BRIEF REPORT

A Polymorphism in *TLR2* Is Associated With Arterial Thrombosis in a Multiethnic Population of Patients With Systemic Lupus Erythematosus

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Objective. Thrombosis is a serious complication of systemic lupus erythematosus (SLE). Studies that have investigated the genetics of thrombosis in SLE are limited. We undertook this study to assess the association of previously implicated candidate genes, particularly Toll-like receptor (TLR) genes, with pathogenesis of thrombosis.

Methods. We genotyped 3,587 SLE patients from 3 multiethnic populations for 77 single-nucleotide polymorphisms (SNPs) in 10 genes, primarily in TLRs 2, 4, 7, and 9, and we also genotyped 64 ancestry-informative markers (AIMs). We first analyzed association with arterial and venous thrombosis in the combined population via logistic regression, adjusting for top principal components of the AIMs and other covariates. We also subjected an associated SNP, rs893629, to meta-analysis (after stratification by ethnicity and study population) to confirm the association and to test for study population or ethnicity effects.

Results. In the combined analysis, the SNP rs893629 in the *KIAA0922*/*TLR2* region was significantly associated with arterial thrombosis (logistic $P = 6.4 \times 10^{-5}$, false discovery rate $P = 0.0044$). Two additional SNPs in *TLR2* were also suggestive: rs1816702 (logistic $P = 0.002$) and rs4235232 (logistic $P = 0.009$). In the meta-analysis by study population, the odds ratio (OR) for arterial thrombosis with rs893629 was 2.44 (95% confidence interval 1.58–3.76), without evidence for heterogeneity ($P = 0.78$). By ethnicity, the effect was most significant among African Americans (OR 2.42, $P = 3.5 \times 10^{-4}$) and European Americans (OR 3.47, $P = 0.024$).

Conclusion. *TLR2* gene variation is associated with thrombosis in SLE, particularly among African

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Americans and European Americans. There was no evidence of association among Hispanics, and results in Asian Americans were limited due to insufficient sample size. These results may help elucidate the pathogenesis of this important clinical manifestation.

Thrombosis is a complication that occurs in up to 37% of systemic lupus erythematosus (SLE) patients (1). It occurs more frequently and at younger ages than in the general population. Prior research has elucidated clinical risk factors associated with thrombosis such as smoking and the presence of antiphospholipid antibodies (aPL) (2). Work to identify the role of genes in this outcome, however, has been limited, especially among patients of non-European ancestry.

Toll-like receptors (TLRs) are a component of the innate immune system. TLRs recognize pathogen-associated molecular patterns such as microbial peptides and viral RNA as well as endogenous ligands released by cells undergoing apoptosis. TLR signaling leads to innate immune activation and an inflammatory response. This inflammatory response can activate the local endothelium (e.g., via tumor necrosis factor α), leading to increased levels of tissue factor and activation of the coagulation cascade (3,4).

Recent work has implicated innate immune activation in the pathogenesis of antiphospholipid syndrome (APS). Innate immune activation, particularly via TLR-2 and TLR-4, may be necessary for initiating aPL production and provides one mechanism for endothelial activation that leads to thrombosis (for review, see refs. 3 and 4). In addition to their importance in the pathogenesis of thrombosis, several TLRs (including TLRs 4, 7, and 9) have been implicated in SLE pathogenesis (5). We investigated single-nucleotide polymorphisms (SNPs) in TLRs 2, 4, 7, and 9 and an additional 6 genes reported in the literature to be associated with thrombosis risk in the general population to assess their association with venous and arterial thrombosis in a multiethnic population of SLE patients.

PATIENTS AND METHODS

Our study population consisted of 3,587 SLE patients enrolled in 1 of 3 North American studies. Characteristics of these patients are shown in Table 1. All subjects met the

Table 1. Characteristics of the patients with systemic lupus erythematosus by study population (post–quality control)*

Characteristic	UCSF (n = 1,748)	HSS (n = 435)	PROFILE (n = 1,404)	Combined (n = 3,587)
Female	1,605 (92)	402 (92)	1,284 (91)	3,291 (92)
Age at diagnosis, mean ± SD years/no. of patients with missing data	33 ± 13/97	30 ± 13/31	33 ± 13/14	33 ± 3/142
Self-reported ethnicity†				
European American	1,015 (58)	201 (46)	660 (47)	1,876 (52)
African American	276 (16)	101 (23)	192 (14)	569 (16)
Hispanic	222 (13)	94 (22)	520 (37)	836 (23)
Asian American	235 (13)	39 (9)	32 (2)	306 (9)
Disease duration, mean ± SD years/no. of patients with missing data	9.0 ± 8.3/102	11.7 ± 9.4/31	12.6 ± 5.9/14	10.7 ± 7.8/147
Smoker, ever	684 (41)/96	88 (39)/209	191 (17)/276	963 (32)/581
Nephritis by ACR renal criteria	554 (32)/16	196 (45)/3	556 (41)/37	1,306 (37)/56
One or more arterial or venous thrombosis	394 (23)	69 (16)	129 (9)	592 (17)
One or more arterial thrombosis‡	258 (15)	85 (20)	104 (7)	447 (12)
One or more venous thrombosis§	147 (8)	42 (10)	32 (2)	221 (6)
aPL positive¶	606 (35)	201 (49)/25	167 (15)/274	974 (30)/299
Prednisone, ever	1,553 (89)	322 (74)	NA	1,875 (86)/1,404
Hydroxychloroquine, ever	1,456 (83)	329 (76)	1,029 (91)/276	2,814 (85)/276
Immunomodulating agents, ever#	991 (57)	237 (54)	602 (43)	1,830 (51)

* Except where indicated otherwise, values are the number of patients (% [based on patients without missing data])/number with missing data. UCSF = University of California, San Francisco; HSS = Hospital for Special Surgery; ACR = American College of Rheumatology; aPL = antiphospholipid antibody; NA = not applicable.
† Genetic outliers were removed from each self-reported ethnicity group prior to analysis (see Patients and Methods).
‡ Cerebrovascular accident, myocardial infarction, recurrent miscarriages (at least 3 in the first trimester or 1 in the second or third trimester).
§ Deep venous thrombosis, pulmonary embolism, or retinal vein thrombosis.
¶ At least 1 positive test result for lupus anticoagulant, anticardiolipin antibodies, or anti–β_2-glycoprotein I antibodies.
# Azathioprine, mycophenolate mofetil, cyclophosphamide, methotrexate, abatacept, rituximab, anti–tumor necrosis factor, cyclosporine, leflunomide, chlorambucil.

American College of Rheumatology (ACR) 1982 revised criteria for SLE (6), and all sites had Institutional Review Board approval.

A total of 1,748 patients were recruited from the Lupus Genetics Project at the University of California, San Francisco (UCSF) (7). The UCSF patients completed an extensive questionnaire, gave permission for medical record review, and provided a DNA sample. Recruitment sources included tertiary care and community hospitals and clinics as well as lupus support groups in northern California and nationwide. Baseline questionnaire data included demographic (e.g., self-reported ethnicity based on origin of 4 grandparents, sex), clinical (e.g., disease duration), and behavioral (e.g., smoking) factors. Thrombotic events were documented in the questionnaire and then confirmed by medical record review (where thromboses not reported were also noted). Events included deep venous thrombosis (DVT), pulmonary embolism, myocardial infarction (MI), cerebrovascular accident (CVA), recurrent miscarriages (at least 3 in the first trimester or 1 in the second or third trimester), and retinal vein thrombosis.

A total of 435 patients were recruited as part of the SLE Registry and Repository at the Hospital for Special Surgery (HSS) in New York, NY. For this collection, patients who met the ACR criteria for SLE were invited to participate at the time of their visit to their HSS rheumatologist, and followup evaluations and sample collections took place at subsequent physician visits. More than 670 data points were collected per visit, including detailed demographics (including self-reported ethnicity), family history, SLE manifestations, activity (SLE Disease Activity Index) (8) and damage (Systemic Lupus International Collaborating Clinics/ACR Damage Index) (9), comorbidities (including arterial and venous thrombotic events and pregnancy outcomes), laboratory test results, and standard lupus and aPL serology results.

Finally, 1,404 SLE patients were recruited from PROFILE, a multicenter prospective cohort based at the University of Alabama at Birmingham (10). Patients in this population met ACR criteria, were at least age 16 years, and had disease duration ≤10 years at enrollment. Phenotype data obtained for these SLE patients included age, sex, ethnicity, smoking, medication use, renal involvement, aPL positivity, and thrombosis outcomes of DVT, CVA, and MI.

Explanatory variables investigated for association with thrombosis risk included ethnicity (European American, Hispanic, African American, and Asian American), age at diagnosis, and SLE disease duration. Other variables available for a majority of patients included smoking (ever versus never exposure), immunomodulating agents (including cyclophosphamide, azathioprine, methotrexate, and mycophenolate mofetil; ever versus never use), other important medications (hydroxychloroquine, prednisone), and the presence of aPL. Positivity for aPL was defined as the presence of at least 1 positive laboratory test result (lupus anticoagulant measured by Russell's viper venom time [including confirmatory studies], IgG and IgM anticardiolipin antibodies, or anti–β_2-glycoprotein I [anti-β_2GPI] antibodies). Our outcome measure was at least 1 arterial or venous thrombosis.

Genotyping and quality control. Primary predictors included 77 SNPs in 10 genes, including genes involved in innate immunity (TLRs 2, 4, 7, and 9), genes for platelet glycoproteins, and genes involved in the coagulation cascade.

Seventy tagging SNPs found 1,000 bp upstream and downstream of TLRs 2, 4, 7, and 9 were included. Tagging SNPs were selected with the web tool SNPinfo (http://snpinfo.niehs.nih.gov/snpinfo/snptag.htm). In order to adjust for confounding by population substructure in our analyses, we also genotyped 64 ancestry-informative markers (AIMs) (11) as well as a 3-marker set as a proxy for north-south European ancestry (12). The complete list of 144 genotyped SNPs is available at http://pages.medicine.ucsf.edu/lupus/supplemental.html.

Genotyping was performed using a custom 144-SNP Illumina GoldenGate Assay in the Genomics Core Facility at UCSF. Four SNPs were dropped because of low genotyping (>10% missing), and 6 SNPs were dropped because of minor allele frequency (MAF) <0.01. Deviations from Hardy-Weinberg equilibrium were assessed for our most strongly associated SNP using an exact test. Of an initial 4,213 participants, 86 were dropped due to genotyping failure or genotyping call rate <10%. Samples were also removed prior to analysis for unexpected duplication or relatedness (n = 85) using PLINK (pngu.mgh.harvard.edu/~purcell/plink), no self-reported ethnicity (n = 5), outliers within self-reported ancestry groups (n = 55; see below), or missing phenotype data (n = 395). Thus, a total of 3,587 participants were analyzed post–quality control (Table 1).

Statistical analysis. Genetic ancestry was analyzed using principal components analysis via EigenStrat software and the 64 continental AIMs. Prior to analysis, genetic outliers with respect to the top 3 principal components were removed from each self-reported ethnicity group ($P = 0.1$ by Stata function hadimvo). The top 3 principal components were also included as covariates in all logistic analyses that included all ethnicities.

Other potential covariates were first analyzed for association with venous and arterial thrombosis (see http://pages.medicine.ucsf.edu/lupus/supplemental.html), and those with $P < 0.1$ were retained for further analysis. SNPs were coded by number of minor alleles. The false discovery rate (FDR) ($\alpha = 0.05$) was used to assess significance to adjust for multiple comparisons.

To further ensure that significance of our top associated SNP, rs893629, was not due to differences in ancestry or population, we performed logistic regression modeling stratified by study population and by ethnicity, again adjusted for additional covariates with association $P < 0.1$. We then combined each set via meta-analysis using random-effects models. We also performed a European-only logistic regression with our 3 north-south European SNPs to test for intra-European confounding of our top association. Logistic regression modeling and meta-analysis (via the Stata metan function) were performed using Stata SE software, version 11.0.

RESULTS

Table 1 shows the characteristics of study subjects by study population. Ninety-two percent of study subjects were female, and the ethnic distribution was 52% European American, 16% African American, 23% Hispanic, and 9% Asian American. The mean ± SD disease

Table 2. SNPs associated with arterial thrombosis in a combined analysis of all study populations*

Chromosome	SNP	Location (basepairs)	Gene	Unadjusted (χ^2) P	Logistic P†	FDR P
4	rs893629	154,604,968	*KIAA0922/TLR2*	0.00042	6.4×10^{-5}	0.0044
4	rs1816702	154,609,523	*TLR2*	0.007	0.002	0.069
4	rs4235232	154,618,084	*TLR2*	0.011	0.009	0.21

* SNP = single-nucleotide polymorphism; FDR = false discovery rate.
† Logistic regression adjusting for top 3 ancestry principal components, disease duration, antiphospholipid antibodies, smoking, and use of immunomodulating agents.

duration was 10.7 ± 7.8 years. A total of 592 patients (17%) experienced at least 1 thrombosis.

In logistic regression models with the combined multiethnic population, 1 SNP—rs893629 in the *KIAA0922/TLR2* region—was significantly associated with arterial thrombosis using the FDR for multiple testing (logistic $P = 6.4 \times 10^{-5}$, FDR $P = 0.0044$). Two additional SNPs in *TLR2* were also suggestive (see Table 2): rs1816702 (logistic $P = 0.002$, FDR $P = 0.069$) and rs4235232 (logistic $P = 0.009$, FDR $P = 0.21$). In the meta-analysis by study population (Table 3), the odds ratio (OR) for arterial thrombosis with rs893629 was 2.44 (95% confidence interval [95% CI] 1.58–3.76), $P = 5.9 \times 10^{-5}$, without evidence for heterogeneity ($P = 0.78$). In the meta-analysis by ethnicity (Table 3) (see http://pages.medicine.ucsf.edu/lupus/supplemental. html), the OR for arterial thrombosis with rs893629 was 2.43 (95% CI 1.58–3.76), $P = 5.3 \times 10^{-5}$, without evidence for heterogeneity ($P = 0.44$); however, the Asian American stratum was not included in this analysis because there were no cases with the minor allele.

The effect was most significant in the African American (OR 2.42, $P = 3.5 \times 10^{-4}$) and European American (OR 3.47, $P = 0.024$) strata and was nonsignificant among Hispanics (OR 0.75, $P = 0.8$). To confirm that the association with European Americans was not due to intra-European population stratification, we performed an additional analysis of that stratum including our 3 north-south European AIMs as covariates, and we obtained similar results (OR 3.54, $P = 0.024$).

The MAF, genotype counts, and Hardy-Weinberg P values for rs893629 by both ethnicity and study population are shown at http://pages.medicine.ucsf.edu/lupus/supplemental.html. For each subgroup, we also show the power to detect an association with an OR of 2.4 at an alpha level of 0.05 (see Table 3).

DISCUSSION

We found that a SNP in *TLR2* was significantly associated with arterial thrombosis in European American and African American patients with SLE. These

Table 3. Logistic regression and meta-analysis results for association of rs893629 with arterial thrombosis, by study population and ethnicity*

	No. of cases/controls	MAF, cases/controls	Logistic regression results†		Power, %‡
			OR (95% CI)	P	
Study population					
UCSF	258/1,488	0.037/0.014	2.83 (1.53–5.27)	0.001	85.1
HSS	85/350	0.053/0.030	2.42 (0.62–9.50)	0.21	64.6
PROFILE	104/1,299	0.067/0.038	2.03 (1.03–4.01)	0.042	90.1
Ethnicity					
European American	254/1,621	0.0098/0.0043	3.47 (1.18–10.2)	0.024	39.6
Hispanic	65/504	0.0077/0.017	0.75 (0.09–6.0)	0.8	40.3
African American	96/738	0.19/0.088	2.42 (1.49–3.93)	3.5×10^{-4}	98.8
Asian American	32/274	0/0.0036	No cases with minor allele		8.3

* In the meta-analysis by study population, the odds ratio (OR) for arterial thrombosis with rs893629 was 2.44 (95% confidence interval [95% CI] 1.58–3.76), $P = 5.9 \times 10^{-5}$, without evidence for heterogeneity ($P = 0.78$). In the meta-analysis by ethnicity, the OR for arterial thrombosis with rs893629 was 2.43 (95% CI 1.58–3.76), $P = 5.3 \times 10^{-5}$, without evidence for heterogeneity ($P = 0.44$). The Asian American stratum was not included in this analysis because there were no cases with the minor allele. MAF = minor allele frequency; UCSF = University of California, San Francisco; HSS = Hospital for Special Surgery.
† Adjusted for covariates with bivariate $P < 0.1$; population strata also adjusted for 3 principal components.
‡ Power to detect an OR of 2.4 at an alpha level of 0.05.

results suggest that variation in genes involved in innate immunity may be important in conferring thrombotic risk in patients with SLE. The associated SNP rs893629 is relatively rare but much more common in our African American subjects (9% in controls) than in other ethnic subgroups (<2% in controls). We did not find any significant association among Hispanics (although power was similar to that of the European American subgroup) or Asian Americans; however, the Asian American analyses were limited by small sample size and very low frequency of the SNP.

TLRs are involved in innate responses to infections as well as inflammatory processes induced by endogenous ligands. TLR activation can lead to production of cytokines that have been implicated in SLE pathogenesis (for review, see ref. 5). TLR polymorphisms have been associated with the development of SLE. *TLR7* and *TLR9* were associated with SLE in a meta-analysis of Asian studies (13) and in a study of Brazilian patients (14).

No studies have investigated *TLR2* polymorphisms in thrombosis specifically in SLE patients; however, TLRs have also been implicated in pathogenesis of thrombosis in the general (non–SLE patient) population. For example, TLR-2 has been found to be an endothelial receptor for β_2GPI, a phospholipid binding protein targeted by aPL in SLE patients and non–SLE patients with thrombosis (15). β_2GPI is a clinically important antigen in the pathogenesis of APS (16). TLR-2 has been found to mediate monocyte and endothelial cell activation by aPL (for review, see refs. 3 and 4). To our knowledge, only 1 study has investigated the relationship between *TLR2* polymorphisms and thrombosis. That study of a polymorphism in *TLR2* in Croatian patients with myocardial infarctions did not find a statistically significant association (17). In contrast, TLR-4 has also been implicated in endothelial signaling and thrombosis induced by aPL, and allelic variants in *TLR4* have been associated with risk of thrombosis in APS (18).

One limitation of our study is that these 3 study populations were not specifically designed to study thrombosis; for example, in PROFILE only MI, DVT, and CVA were identified. Thus, we may have underestimated the number of arterial thrombosis events in this cohort, which would attenuate associations in this population; indeed, the PROFILE stratum had the lowest magnitude of effect for the association of rs893629 with arterial thrombosis risk. Nevertheless, the combined meta-analysis of the 3 populations and low heterogeneity support the validity of the association results. Further studies of *TLR2* polymorphisms in SLE are required to confirm these findings and further elucidate the role of these SNPs in thrombosis.

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