Association of Kallikrein Gene Polymorphisms With Intracranial Aneurysms

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Background and Purpose—Genomewide DNA linkage analysis identified a susceptibility locus for intracranial aneurysm (IA) on chromosome 19q13 in the Finnish population, a region including the kallikrein gene cluster. We investigated the association of single nucleotide polymorphisms (SNPs) in the kallikrein gene cluster with IA in the Finnish population.

Methods—We genotyped 18 haplotype-tagging SNPs spanning a 244 kbp region in the kallikrein gene cluster for 266 Finnish IA cases and 290 Finnish control subjects. In a second phase, we genotyped 2 SNPs (rs1722561 and rs1701946) in an additional set of 102 Finnish IA cases and 102 Finnish control subjects; and in a third phase, we genotyped these 2 SNPs in 156 Russian IA cases and 186 Russian control subjects. Both single-marker and haplotype-based tests of association were performed.

Results—In phase I, SNPs rs1722561 and rs1701946 were significantly associated with IA in the Finnish population for single locus models (rs1722561: $P=0.0395$; rs1701946: $P=0.0253$). A 2-SNP haplotype block (rs1722561–rs1701946) identified in phase I was also associated with IA in the expanded Finnish (phase II) data set (asymptotic $P=0.012$; empirical $P=0.019$). In the Finnish and Russian combined data set (phase III) with 524 cases and 578 control subjects, the same 2 SNPs (OR: 1.35, 95% CI: 1.14, 1.60; $P=0.0005$ for rs1722561 and OR: 1.32, 95% CI: 1.12, 1.57; $P=0.0011$ for rs1701946) were significantly associated with IA. These SNPs are located in the intronic region of *KLK8*, although linkage disequilibrium could extend from rs268912–rs2250066, a ≈76-kbp region that includes KLK5–KLK10.

Conclusions—Polymorphisms within the kallikrein gene cluster are associated with IA suggesting that the kallikreins are important candidate genes for IA. **(*Stroke*. 2007;38:2670-2676.)**

Key Words: cerebrovascular disease ■ genetic association ■ genetics ■ polymorphism ■ subarachnoid hemorrhage

Saccular intracranial aneurysm (IA) occurs in approximately 2% of the population and is a complex disease with both environmental and genetic components. Despite intensive study, the pathogenesis is still poorly understood.[1,2] A number of environmental risk factors, including cigarette smoking, heavy alcohol consumption, and caffeine intake, have been identified.[3,4] Genetic epidemiological and DNA linkage studies provide evidence for a genetic component to IA. First-degree relatives of patients with IA have a higher risk of developing an IA and inheritance in familial IA is autosomal; however, inheritance patterns are not easily defined.[5,6]

IAs also occur in individuals with some heritable disorders of the extracellular matrix (ECM).[2] This suggests that disruption of the ECM of the arterial wall is likely a factor in the pathogenesis of IA. A recent histological analysis of ruptured and unruptured IAs illustrated that the aneurysmal artery wall undergoes morphological changes associated with vascular remodeling.[7] Apoptosis, deendothelialization, luminal thrombosis, smooth muscle cell proliferation, and T-cell and macrophage infiltration were processes associated with IA rupture.[7]

The identification of susceptibility genes is complicated by multigenic inheritance, environmental factors, and genetic

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Table 1. Characteristics of Finnish and Russian Cases and Control Subjects Included in Phase III

	Finnish			Russian			*P*
	Cases	Controls	*P*	Cases	Controls	*P*	Population
Total no.	368	392		156	186		
Female (%)	193 (52.4)	195 (49.7)	0.5	77 (49.4)	82 (44.3)	0.4	0.4
Age,* years							
Mean (SD)	47.5 (12.3)	58.2 (12.2)	<0.001	43.5 (11.6)	41.8 (11.5)	0.2	<0.001
Range	18–80	32–90		14–73	22–63		
Ruptured IA (%)	252 (69.4)	...		136 (88.9)	...		<0.001
Family history of IA or subarachnoid hemorrhage (%)	230 (62.5)	...		3 (2)	...		<0.001

*Age cases: age at diagnosis; age controls: age at recruitment. 91.3% of the Finnish cases had age at diagnosis information and 98.6% had rupture status information. Family history of IA is indicated when an individual has one or more affected parents, siblings, or offspring. Unrelated Finnish cases (n=368) were ascertained from 368 independent families with IA; Russian cases were mostly sporadic.

heterogeneity. Several genomewide DNA linkage analyses have been performed.[8–15] Some positional candidate genes that have been examined for association with IA include genes involved in vascular biology or homeostasis[2,16]; these include proα2 chain of type I collagen (*COL1A2*), apolipoprotein E (*APOE*), elastin (*ELN*), LIM domain kinase 1 (*LIMK1*), endoglin (*ENG*), lysyl oxidase (*LOX*), endothelial nitric oxide synthase (*NOS3*), nitric oxide synthetase 2A (*NOS2A*), angiotensin I converting enzyme 2 (*ACE2*), and heme oxygenase 1 (*HO1*). The studies have frequently yielded conflicting results and associations have often not been replicated in other populations or at all. Discrepancies may be a result of population stratification, misclassification, inappropriate statistical methods, allelic and locus heterogeneity, or underpowered studies due to small sample sizes.

Previously, we identified a linkage interval in a Finnish population, which spans 6.6 cM on 19q13.3 between markers D19S545 and D19S246,[10,11] and was confirmed in a Japanese sample set.[9] Positional candidate genes on chromosome 19q13 for IA include growth factors and genes involved in regulation of the vascular system and tissue remodeling. An unusual cluster of all 15 kallikrein (*KLK*) genes is located at the telomeric end of 19q13. *KLK1–KLK15* encode serine proteases known to be involved in a wide range of physiological processes, including regulation of blood pressure, smooth muscle contraction, vascular permeability, cell growth, tissue remodeling, neural plasticity, and Alzheimer disease.[17,18]

To evaluate the role of the KLK genes in IA, we carried out a case–control study using single nucleotide polymorphisms (SNPs) spanning a 244-kbp region in the KLK gene cluster. We found 2 SNPs to be associated with IA in a Finnish sample set.

Subjects and Methods

Study Population

This is a 3-phase case–control study with samples from the Finnish and Russian populations (Table 1). In phase I, Finnish unrelated IA cases (n=266; 133 males, 133 females) and unrelated control subjects (n=290; 144 males, 146 females) were genotyped for SNPs that map to the kallikrein gene cluster on chromosome 19q13. In phase II, additional Finnish IA cases and control subjects were added to the study and a total of 368 unrelated Finnish IA cases and 392 unrelated Finnish IA control subjects were analyzed for 2 SNPs (rs1722561 and rs1701946). Finnish control samples included spouses of IA cases (n=193) and other unrelated individuals (n=199). In phase III, Russian unrelated IA cases (n=156) and control subjects (n=186) were genotyped for SNPs rs1722561 and rs1701946, and a joint analysis[19] of 524 cases and 578 control subjects was performed.

Aneurysms in the Finnish cases were detected by subarachnoid hemorrhage (approximately 70%), or by magnetic resonance angiography and confirmed by digital subtraction angiography before operations. Most (>95%) of the IAs discovered by magnetic resonance angiography were repaired by operation. Only the few inoperable (age, health, location) were not. Aneurysms in the Russian cases were detected by MRI or CT and cerebral angiography. Control subjects were not screened for IA.

The study was approved by the Human Investigation Committee of Wayne State University, Detroit, Mich, as well as the Ethics Committees of Helsinki University Hospital, Helsinki, Finland; Kuopio University Hospital, Kuopio, Finland; and The Urals State Medical Academy, Ekaterinburg, Russia.

Single Nucleotide Polymorphism Selection

We selected 19 validated haplotype tagging SNPs that span a 244-kb region of the KLK gene cluster using SNPBrowser software version 2.0[20] (Applied Biosystems supplemental Table I, available online at http://stroke.ahajournals.org). All SNPs had a minor allele frequency $\geq$0.12 and $r^2 \geq$0.99 in SNPBrowser.

Genotyping

We isolated genomic DNA from peripheral blood using a Puregene kit (Gentra Systems) and performed a whole-genome amplification using primer extension preamplification.[21] One microliter of 100-fold diluted primer extension preamplification products was used for each genotyping reaction. We performed genotyping using predesigned 5′-nuclease assays (Taqman Assay; Applied Biosystems) in an ABI PRISM Sequence Detection System 7900. One SNP, rs2075690, did not perform well and was eliminated from the study.

Power Calculations

Power calculations were performed using the Genetic Power Calculator.[22] We assumed that the polymorphism and the disease locus were in complete linkage disequilibrium (LD) and that they had the same allele frequencies, ie, the polymorphism was the disease locus. Assuming a recessive disease locus and a disease prevalence of 0.02, our exploratory sample (phase I) size of 266 cases and 290 control subjects had an 80% power to detect a susceptibility locus with a genotypic relative risk $\geq$3 at $\alpha \leq$0.10 for SNPs with minor allele frequencies $\geq$0.2. A dominant model had 80% power to detect a

Table 2. Single Marker Tests of Association for KLK SNPs

		Major Allele			*P*	
No.	dbSNP rs ID	Cases	Controls	χ^2	Nominal	Corrected
Phase I						
1	rs2659101	C	C	0.038	0.8451	1
2	rs266115	A	A	1.787	0.1813	0.97
3	rs266856	C	C	0.136	0.7126	1
4	rs198977	C	C	0.217	0.6415	1
5	rs2235091	T	T	0.393	0.5306	1
6	rs198968	G	G	0.028	0.8664	1
7	rs870361	A	A	0.373	0.5414	1
8	rs268912	G	G	0.646	0.4215	1
9	rs1722561	C	T	4.239	0.0395*	0.5
10	rs1701946	C	T	5	0.0253*	0.35
11	rs1701947	A	A	2.101	0.1472	0.93
12	rs2659096	A	A	4.069	0.0437*	0.53
14	rs2250066	C	C	0.124	0.7245	1
16	rs8104577	T	T	0.045	0.8323	1
17	rs3760739	G	G	0.999	0.3177	1
18	rs1880413	T	T	0.037	0.8484	1
19	rs2736433	C	C	1.854	0.1733	0.96
Phase II						
9	rs1722561	C	T	8.805	0.003*	0.05*
10	rs1701946	C	T	9.701	0.0018*	0.03*
Phase III						
9	rs1722561	C	T	12.06	0.0005*	0.008*
10	rs1701946	C	T	10.704	0.0011*	0.019*

*$P \leq 0.05$.

locus with a genotypic relative risk ≥ 1.7 and a SNP minor allele frequency ≥ 0.1 at $\alpha \leq 0.1$.

Post hoc power was calculated using a recessive model with a disease allele frequency of 0.52, a population prevalence of 0.02, and a genotypic relative risk=1.68, for $\alpha=0.05$ and 0.003 (Bonferroni threshold). The power for phase II was 85% and 52%; for the Russian sample set alone, it was 52% and 17%; and for the joint analysis, it was 95% and 74%.

Statistical Analyses

For all SNPs in the study, Haploview[23] was used to perform tests of Hardy-Weinberg equilibrium (HWE) by comparing the observed genotype frequencies in IA cases and control subjects with their expected frequencies at equilibrium based on the χ^2 goodness-of-fit test to conduct association analyses by comparing allele and genotype frequency distributions between cases and control subjects for each population using a χ^2 test and to estimate LD between polymorphisms used in the study by computing Hedrick's multiallelic D′.[24] We used S-PLUS (Version 14; Insightful Corp) for logistic regression with dominant and recessive models. Haplotype frequencies were estimated and then compared using a likelihood ratio test (as implemented in Decipher[25]) or a score test (as implemented in Haplo.stats[26]) to validate results using 2 different statistical methods. Probability values were obtained for both methods using the asymptotic distribution or empirically using a permutation test.

Correction for multiple testing was applied to the most significant probability values using the Sidak correction as implemented in R: A Language and Environment for Statistical Computing (Version 2.4.1). We calculated Reynolds' distance to compare the Finnish and Russian populations and also performed neutrality tests using Arlequin.[27]

Results

In phase I, we analyzed 266 Finnish IA cases and 290 Finnish control subjects for 18 SNPs in the KLK gene cluster. SNPs were successfully genotyped in >93% of the total sample. One SNP, rs3865443, was removed from the analysis because it did not conform to HWE proportions in the control sample, leaving 17 SNPs for association analyses. The observed heterozygosity ranged from 0.1 to 0.5, and all SNPs were polymorphic (minor allele frequency >1%) in both cases and control subjects (supplemental Table I, available online at http://stroke.ahajournals.org). Among cases, the genotype distribution at SNP rs198968 deviated from HWE with an excess in both homozygous genotypes; however, because the control subjects were in HWE, this SNP was included in the analysis. The remaining 16 SNPs all conformed to HWE among control subjects after Bonferroni correction (supplemental Tables I and II, available online at http://stroke.ahajournals.org).

Analysis of Finnish IA cases versus control subjects in phase I indicated that 3 markers (rs1722561, rs1701946, and rs2659096) were nominally associated with IA at the 0.05 significance level (Table 2; Figure 1; supplemental Table I).

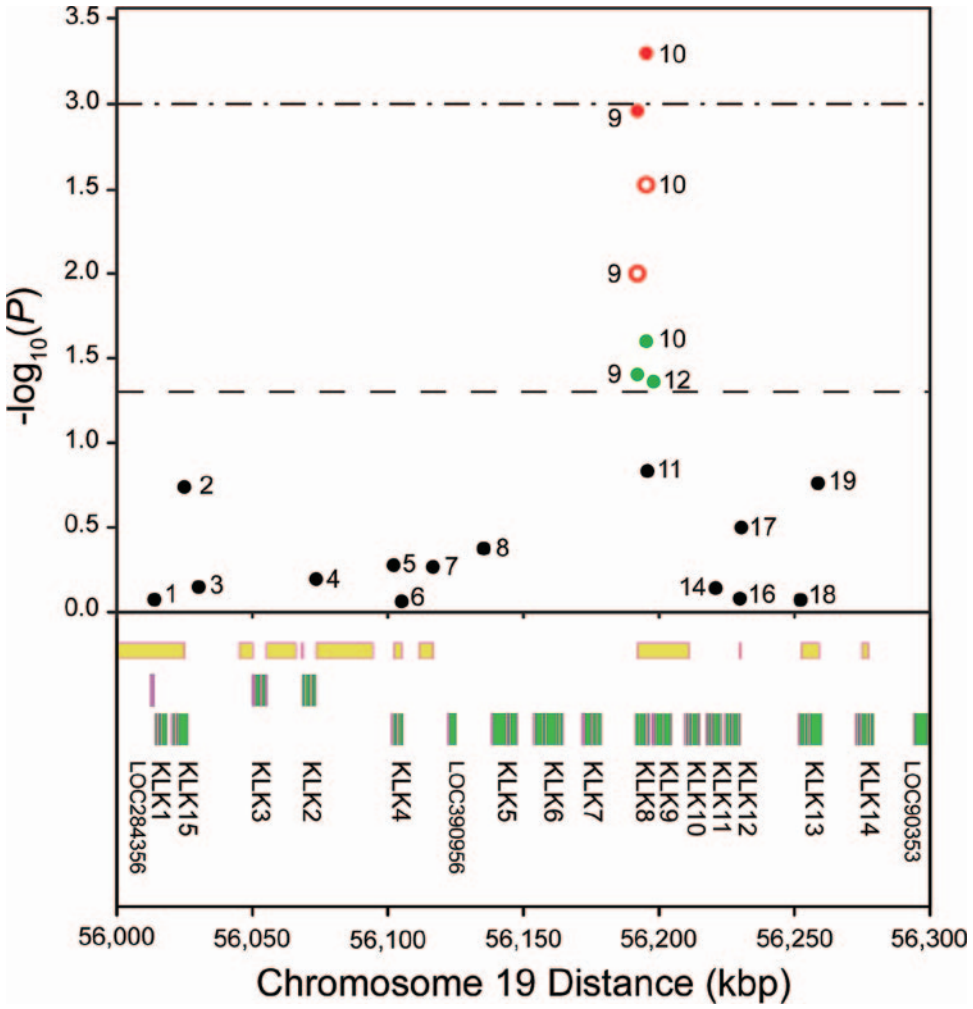

Figure 1. Genetic association of KLK SNPs with IA. Negative $\log_{10}$(P) values for association plotted against physical distance in the context of the gene location and LD structure. Symbols: filled green circles, phase I $P \leq 0.05$; filled black circles, phase I $P > 0.05$; open red circles, phase II probability values; filled red circles, phase III probability values; dashed line, $P=0.05$; dot-dashed line, $P=0.001$; horizontal yellow bars, white LD blocks; green and magenta rectangles, genes with magenta exons and green introns. SNPs numbered as in Table 2.

In additional analyses using a recessive model, rs1722561 and rs1701946 were significantly associated with IA (nominal $P=0.031$ and 0.026, respectively). Under the dominant model, only rs2659096 was significantly associated with IA (nominal $P=0.019$).

Two haplotype blocks were identified in the phase I data set as shown in Figure 2. We conducted haplotype-based tests of association using Haplo.Stats and DECIPHER for the 2 blocks with IA in the Finnish population. Block 1 includes SNPs 9 and 10 (rs1722561-rs1701946, $D' \geq 0.95$), and block 2 includes SNPs 16 and 17 (rs8104577–rs3760739, $D' \geq 0.95$). Haplotype block I showed a marginal association with IA (asymptotic $P=0.13$; empirical $P=0.07$, DECIPHER), but there was no evidence of association for haplotype block 2 (asymptotic $P=0.33$; empirical $P=0.34$, DECIPHER).

In phase II, SNPs rs1722561 and rs1701946 were genotyped in the second cohort of Finnish cases and control subjects and a joint analysis was carried out. Comparison of allele frequencies in this expanded Finnish sample set, with a total of 368 cases and 392 control subjects, resulted in significant association with IA for both SNPs rs1722561 and rs1701946 (nominal $P=0.003$ and 0.0018, respectively; Sidak corrected $P=0.05$ and 0.03) (Table 2; Figure 1). Haplotype-based association tests yielded asymptotic $P=0.012$ and empirical $P=0.019$ with block 1.

To determine if the genetic association extends to another population, we genotyped 156 Russian IA cases and 186 Russian control subjects for SNPs rs1722561 and rs1701946. The allele and genotype frequencies in control subjects were similar to those in the Finnish population (supplemental Table II, available online at http://stroke.ahajournals.org). Both SNPs were in HWE in the Russian cases and control subjects. We used logistic regression to determine if there was genotypic association for these 2 SNPs for IA in the Russian population and identified that rs1722561 ($P=0.1027$) was marginally significant at the nominal 0.1 level. Because the allele and genotype frequencies did not differ significantly (χ^2 test, data not shown) between the Finnish and Russian control subjects, and the Reynolds' distance between

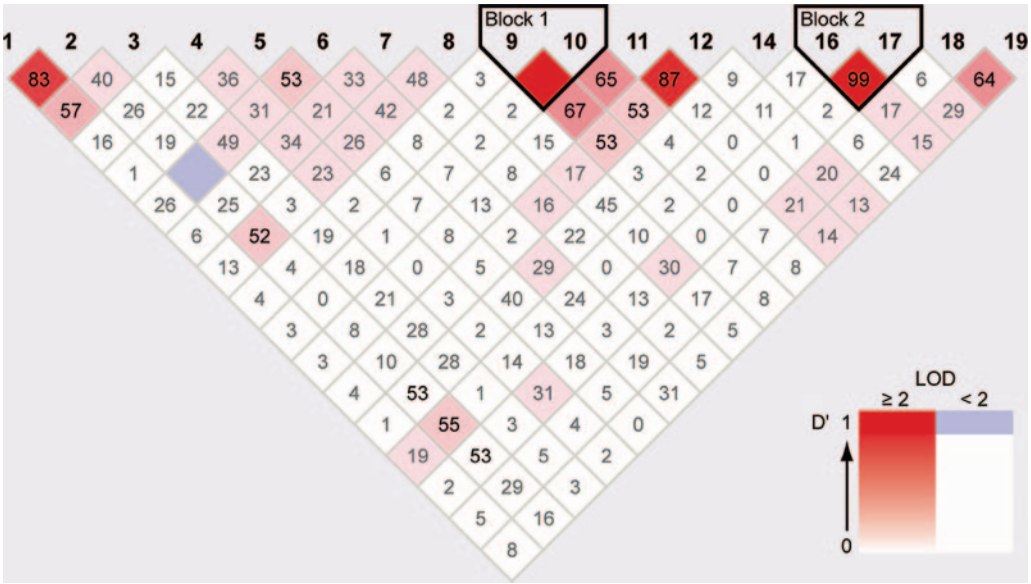

Figure 2. LD structure for KLK SNP region in the Finnish population. KLK SNPs are represented in order on the chromosome as markers 1 to 19 (excluding SNPs 13 for technical reasons and 15, which was not in HWE). D′ values are stated in each box and describe the LD for each SNP combination. Two haplotype blocks with D′ ≥0.95 exist in this KLK region: block 1, SNPs 9 to 10, and block 2, SNPs 16 to 17.

the populations was small (control subjects 0.009; cases 0.003; $P<0.05$), we subsequently evaluated the Finnish and Russian case and control data together as a combined data set (phase III), which included 524 cases and 578 control subjects. Tests of association for the 2 SNPs in the combined sample set indicated that SNPs rs1722561 ($P=0.0005$) and rs1701946 ($P=0.0011$) were significantly associated with IA ($P=0.008$ and $P=0.019$, respectively, Sidak correction) (Table 2). The ORs from the additive model in the combined sample set for SNPs rs1722561 and rs1791946 were: 1.35 (95% CI: 1.14 to 1.60, $P=0.0005$) and 1.32 (95% CI: 1.12 to 1.57, $P=0.0011$), respectively. Haplotype analysis for the Finnish and Russian combined data set (phase III) indicated that the SNP9–SNP10 haplotype was significantly associated with IA (asymptotic $P=0.006$; empirical $P=0.008$). Haplotype frequency estimates for the combined data set with the SNP9–SNP10 haplotype indicated that the C-C haplotype might be a possible IA disease haplotype ($C\text{-}C_{case}=0.4971$; $C\text{-}C_{control}=0.4248$) (Table 3). Using neutrality tests, which may have low power with these data, we found that there was no evidence for selection ($P>0.5$) on either the SNPs or the haplotypes, suggesting that they are merely markers, and also that there was no evidence of admixture (amalgamation; Chakraborty's test of neutrality).

Table 3. Haplotype SNP9–SNP10 Frequency Estimates in Cases and Control Subjects

	Finnish (phase II)		Russian		Combined (phase III)	
Haplotype	Case (n=368)	Control (n=392)	Case (n=156)	Control (n=186)	Case (n=524)	Control (n=578)
T-T	0.4659	0.5446	0.5562	0.6147	0.4933	0.5673
C-C	0.5286	0.4502	0.4240	0.3716	0.4971	0.4248
T-C	0.0055	0.0038	0.0098	0.0111	0.0067	0.0061
C-T	<0.0001	0.0013	0.0100	0.0027	0.0029	0.0018

Discussion

We undertook the current association study after we had previously identified linkage with a lod score of 5.7[11] in an interval that was confirmed in a second linkage study[9] and that spans the region containing the KLK gene cluster. We identified 2 SNPs (rs1722561 and rs1701946) in the KLK gene cluster, which were associated with IA in a Finnish, and in a combined Finnish and Russian, data set using a case–control design with a total of 524 cases and 578 control subjects. Although no statistically significant association was detected for SNPs rs1722561 and rs1701946 in the Russian population alone, probably due to small sample size and low power (post hoc power for the Russian sample set was 52% and 17% at $\alpha=0.05$ and $\alpha=0.003$, respectively), the more significant association observed in the combined Finnish and Russian data set suggests that one or both SNPs are associated with IA. When analyzing SNPs in more than one population, 2 issues arise, population stratification and locus heterogeneity. Population stratification may lead to spurious association when both the disease and SNP allele frequencies differ between the populations. When there is locus heterogeneity in a disease, the contribution of loci can differ substantially between populations. The allele frequencies did not differ significantly between the populations and one of the SNPs was marginally significant in the Russians, suggesting that neither stratification nor locus heterogeneity is a concern. Also, the Reynolds' genetic distance between the population samples was small (control subjects: 0.009; cases 0.003; $P<0.05$). The differences between populations in terms of the prevalence of a positive family history could cause bias. We would anticipate this to be relevant in the presence of genetic heterogeneity and a possible explanation

when the results differ between subpopulations. However, our interpretation of the study findings is that our results were consistent between the subpopulations. Thus, although the populations differed in this aspect, we do not consider this a cause for concern. We do not expect population differences to be an explanation for consistency in the findings, especially because the genetic distance between the populations is small.

The SNPs are located in introns of *KLK8* and are likely not the true risk variants, but surrogate markers. Both SNPs have C and T alleles with the major allele being T in the control subjects. The C allele for both SNPs was more frequent in the cases compared with their respective control subjects.

KLK8 encodes a secreted serine protease with trypsin-like specificity that has been implicated in the function of keratinocytes, including migration, differentiation, and desquamation; and in the central nervous system, controlling processes such as synaptogenesis, neural development, regulation of long-term potentiation and seizures.[17,28] The KLK8 protease has the ability to degrade the ECM protein fibronectin and the basement membrane component type IV collagen.[29,30] Additional substrates for KLK8 include tissue-type plasminogen activator involved in the activation of plasmin, which can catalyze the degradation of proteins in the basement membrane and ECM; and kininogen, which releases kinins known to have vascular effects as described previously.[29] Taken together, these reports demonstrate that *KLK8* encodes a protease that, although not specifically studied in the vascular context, has the ability to cleave ECM components, a characteristic that suggests *KLK8* could play a regulatory role in maintaining the integrity of the vascular wall through ECM remodeling.

We can hypothesize that dysregulated KLK function, caused either by structural changes in the KLK proteins or by changes in the protein expression, activation, or inhibition, could result in intracranial vessel abnormalities that facilitate the development or rupture of IAs. A KLK8-deficient ($KLK8^{-/-}$) mouse model exists and could be a useful tool for investigating the putative role of KLK8 in IA formation.[31]

The joint analysis conducted here is not considered a replication. Therefore, the genetic association reported here needs to be replicated in additional sample sets to verify the association and, if present, to provide a more reliable estimate of the effect size at the KLK locus.

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Disclosures

None.

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