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Functional polymorphisms of the coagulation factor II gene (*F2*) and susceptibility to systemic lupus erythematosus (SLE)

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Abstract

Objective—Two *F2* functional polymorphisms, rs1799963 (G20210A) and rs3136516 (A19911G), are known to be associated with elevated prothrombin (encoded by *F2*) levels/activity and thrombosis risk. Since systemic lupus erythematosus (SLE) patients 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 SLE risk.

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 controls (48.4% *vs.* 43.7%) with a covariate-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). Stratification of Caucasian SLE patients based on the presence or absence of cardiac and vascular events (CVE) revealed stronger association with the CVE-positive SLE subgroup than the CVE-negative SLE subgroup (OR: 1.42 *vs.* 1.20). Prothrombin activity measurements in a subset of SLE cases demonstrated higher activity in the carriers of the rs3136516 G allele.

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 warrant further investigation in independent samples.

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Keywords

lupus; prothrombin; *F2*; polymorphism; A19911G; G20210A

INTRODUCTION

Prothrombin is a vitamin K-dependent glycoprotein that is primarily synthesized in the liver and secreted into circulating plasma. Upon activation by the prothrombinase complex (activated factor X, factor V, calcium and phospholipids), prothrombin (factor II) is converted to its enzymatically active form thrombin (factor IIa). In the coagulation cascade, thrombin exerts its procoagulant activity by converting soluble fibrinogen into insoluble fibrin strands as well as by activating other coagulation factors (1). Thrombin acts also as an indirect anticoagulant by activating protein C on the surface of endothelial cells in the presence of thrombomodulin. Thrombin is a multifunctional protein and, in addition to its well-known role in the coagulation cascade, it is involved in platelet aggregation, thrombus formation and fibrinolysis, endothelial barrier integrity, immune cell adhesion/activation, inflammation, and tissue reparative processes (2–4). Thrombin is among the key factors that mediate the extensive crosstalk between inflammation and hemostasis, the 2 major processes of defensive host response (5).

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease that predominantly targets women of reproductive age. Immunological and genetic studies suggest that SLE-related pathogenic mechanisms involve various abnormalities in both innate and adaptive immune responses (6–8). It has been well-recognized that SLE patients are at high risk of thrombosis and premature atherosclerosis (9–11). Elevated plasma prothrombin levels/activity are common in the general population and have been shown to be a risk factor for cardiac and vascular events, especially for venous thrombosis (12–14). Moreover, prothrombin is among major antigens recognized by antiphospholipid antibodies that are also associated with cardiovascular thrombotic events in SLE and other conditions (15–19).

Prothrombin levels are under significant genetic influence with heritability estimates reaching 50% and higher (20–22). The gene encoding prothrombin (*F2*) spans ~21 kb on chromosome 11p11 and harbors 14 exons. Two functional *F2* single nucleotide polymorphisms (SNPs), rs3136516 (A19911G) and rs1799963 (G20210A), have been reported to be associated with elevated prothrombin levels/activity and increased thrombosis risk, with the results being more consistent and stronger for the less common G20210A variant (12,23–28). The rs1799963 (located at 3'UTR) minor allele A is believed to affect the RNA metabolism by increasing the mRNA 3'-end formation efficiency (enhanced cleavage and processing) and the mRNA stability (23,29,30). An *in vitro* study (24) demonstrated that the rs3136516 polymorphism (located within the 13th intron that is only 146 bp) is also functional through its effect on an intronic splicing enhancer motif (CAG**G**G); that is, the G allele was found to cause more efficient splicing of intron 13 than the A allele (~30% higher efficiency) due to the disruption of the intronic enhancer motif by the A allele.

The extensive crosstalk between hemostasis and inflammation, 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 SLE patients strongly support the prothrombin/thrombin gene (*F2*) as a plausible candidate for susceptibility to SLE and related phenotypes. Prior studies investigated functional *F2*

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polymorphisms mainly for their effects on cardiovascular events in non-SLE individuals. Some reports examined the G20210A single nucleotide polymorphism (SNP) in relation to SLE-associated cardiovascular events (31–35), 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 been undertaken to examine both *F2* rs3136516 (A19911G) and rs1799963 (G20210A) SNPs (the two well-known genetic determinants of plasma prothrombin levels/activity) in relation to SLE risk, which is the focus of this study.

SUBJECTS AND METHODS

Subjects and data collection

A total of 1,284 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 ± SD = 42.5 ± 11.3 yrs) and 447 age-matched female controls with no apparent history of SLE (411 Caucasian Americans and 36 African Americans, mean age ± SD = 45.5 ± 13.5 yrs). In addition, 76 African American older female controls (mean age ± SD = 67.1 ± 8.0 yrs) from Pittsburgh were included in the study in order to increase the African American sample size, after confirming that allele frequencies of the SNP(s) of interest were almost identical in the two control age groups. The Chicago sample comprised 153 women with SLE (107 Caucasian Americans and 46 African Americans, mean age ± SD = 44.2 ± 10.5 yrs) and 134 age-matched female controls (102 Caucasian Americans and 32 African Americans, mean age ± SD = 47.4 ± 10.0 yrs).

All SLE patients (18 years of age or older) met the 1982 or revised 1997 American College of Rheumatology (ACR) classification criteria for SLE (36,37). Data were collected at both recruitment sites using identical protocols and laboratory tests. More detailed description of the SLE sample can be found elsewhere (38–40). Of the 524 Caucasian SLE women included 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%), percutaneous 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 (Qiagen, Chatsworth, CA). Genotyping of *F2* SNPs was performed by TaqMan® allelic discrimination (Applied Biosystems, Foster City, CA) using the pre-made SNP Genotyping Assays (C__11661574_10 for rs3136516 and C___8726802_20 for rs1799963) and end-point 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). Briefly, 10 μl plasma was diluted 1:40 with tris-BSA buffer and mixed with Ecarin to activate the prothrombin to

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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) 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 two 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, BMI, and Warfarin use as covariates. Association analyses were performed using R statistical software (http://www.r-project.org) packages (SNPassoc, genetics, plotrix). Haplotype distribution was determined using Haploview (http://www.broad.mit.edu/mpg/haploview/).

RESULTS

Association analyses of *F2* rs3136516 and rs1799963 SNPs 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%) 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 *vs.* 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% and $P = 0.593$ in the combined sample). Haplotype analysis revealed three of the four expected haplotypes (GG, AG, AA); the 4th haplotype carrying the rs3136516 G and rs1799963 A alleles that are both associated with elevated prothrombin levels/activity was absent (D'=1, r^2=0.019). The common haplotype carrying the rs3136516 risk allele G was over-represented in cases (GG frequency: 0.484 in cases *vs.* 0.437 in controls) while the one carrying the protective allele A was over-represented in controls (AG frequency: 0.543 in controls *vs.* 0.493 in cases).

Next, we wanted to determine whether the association of the rs3136516 SNP with SLE risk might have been influenced by the cardiovascular status of SLE patients. For this purpose, we stratified the Caucasian SLE patients who had been characterized for cardiac and vascular events (CVE) into two subgroups based on the presence or absence of CVE and compared them separately with the controls (Table 1). Although the association trend was present in both SLE subgroups, the association of the rs3136516 SNP was stronger with the CVE-positive group (n=100, OR: 1.42) than with the CVE-negative group (n=228, OR: 1.20) as compared to the healthy controls (n=509) and remained significant ($P = 0.024$ *vs.* 0.114) despite more dramatically reduced sample size. The comparison between SLE patients with and without CVE did not yield a significant result, although the numbers were relatively small (100 *vs.* 228 patients) for meaningful analysis considering the modest effect sizes observed in Caucasians.

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Association analysis of *F2* rs3136516 SNP with SLE risk in African Americans

Following the observation of a significant association of the rs3136516 SNP with SLE in Caucasian Americans, we sought for a similar association in African Americans. As in Caucasian Americans, the rs3136516 G allele frequency was higher in African American women with SLE than in controls at both Pittsburgh (92.9% *vs.* 82.0%) and Chicago (89.1% *vs.* 82.8%) sites. In the combined sample (Table 1), the rs3136516 G allele frequency was 91.2% in SLE cases *vs.* 82.2% in controls ($P = 0.003$). The OR for the rs3136516 G allele carriers (AA=0, GA=1, GG=2) was 1.96 (95% CI: 1.08–3.58; $P = 0.022$) after adjusting for the effects of the recruitment site and age. Only two African American individuals were found to carry the rs1799963 A allele (in the heterozygous state), thus the association analysis was not feasible for this SNP in the African American sample.

Association analysis of *F2* rs3136516 SNP with plasma prothrombin activity in Caucasian American SLE cases

Among Caucasian American SLE cases with available prothrombin activity measurements at the Pittsburgh site, the rs3136516 G allele was significantly associated with a modest increase in plasma prothrombin activity ($P = 0.039$ after adjusting 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 1996 (12), 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 polymorphism and arterial thrombosis risk (i.e. myocardial infarction and stroke) was also evaluated but yielded inconsistent results (14). More recently, a common *F2* SNP (rs3136516 - A19911G) was also reported to be associated with increased plasma prothrombin activity and thrombosis risk in Caucasians, although at a lesser degree (27,28,41). Large studies and meta-analysis suggested that the rs3136516 G allele is associated with a slight increase in prothrombin activity and thrombotic risk (26,42,43). Prior studies (13,26–28) indicated that chromosomes carrying the rs1799963 A allele almost always had the rs3136516 A allele, which was also confirmed in our study.

While no significant effect of the uncommon rs1799963 variant on SLE was observed in our study, a significant association of the common rs3136516 SNP with SLE susceptibility was detected in both Caucasian and African Americans (Table 1). The rs3136516 G allele frequency was significantly higher in Caucasian American SLE cases than in controls (48.4% *vs.* 43.7%) with a covariate-adjusted OR of 1.22 (95% CI: 1.03–1.46, $P = 0.023$) indicating a modest effect size. The effect seemed to be stronger in the CVE-positive SLE subgroup than in the CVE-negative SLE subgroup (OR: 1.42 *vs.* 1.20) which warrants further confirmation in larger SLE samples characterized for CVE. The association was replicated in the African American sample where the rs3136516 G allele frequency was also significantly higher in SLE cases than in controls (91.2% *vs.* 82.2%) with an adjusted OR of 1.96 (95% CI: 1.08–3.58, $P = 0.022$). In public databases (dbSNP and HapMap), African populations show absence or varying low frequencies of the rs3136516 A allele, suggesting that the G allele is the ancestral allele and that the presence of the A allele maybe due to varying degrees of Caucasian admixture. Interestingly, a major SLE locus was identified on the short arm of chromosome 11 by genome-wide linkage scan of African American families (44), although the maximum LOD score was reported at a marker (D11S1392 - 11p13) located ~12.1 Mb telomeric to *F2* (11p11). It may still be worthwhile to evaluate *F2*

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in these families given that the location estimates may substantially vary in linkage studies of complex disorders, with 95% confidence intervals covering 10s of cM, even in samples including relatively large numbers of families (45).

The mechanism of action of the *F2* 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 under-powered 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 yet 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 *F2* or a nearby gene. Currently available information in the literature (13) and public databases (SeattleSNPs and HapMap) does not indicate the presence of other *F2* common SNP(s) strongly correlated with the rs3136516 SNP. Comprehensive resequencing-based analysis of the entire *F2* gene and its flanking regions, in conjunction with the analysis of the 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 (2,3,5). 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 (5,46). A number of biological pathways are being implicated in SLE pathogenesis and our study indicates that the ‘hemostasis and its crosstalk with immunity & inflammation’ can be added to this growing list.

To the best of our knowledge, this is the first study to evaluate the role of *F2* rs3136516 common SNP in relation to SLE susceptibility. 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 ORs 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 (47–49) nor strongly correlated with any common *F2* SNP(s) 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.

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Table 1

Allele frequencies and association statistics for *F2* rs3136516 SNP in SLE women *vs.* the control women

rs3136516	*Caucasian Americans*[*]				*African Americans*[*]	
	Controls (*n*=509)	**All Cases (*n*=519)**	**CVE(−) Cases[**] (*n*=228)**	**CVE(+) Cases[**] (*n*=100)**	**Controls (*n*=143)**	**All Cases (*n*=102)**
Alleles						
A	0.563	0.516	0.515	0.470	0.178	0.088
G	0.437	0.484	0.485	0.530	0.822	0.912
$P^{\#}$	-	*0.034*	*0.091*	*0.016*	-	*0.003*
$OR^{\dagger}$ (95%CI; $P^{\dagger}$)	-	1.22 (1.03–1.46; *0.023*)	1.20 (0.96–1.49; *0.114*)	1.42 (1.05–1.92; *0.024*)	-	1.96 (1.08–3.58; *0.022*)

[*] Only successfully genotyped individuals were included in the 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 two binomial proportions

[†] Odds ratios and p-values under additive genetic effect model (AA=0, GA=1, GG=2); adjusted for recruitment site and age

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Table 2

Relation between *F2* rs3136516 genotypes and plasma prothrombin activity (PPA) in a subset of Caucasian SLE cases (analysis was performed regardless of *F2* rs1799963 status as well as only in individuals with wild-type GG genotype for rs1799963).

	Caucasian Americans*	
rs3136516	**All cases (*n*=117)**	**Cases with GG genotype for rs1799963 (*n*=111)**
Genotypes	PPA Mean±SE** [NIH-U/ml (%)]	PPA Mean±SE** [NIH-U/ml (%)]
AA	15.17 ± 0.88 (86.69 ± 5.05)	14.79 ± 0.88 (84.53 ± 5.00)
GA	15.65 ± 0.46 (89.45 ± 2.61)	15.62 ± 0.47 (89.28 ± 2.69)
GG	17.70 ± 1.01 (101.12 ± 5.76)	17.73 ± 1.00 (101.30 ± 5.73)
*P***	*0.039*	*0.015*

*Only successfully genotyped individuals with available PPA measurements were included in the table

**Mean and p-values adjusted for age, BMI, and Warfarin use; p-values under additive genetic effect model