Toll-like Receptor 4 Asp299Gly Gene Polymorphism and Risk of Atherothrombosis

Robert Y.L. Zee, PhD; Hillary H. Hegener, BS; Jessica Gould, BS; Paul M. Ridker, MD

Background and Purpose—Recent findings of an association between a functional toll-like receptor 4 (TLR4) D299G gene variant and reduced risk of atherothrombotic disorders have generated great interest.

Methods—We evaluated the TLR4 D299G polymorphism among 695 individuals with incident myocardial infarction (MI) or stroke and among 695 age- and smoking-matched individuals who remained free of reported cardiovascular disease during follow-up within the Physicians’ Health Study.

Results—Overall, we observed little evidence of association between the D299G polymorphism and risk of any atherothrombotic event (P=0.25), incident MI (P=0.89), or stroke (P=0.09), assuming an additive model. Adjusting for traditional cardiovascular risk factors or assuming a dominant model yielded similar null findings. Whereas the observed carrier frequency of the D299G polymorphism in our data (13.0%) is consistent with those observed in most other studies, it was higher than the 6.8% carrier frequency observed in the initial study that suggested a protective effect for this gene variant. Thus, this former association may have been caused, in part, by an underestimation of the control frequency.

Conclusion—in contrast to previous data, the D299G TLR4 polymorphism was not associated with risk of incident MI or stroke in this large prospective study of US men. (Stroke. 2005;36:154-157.)

Key Words: cardiovascular diseases ■ genetics ■ thrombosis ■ toll-like receptors

Data from human and animal studies have shown an upregulated expression of toll-like receptors, particularly toll-like receptor 4, in cardiomyopathies and experimental cardiac dysfunction, as well as in neurodegeneration. Mutations in the toll-like receptor 4 gene have been associated with differences in lipopolysaccharide responsiveness, resulting in a differential production of pro-inflammatory cytokines. Recently, a D299G polymorphism (rs4986790) in the TLR4 gene (LocusID 7099) has been found to be associated with altered risk of atherosclerosis in some, but not all, studies. We investigated the role of the TLR4 D299G polymorphism as a risk marker for atherothrombosis in the Physicians’ Health Study.

Materials and Methods

Study Design
We used a nested case-control design within the Physicians’ Health Study. Before randomization, 14,916 participants provided an EDTA-anticoagulated blood sample that was stored for genetic analysis. All participants were free of previous myocardial infarction (MI), stroke, transient ischemic attacks, and cancer at study entry. Yearly follow-up self-report questionnaires provide reliable information on newly developed diseases and the presence/absence of other cardiovascular risk factors. History of cardiovascular risk factors, such as hypertension, diabetes or hyperlipidemia, was defined by self-report of diagnosis at entry into the study. For all reported incident vascular events occurring after study enrollment, hospital records, death certificates, and autopsy reports were requested and reviewed by an endpoint committee using standardized diagnostic criteria.

The diagnosis of MI was confirmed by evidence of symptoms in the presence of either diagnostic elevations of cardiac enzymes or diagnostic changes on electrocardiograms. In the case of fatal events, the diagnosis of MI was also accepted based on autopsy findings. Stroke was defined by the presence of a new focal neurological deficit, with symptoms and signs persisting for >24 hours, and was ascertained from blinded review of medical records, autopsy results, and the judgment of a board-certified neurologist. Stroke was classified as ischemic, hemorrhagic, or unknown, on the basis of clinical reports, computed tomographic, or magnetic resonance image scanning.

Six hundred ninety-five cases were identified. For each case, a control matched by age, smoking history, and length of follow-up were chosen among those subjects who remained free of vascular diseases. The study was approved by the Brigham and Women’s Hospital Institutional Review Board for Human Subjects Research.

Genotype Determination
Genotyping was performed using an ABI Assay-by-Demand allelic discrimination method (Applied Biosystems, Foster City, CA) and is available at http://www.strokeaha.org DOI: 10.1161/01.STR.0000149948.31879.f0
Calif) with: forward primer, 5'-tga cca ttg aag aat tcc gat tagca-3'; reverse primer, 5'-ACA CTC ACC AGG GAA AAT GAA GAA-3'; D-allele specific probe, 5'-VIC TAC CTC GAT GAT ATT ATT-3'; and G-allele specific probe, 5'-FAM CCT CGA TGG TAT TAT T-3'. Amplification reactions were performed on an ABI7900 Sequence Detection System according to the manufacturer's specifications. Genotype scoring was performed by 2 independent observers. Discordant results (<1% of all scoring) were resolved by a joint reading and, when necessary, a repeat genotyping. Results were scored blinded to case-control status.

Statistical Analysis

Genotype frequencies among cases and controls were compared with values predicted by Hardy–Weinberg equilibrium using the \( \chi^2 \) test. Relative risks associated with each genotype, with 95% confidence intervals, were calculated by conditional logistic regression analysis—an adaptation of the proportional hazards failure time model of Cox for a nested case-control design—conditioning on the matching by age, smoking status, and length of follow-up since randomization, and further controlling for randomized treatment assignment, hypertension, diabetes, and body mass index. Prespecified subgroup analyses were also performed on the basis of smoking status, and the presence/absence of other cardiovascular risk factors. A 2-tailed probability value of 0.05 was considered a statistically significant result.

Results

Baseline characteristics of cases and controls are shown in Table 1. As expected, the case participants had a higher prevalence of hypertension and diabetes at baseline as compared with controls.

<table>
<thead>
<tr>
<th>Genotype Distribution and Conditional Logistic Regression Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case Events</td>
</tr>
<tr>
<td>Genotype</td>
</tr>
<tr>
<td>D299D</td>
</tr>
<tr>
<td>D299G</td>
</tr>
<tr>
<td>Allele</td>
</tr>
<tr>
<td>D299</td>
</tr>
<tr>
<td>G299</td>
</tr>
<tr>
<td>Additive mode</td>
</tr>
<tr>
<td>Any thrombotic event</td>
</tr>
<tr>
<td>MI</td>
</tr>
<tr>
<td>Stroke</td>
</tr>
<tr>
<td>Dominant mode</td>
</tr>
<tr>
<td>Any thrombotic event</td>
</tr>
<tr>
<td>MI</td>
</tr>
<tr>
<td>Stroke</td>
</tr>
</tbody>
</table>

*All genotype distributions in Hardy–Weinberg equilibrium. All \( P \) values for the differences between patients (or subgroups of cases) and controls in genotype distribution were nonsignificant.

Crude indicates matched on age and smoking; adjusted, further controlling for randomized treatment group, body mass index, history of hypertension, and presence or absence of diabetes.
The genotype frequencies for the control study population were in Hardy–Weinberg equilibrium. Thus, the genotype frequencies were similar between any arterial event, MI, or stroke, and controls (Table 2). The crude conditional logistic regression analysis showed no significant association with risk of atherothrombosis (Table 2). Further adjustment for body mass index, hypertension, diabetes, and randomized treatment assignment yielded similar null findings (Table 2). Although statistically nonsignificant, we observed a trend of risk reduction among stroke cases (Table 2). In the prespecified subgroup analyses, we found no evidence of an effect modification by age, smoking status, or other cardiovascular risk factor (data not shown), nor in analyses limited to ischemic stroke cases (data not shown).

Discussion

In our prospective investigation, we found little evidence of an association between the D299G polymorphism and risk of future MI or stroke, nor any effect modification by known cardiovascular risk factors. For the endpoint of MI, our data indicate a completely null effect (Table 2), an outcome at odds with the findings by Kiechl et al. It is thus important to consider alternative explanations for these apparent discrepancy.

The sample-size of our study, its prospective design, and the use of a closed population sampling scheme in which subsequent case status was determined solely by the development of disease rather than any selection criteria designed by the investigators all strongly reduce the possibility that our null result is caused by chance or bias. Further, the wide availability of covariate information in our study greatly reduces the possibility of residual uncontrolled confounding. Nonetheless, our study cohort consists of predominantly white males with distinct socioeconomic status (physicians), so our data cannot be generalized to other ethnic groups, women, and other populations. Because there are no in vivo/functional data related to D299G presented, further discussion regarding its biological implication is unwarranted.

Given this situation, an alternative hypothesis is that the D299G allele frequencies may differ importantly between studies, which could be caused by population/ethnic differences. In our study, the G299 allele frequency in our control group was 12.95%, a rate consistent with the overall control frequency of 12.36% observed in a cumulative analysis (Table 3). By contrast, the control carrier frequency in the study by Kiechl et al was only 6.79%. Thus, one possibility is that the “protective” effect of the G299 allele observed in previous data are caused more by an underestimate of the background allele frequency than by any true increase in frequency among those with atherothrombotic diseases. This alternative explanation also helps explain 2 other null studies of the D299G polymorphism that have recently been presented (Table 3). Of note, Kiechl et al reported an interaction of D299G with T399I variant, and further investigation of the interaction between these 2 variants is warranted.

In summary, these prospective data show little evidence of association between the TLR4 D299G gene polymorphism and risks of incident MI or stroke.

References


Toll-like Receptor 4 Asp299Gly Gene Polymorphism and Risk of Atherothrombosis
Robert Y.L. Zee, Hillary H. Hegener, Jessica Gould and Paul M. Ridker

*Stroke*. 2005;36:154-157; originally published online December 2, 2004;
doi: 10.1161/01.STR.0000149948.31879.f0

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/36/1/154

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org//subscriptions/