Functional Polymorphisms of the Coagulation Factor II Gene (F2) and Susceptibility to Systemic Lupus Erythematosus

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ABSTRACT. Objective. Two F2 functional polymorphisms, rs1799963 (G20210A) and rs3136516 (A19911G), are known to be associated with elevated levels/activity of prothrombin (encoded by F2) and risk of thrombosis. Since patients with systemic lupus erythematosus (SLE) have high risk of thrombosis and accelerated atherosclerosis and also high prevalence of anti-prothrombin antibodies, we hypothesized that these two F2 polymorphisms could affect risk of SLE.

Methods. We investigated these polymorphisms in 627 women with SLE (84% Caucasian Americans, 16% African Americans) and 657 female controls (78% Caucasian Americans, 22% African Americans).

Results. While the rs1799963 A allele was almost absent in African Americans, it was present at ~2% frequency in Caucasian Americans and showed no significant association with SLE. The rs3136516 G allele frequency was significantly higher in Caucasian SLE cases than in controls (48.4% vs 43.7%, respectively) with a co-variate-adjusted odds ratio (OR) of 1.22 (95% CI 1.03–1.46, p = 0.023). The association was replicated in African Americans (rs3136516 G allele frequency 91.2% in cases vs 82.2% in controls) with an adjusted OR of 1.96 (95% CI 1.08–3.58, p = 0.022).

Conclusion. Our results suggest a potential role for prothrombin and the crosstalk between hemostatic and immune/inflammatory systems in SLE and SLE-associated cardiovascular events, which warrants further investigation in independent samples.

Key Indexing Terms:
LUPUS PROTHROMBIN F2 POLYMORPHISM A19911G G20210A
ed phenotypes. Previous studies investigated functional events \(^3\), although the sample sizes were under-
G20210A SNP in relation to SLE-associated cardiovascular events in non-SLE individuals. Some reports examined the as a plausible candidate for susceptibility to SLE and relat-
thrombosis and accelerated atherosclerosis), and the high (1.5%–2% frequency in the general Caucasian population). The extensive crosstalk between hemostasis and inflam-
mation, multiple functions of prothrombin/thrombin that are highly relevant to SLE and SLE-associated microvascular disease and/or cardiovascular events (increased risk of thrombosis and accelerated atherosclerosis), and the high prevalence of anti-prothrombin antibodies in patients with SLE strongly support the prothrombin/thrombin gene (F2) as a plausible candidate for susceptibility to SLE and relat-
ed phenotypes. Previous studies investigated functional F2 polymorphisms mainly for their effects on cardiovascular events in non-SLE individuals. Some reports examined the G20210A SNP in relation to SLE-associated cardiovascular events \(^3\), although the sample sizes were under-
powered to detect the effects of such an uncommon variant (1.5%–2% frequency in the general Caucasian population). To our knowledge, no study has previously examined both F2 rs3136516 (A19911G) and rs1799963 (G20210A) SNP (the 2 well known genetic determinants of plasma pro-
thrombin levels/activity) in relation to risk for SLE, which is the focus of this study.

**Materials and Methods**

**Subjects and data collection.** A total of 1284 women (997 from Pittsburgh, PA, and 287 from Chicago, IL) were included in this study. The Pittsburgh sample comprised 474 women with SLE (417 Caucasian Americans and 57 African Americans, mean age 42.5 ± 11.3 SD y) and 447 age-matched female controls with no apparent history of SLE (411 Caucasian Americans and 36 African Americans, mean age 45.5 ± 13.5 y). In addition, 76 older female African American controls (mean age 67.1 ± 8.0 y) from Pittsburgh were included in the study in order to increase the African American sample size, after confirming that allele frequencies of the SNP of interest were almost identical in the 2 control age groups. The Chicago sample comprised 153 women with SLE (107 Caucasian Americans and 46 African Americans, mean age 44.2 ± 10.5 y) and 134 age-matched female controls (102 Caucasian Americans and 32 African Americans, mean age 47.4 ± 10.0 y).

All SLE patients (≥ 18 yrs of age) met the 1982 or revised 1997 American College of Rheumatology classification criteria for SLE \(^3\). Data were collected at both recruitment sites using identical protocols and laboratory tests. Detailed description of the SNP sample can be found elsewhere \(^3\). Of the 524 Caucasian SLE women in this study, 332 were also characterized for the occurrence of cardiac and vascular events, of which 101 (30.4%) had experienced one or more of the following physician-confirmed events in medical records: myocardial infarction (5.5%), coronary artery bypass graft surgery (3.4%), percutane-
ous transluminal coronary angioplasty (5.8%), angina pectoris (13.4%), cardiac death (1.2%), stroke (5.5%), transient ischemic attack (6.4%), congestive heart failure (4.0%), blood clots (9.7%), or vascular surgery (0.9%). All participants provided written informed consent for genetic research approved by the University of Pittsburgh and the Northwestern University institutional review boards.

**DNA extraction and genotyping.** Buffy coat samples from both recruitment sites were processed for genomic DNA isolation at the same laboratory (University of Pittsburgh Human Genetics Department) using QIAamp DNA Kits (Quagen, Chatsworth, CA, USA). Genotyping of F2 SNP was performed by TaqMan \(^6\) allelic discrimination (Applied Biosystems, Foster City, CA, USA) using ready-made SNP Genotyping Assays (C\(_{11661574_10}\) for rs3136516 and C\(_{8726802_20}\) for rs1799963) and endpoint fluorescence readings on an ABI Prism 7900HT instrument (Applied Biosystems).

**Prothrombin activity measurement.** Plasma prothrombin activity measurements were available for analysis in a subset of Caucasian SLE women (n = 120) at the Pittsburgh site. Prothrombin activity was determined using a chromogenic assay (DiaPharma, West Chester, OH, USA). Briefly, 10 µl plasma was diluted 1:40 with Tris-BSA buffer and mixed with Ecarcin to activate the prothrombin to meizothrombin, which in turn cleaved the thrombin selective chromogenic substrate S-2238. The absorbance, which is proportional to prothrombin activity in the sample, was measured at 405 nm. Serial dilutions of pooled human plasma (Innovative Research, Novi, MI, USA) were used as the standard.

**Statistical methods.** Allele and genotype frequencies were determined by direct counting. Allele frequencies were compared between cases and controls using a standard Z-test of 2 binomial proportions. Recruitment site and age were included as covariates in the logistic regression analysis of genotype distribution differences between cases and controls. Genotype associations were tested under the additive model for the common rs3136516 SNP and the dominant model for the uncommon rs1799963 SNP. Linear regression analysis of the effects of genotypes on prothrombin activity was also performed under the additive model, which included age, body mass index (BMI), and warfarin use as covariates. Association analyses were performed using R statistical software (available from: http://www.r-project.org) packages (SNPassoc, genetics, plotrix). Haplotype distribution was determined using Haplovie (available from: http://www.broad.mit.edu/mpg/haplovie/).
RESULTS

Association analyses of F2 rs3136516 and rs1799963 SNP with SLE risk in Caucasian Americans. The frequency of the rs3136516 G allele was higher in SLE patients than in controls at both Pittsburgh (47.9% vs 43.4%, respectively) and Chicago (50.0% vs 45.0%) sites. In the combined Pittsburgh + Chicago sample (Table 1), the rs3136516 G allele frequency was 48.4% in SLE cases versus 43.7% in controls (p = 0.034). The recruitment site- and age-adjusted OR for the rs3136516 G allele carriers (AA = 0, GA = 1, GG = 2) was 1.22 (95% CI 1.03–1.46, p = 0.023), indicating a modest effect. No significant association was observed for the rs1799963 SNP, which showed comparable allele frequencies between SLE cases and controls (A allele: 2.4% vs 2.0%; p = 0.593, in the combined sample). Haplotype analysis revealed 3 of the 4 expected haplotypes (GG, AG, AA); the fourth haplotype carrying the rs3136516 G and rs1799963 A alleles that are both associated with elevated prothrombin levels/activity was absent (D’ = 1, r² = 0.019).

For hyperprothrombinemia and venous thrombosis in Caucasian populations. The relationship between this poly-

The frequency of the rs3136516 G allele was significantly associated with a modest increase in plasma prothrombin activity (p = 0.039 after adjustment for age, BMI, and warfarin use; Table 2). The effect of the rs3136516 G allele remained significant (p = 0.015) after excluding the cases carrying the rs1799963 A allele (by evaluating only the individuals with wild-type GG genotype for rs1799963).

DISCUSSION

Since first reported in 199612, the uncommon F2 variant, rs1799963 (G20210A), has been established as a risk factor for hyperprothrombinemia and venous thrombosis in Caucasian populations. The relationship between this poly-

Table 1. Allele frequencies and association statistics for F2 rs3136516 SNP in SLE women compared to control women. Only data for successfully genotyped individuals were included in the table.

<table>
<thead>
<tr>
<th>rs3136516</th>
<th>Caucasian Americans</th>
<th>African Americans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls, n = 509</td>
<td>All Cases, n = 519</td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.563</td>
<td>0.516</td>
</tr>
<tr>
<td>G</td>
<td>0.437</td>
<td>0.484</td>
</tr>
<tr>
<td>p***</td>
<td>—</td>
<td>0.034</td>
</tr>
<tr>
<td>OR (95% CI; p³)</td>
<td>1.22 (1.03–1.46; 0.023)</td>
<td>1.20 (0.96–1.49; 0.114)</td>
</tr>
</tbody>
</table>

* Caucasian SLE cases with available cardiovascular data were stratified by the occurrence of cardiac and vascular events (CVE): myocardial infarction, coronary artery bypass graft surgery, percutaneous transluminal coronary angioplasty, angina pectoris, cardiac death, stroke, transient ischemic attack, congestive heart failure, blood clots, or vascular surgery. ** Comparison of the allele frequencies between cases and controls using a standard Z-test of 2 binomial proportions. ³ Odds ratios and p values under additive genetic effect model (AA = 0, GA = 1, GG = 2), adjusted for recruitment site and age.
The mechanism of action of the $F_2$ rs3136516 SNP on SLE risk remains to be determined. The rs3136516 G allele, which was shown to cause more efficient RNA splicing than the A allele, was reported to cause slightly higher prothrombin activity. Consistently, we found in our SLE cases that the rs3136516 G allele was associated with higher plasma prothrombin activity and its effect remained significant after excluding the cases carrying the rs1799963 A allele, which is an established genetic determinant of elevated prothrombin levels and activity (Table 2). The rs1799963 A allele did not appear to increase SLE risk in our sample, although our study was underpowered to detect a small to moderate effect of this uncommon variant, thus its effect on SLE still remains a possibility. Alternatively, the rs3136516 SNP may be influencing the SLE risk by another currently unknown mechanism (i.e., not only affecting the splicing efficiency but also changing the splicing behavior and yielding different isoforms). A direct analysis of the RNA samples from primary liver cells of individuals carrying different rs3136516 genotypes will be necessary to unravel the exact functional effect of this SNP. Another possibility is that the rs3136516 SNP may not be causative itself, but may simply be in strong linkage disequilibrium (LD) with a true causative (yet to be identified) variant residing in $F_2$ or a nearby gene. Current information in the literature and public databases (dbSNP and HapMap) does not indicate the presence of other $F_2$ common SNP strongly correlated with the rs3136516 SNP. Comprehensive resequencing-based analysis of the entire $F_2$ gene and its flanking regions, in conjunction with analysis of prothrombin activity and levels, will help to characterize the true SLE-related causative effects.

The role of prothrombin/thrombin in hemostasis, thrombosis, and occurrence of antiphospholipid antibodies (that are also associated with increased thrombosis risk) has long been recognized. Studies increasingly emphasize that prothrombin has actually a plethora of biological functions that...
also include an important role in inflammation and immune activation. Thrombin, the active form of prothrombin, was shown to be chemotactic for monocytes and neutrophils and can induce several inflammatory responses, including cytokine production and apoptosis. A number of biological pathways are being implicated in SLE pathogenesis and our study indicates that the "hemostasis and its crosstalk with immunity and inflammation" can be added to this growing list.

To our knowledge, this is the first study to evaluate the role of F2 rs3136516 common SNP in relation to susceptibility for SLE. The significant and consistent association of the rs3136516 G allele with SLE risk in both Caucasians and African Americans suggests that this F2 polymorphism might play a role in SLE pathogenesis. Its effect size seems to be modest, although more pronounced among SLE patients who had experienced cardiac and/or vascular events. Nevertheless, replication by independent groups is essential in establishing genetic associations with complex disorders due to various factors that may lead to false-positive associations (i.e., by chance, power issues, population stratification). Our study had more than 60% but less than 80% power to detect the odds ratios reported in our Caucasian and African American samples. Although our sample size was reasonable in Caucasians, it was relatively small in African Americans. The rs3136516 SNP was neither part of the high-density genotyping panels used by recently published genome-wide association studies of SLE nor strongly correlated with any common F2 SNP included in those panels. Therefore, other groups will need to genotype this SNP in their independent large samples in order to replicate our findings, and the cardiovascular status of the participants (cases and controls) is likely to influence the results.

REFERENCES