)</td>
<td></td>
</tr>
<tr>
<td>TT (IleIle)</td>
<td>5 (0.4)</td>
<td>15 (0.4)</td>
<td></td>
</tr>
<tr>
<td>Minor allele</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>896G (Gly)</td>
<td>146 (6.0)</td>
<td>388 (5.3)</td>
<td>0.18</td>
</tr>
<tr>
<td>1196T (Ile)</td>
<td>150 (6.2)</td>
<td>405 (5.5)</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Data are presented as number (%) of genotypes or major alleles in the control (n = 1211) and MI (n = 3657) groups.

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**Table 3** Distributions of haplotypes of the TLR4 896A/G (Asp299Gly) and 1196C/T (Thr399Ile) polymorphisms in the control and MI groups

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Control group</th>
<th>MI group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC (AspThr)</td>
<td>2270 (93.7)</td>
<td>6901 (94.4)</td>
<td>0.25</td>
</tr>
<tr>
<td>GT (GlyIle)</td>
<td>144 (5.9)</td>
<td>380 (5.2)</td>
<td>0.16</td>
</tr>
<tr>
<td>AT (AspIle)</td>
<td>6 (0.2)</td>
<td>25 (0.3)</td>
<td>0.48</td>
</tr>
<tr>
<td>GC (GlyThr)</td>
<td>2 (0.1)</td>
<td>8 (0.1)</td>
<td>0.94</td>
</tr>
</tbody>
</table>

Data are presented as number (%) of haplotypes in the control and MI groups.

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**Table 4** Distributions of diplotypes of the TLR4 896A/G (Asp299Gly) and 1196C/T (Thr399Ile) polymorphisms in the control and MI groups

<table>
<thead>
<tr>
<th>Diplotype</th>
<th>Control group</th>
<th>MI group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC/AC (AspThr/AspThr)</td>
<td>1064 (87.9)</td>
<td>3259 (89.1)</td>
<td>0.23</td>
</tr>
<tr>
<td>AC/GT (AspThr/GlyIle)</td>
<td>135 (11.1)</td>
<td>351 (9.6)</td>
<td>0.12</td>
</tr>
<tr>
<td>AC/AT (AspThr/AspIle)</td>
<td>5 (0.4)</td>
<td>24 (0.7)</td>
<td>0.34</td>
</tr>
<tr>
<td>AT/GT (GlyIle/GlyIle)</td>
<td>4 (0.3)</td>
<td>14 (0.4)</td>
<td>0.96</td>
</tr>
<tr>
<td>AT/GC (AspThr/GlyThr)</td>
<td>2 (0.2)</td>
<td>8 (0.2)</td>
<td>0.94</td>
</tr>
<tr>
<td>AT/GT (AspIle/GlyIle)</td>
<td>1 (0.1)</td>
<td>1 (0.03)</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Data are presented as number (%) of diplotypes in the control and MI groups.
15% of the genetic variance, calculated as heterozygosity, at the TLR4 locus.

We addressed the question whether sex-specific relationships existed between the 896A/G and 1196C/T SNPs and MI. The genotype distributions of the SNPs were not essentially different between the women and men (\( P = 0.68 \); 896A/G SNP and 1196C/T SNP). Comparisons of the genotype distributions between the women in the control group and the patient group and between the men in the two groups did not reveal significant differences (\( P / C 0.11 \)). Haplotype frequencies were not substantially different between the women in the control and patient groups (overall \( P = 0.86 \)) and between the men in the two groups (overall \( P = 0.55 \)). Finally, diplotype distributions were not associated with MI in the women (overall \( P = 0.83 \)) and men (overall \( P = 0.30 \)).

**Discussion**

The present results show that the 896A/G and 1196C/T SNPs of TLR4 are not associated with MI. Lack of association relates to alleles, genotypes, haplotypes, and diplotypes, and applies to women and men. The probability of a false negative association result because of population stratification is relatively low, because the study participants were consecutively recruited from a defined geographic area of southern Germany with limited recent immigration. The sample size provided the analysis with 93% power to detect a 30% decrease in the risk of MI among the carriers of the 896G allele at a two-sided \( \alpha \)-error of 0.05. Because all control subjects had some indication for coronary angiography, they did not represent a typical sample of healthy controls. However, the control group appears not to be fundamentally different from typical control groups, because the genotype distributions were not significantly different from the genotype distributions observed in other control groups that consisted predominantly or completely of Whites.\(^{13,14,16}\)

In agreement with one of the negative findings of this study, no association of the 896A/G SNP with MI was observed in a sample of predominantly white US men that included 370 MI patients and 695 individuals free from vascular diseases [adjusted OR 0.97, 95% CI 0.62–1.52; \( P = 0.89 \) (additive model)].\(^{13}\) The present study extends...
the prior negative result\textsuperscript{13} to women, the 1196C/T SNP, and haplotypes of the 896A/G and 1196C/T SNPs.

We compared the risk of MI between carriers of the 896G allele and carriers of the 896AA genotype in the present sample and in samples examined in six prior studies from Europe.\textsuperscript{14–19} No significant risk effects were found in the studies, except in one prior study,\textsuperscript{16} in which the 896G carriers had a significantly higher risk of MI than the 896AA carriers (Figure 2). Combined analysis of the study samples (5926 cases and 4375 control individuals) yielded an OR of 0.90 (95% CI 0.68–1.19) under the random effects model (Figure 2). There was significant heterogeneity between the sample sets ($P = 0.01$). In one of the prior studies with a negative result in the present comparison, a French study that included 183 patients and 216 healthy control subjects, a significant association was observed between the 896G allele and a lower risk of acute coronary events (MI or unstable angina pectoris) after adjustments had been made for cardiovascular risk factors (adjusted OR 0.41; 95% CI 0.18–0.95; $P = 0.037$).\textsuperscript{14}

In some of the prior studies, an association of the 896G allele or GT haplotype with MI was only found in specific subgroups but not when the study population was assessed on the whole.\textsuperscript{17–19} The effect of treatment with statins on the association of the 896G allele with cardiovascular events, including MI, was examined in a cohort of Dutch men with angiographically documented coronary atherosclerosis.\textsuperscript{17} Among the patients with statin therapy ($n = 329$), the carriers of the 896G allele showed a lower rate of adverse cardiovascular events during a 2-year follow-up period than the group without this allele (2.0 vs. 11.5%, $P = 0.045$), whereas allele-specific event rates were not significantly different among the patients with placebo ($n = 326; P = 0.10$).\textsuperscript{17} Similarly, the influence of statins on the relationship between the 896G allele and MI was examined in a sample of UK Caucasians with angiographic evidence of coronary artery disease (CAD).\textsuperscript{18} In the subgroup with statins ($n = 588$), the patients with the 896G allele had a lower frequency of MI than the patients without this allele (8.8 vs. 14.2%; adjusted OR 0.49; 95% CI 0.27–0.78; $P = 0.015$).\textsuperscript{18} No significant effect of the 896G allele on MI risk was found among the patients who were not treated with statins ($n = 490$; adjusted OR 1.28; 95% CI 0.64–2.57; $P = 0.49$).\textsuperscript{18} In a study with participants from the Stockholm (Sweden) region, the GT haplotype showed borderline association with an increased risk of MI in the subgroup of men ($n = 1852$; adjusted OR 1.4, 95% CI 1.0–2.0), but no association was found in the subgroup of women ($n = 837$; adjusted OR 0.9, 95% CI 0.5–1.5).\textsuperscript{19} Such a sex-specific relationship between the GT haplotype and MI was not detected in the present study.

The relatively low prevalence of the 896G allele is a potential limitation in association studies, especially when small samples are analyzed. There are examples with other polymorphisms that underpowered studies with significant effects were misleading. Such examples include studies on the association of polymorphisms in the angiotensin I-converting enzyme gene, paraoxonase gene, and genes of haemostatic factors with the risk of MI or coronary heart disease.\textsuperscript{31–33}

In conclusion, the results of this study argue against an association of the 896A/G and 1196C/T SNPs of TLR4 with MI. We infer from the present findings that the 896A/G and 1196C/T SNPs are not useful as molecular markers of MI. However, despite the negative results, a causal relationship may exist between TLR4, known as a mediator of inflammatory reactions,\textsuperscript{1–3} and MI, a disease with an inflammatory background.\textsuperscript{34–36}

\textbf{Supplementary material}

Supplementary material is available at European Heart Journal online.
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Conflict of interest: none declared.

References

14. Yang IA, Holloway JW, Ye S. TLR4 Asp299Gly polymorphism is not associ-
17. Arbour NC, Lorenz E, Schutte BC, Zanber J, Kline JN, Jones M, Frees K, Watt JL, Schwartz DA. TLR4 mutations are associated with endotoxin hy-