European Journal of Human Genetics (2009) 17, 1448–1453

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ARTICLE

Phosphodiesterase 4D and 5-lipoxygenase activating protein genes and risk of ischemic stroke in Sardinians

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Genetic factors contribute to the risk of ischemic stroke (IS). The phosphodiesterase-4D (*PDE4D*) and the 5-lipoxygenase activating protein (*ALOX5AP*) genes were identified as contributors to stroke in an Icelandic population. In an attempt to better define the contributory role of *PDE4D* and *ALOX5AP* genes to the risk of IS in humans, we carried out the present association study in a well-characterized, earlier published, genetically homogenous population from the island of Sardinia, Italy. In this cohort, including 294 cases and 235 controls, age, hypertension, hypercholesterolemia, and atrial fibrillation represent risk factors for IS. The *PDE4D* gene was evaluated by four single nucleotide polymorphisms (SNP32, SNP45, SNP83, SNP87) and by the microsatellite AC008818-1; the *ALOX5AP* gene was characterized by three SNPs (SG13S32, SG13S89, ALO2A). The results of our study provide no evidence of association between any single *PDE4D* and *ALOX5AP* gene variant with the risk of IS in the Sardinian cohort. Haplotype analysis, including that constructed with allele 0 of microsatellite AC008818-1 and SNP45 of the *PDE4D* gene, was also negative. In conclusion, we found no evidence of association between *PDE4D* and *ALOX5AP* genes and the risk of IS in a genetically homogenous population from Sardinia.
European Journal of Human Genetics (2009) **17,** 1448–1453; doi:10.1038/ejhg.2009.71; published online 6 May 2009

Keywords: phosphodiesterase 4D; 5-lipoxygenase activating protein; ischemic stroke

Introduction

Stroke is still the leading cause of disability and the third cause of mortality in developed countries.[1] Ischemic stroke (IS) is the most common form and accounts for about 80% of all strokes, being one of the most devastating consequences of atherosclerotic disease. Twin studies, family studies and case–control association studies,[2] have provided broad evidence that genetic factors substantially contribute, together with environmental factors, to the risk of IS.

Recently, inflammation has emerged as a key element in all critical steps of atherosclerosis, such as in the development of atherosclerotic lesions and in the progression to mature atheroma. Moreover, inflammatory activation promotes thrombosis, the most dreadful complication of atherosclerosis, which can result in stroke and/or myocardial infarction (MI).[3] Several genetic studies exploring the contributory role of genes encoding inflammatory

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Received 1 December 2008; revised 24 March 2009; accepted 29 March 2009; published online 6 May 2009

proteins in the risk of cardiovascular diseases have been reported.[2,4–7]

A genome-wide linkage analysis conducted by the deCODE group in an Icelandic population had suggested a possible role of two genes encoding proteins implicated in inflammation on the risk of stroke and MI: the phosphodiesterase 4D (*PDE4D*) gene[8] and the 5-lipoxygenase activating protein (*ALOX5AP*) gene.[9] PDE4D is a member of the superfamily of cyclic nucleotide phosphodiesterases and is involved in degradation of second messenger cAMP, a key signal transduction molecule located in several cell types, including smooth muscle, vascular endothelial and inflammatory cells.[10] ALOX5AP is an essential regulator of the biosynthesis of leukotriene A4 (LTA4), a pro-inflammatory mediator implicated in the pathogenesis and progression of atherosclerosis.[11] Extension of the findings to non-Icelandic populations had subsequently led to controversial and conflicting results.[12,13]

In the attempt to further elucidate the role of these two genes in the risk of IS, we carried out a case–control association study in a well-characterized, earlier published[14,15] genetically homogenous population from the island of Sardinia, Italy. Based on the earlier reports, we characterized common *PDE4D* and *ALOX5AP* gene polymorphisms, for some of which a relevant strength of association with stroke had been described in both Icelandic and other populations.

Materials and methods

Study population

The characteristics of our case–control study, including description of selection criteria for both cases and controls, have been described earlier.[14,15]

Briefly, unrelated patients were enrolled at the Neurological Department of the University of Sassari (Sardinia, Italy). From all consecutive patients with cerebrovascular events admitted between September 1998 and March 2003, we diagnosed 294 cases of IS. The diagnosis of IS was based on clear, unequivocal clinical parameters, with signs and symptoms persisting for >24 h, and confirmed in each individual case by computed tomography scan and/or nuclear magnetic resonance imaging. Unrelated control subjects ($n=235$) were drawn from the same geographical area and were selected from among the whole patients population admitted to the same hospital, mainly from surgical, urological, dermatological and ophthalmological inpatient and outpatient clinics. Exclusion criteria for selection of controls for the present study were either current or earlier cerebrovascular disease and presence of cardiovascular disease (MI, earlier coronary revascularization procedure, peripheral vascular disease). Consanguineous subjects were excluded based on an accurate and careful family history of each individual.

Cardiovascular risk factors were assessed both in patients and in controls. Hypertension was defined as being present if subjects had been diagnosed earlier according to the World Health Organization/International Society of Hypertension (WHO/ISH) guidelines and were routinely receiving antihypertensive therapy. Hypercholesterolemia was defined as a total cholesterol blood level >220 mg per 100 ml or if the patients were taking cholesterol-lowering drugs. Smoking was categorized as either past (when subjects had stopped smoking >2 months before the study) or current. Alcohol consumption was defined as an intake of >30 g/day. Presence or absence of diabetes was recorded. Among cases, history of MI and peripheral arteriopathy (PAD) were recorded.

The study received institutional approval for research involving human subjects and all participants gave informed written consent.

DNA isolation and PDE4D and ALOX5AP genotyping

Genomic DNA was isolated from peripheral blood using a commercially available kit (Qiagen, Chicago, IL, USA). *PDE4D* gene was characterized by the following SNPs: SNP32, SNP45, SNP83, SNP87, and by microsatellite AC008818-1. The PCR and restriction digestion procedures have been described earlier.[16–18] The following SNPs were used to genotype ALOX5AP: SG13S89, SG13S32, ALO2A, by using standardized procedures.[17,19,20]

Statistical analysis

Age is expressed as median value with inter-quartile range (IQR). Categorical variables were compared with χ^2-test or Fisher's exact test, as appropriate. Continuous variables were compared by use of Mann–Whitney *U* test.

Genotype and allele frequencies were computed for each locus and their distribution in cases and controls was analyzed by χ^2-test with 2 DF and 1 DF. Concordance to the frequency predicted by the Hardy–Weinberg equilibrium (HWE) was assessed by χ^2-test with 1 DF. The risk associated with each genotype to the occurrence of IS was estimated by logistic regression analysis under the assumption of an additive inheritance model (ie, fitting the three genotypes assuming one-step increase in odds per mutated allele). A multivariable logistic model was carried out including the genetic predictor and potential confounders**.** In particular, the multivariate model included variables that were significant ($P<0.2$) in the univariate analysis, any potential confounder that changed the unadjusted OR for genotype by $>5\%$ after adjustment[21] or earlier-recognized risk factors associated with IS. Based on these criteria, age (added to the model as continuous variable), gender, history of smoking, diabetes, hypercholesterolemia, hypertension and atrial fibrillation were included in the model. Likelihood ratio tests were used to assess the significance of the model.

SPSS statistical software (SPSS Inc., Chicago, IL, USA, version 12.0) was used for most of the statistical analysis. Haplotype frequencies and association testing were assessed using the R library HaploStat. Pairwise linkage disequilibrium (LD) was calculated as reported by Devlin and Risch.[22] The power of our case–control sample was calculated by QUANTO statistical software. This study had the ability to detect, assuming 80% power and a two-sided 0.05 significance level with a minor allele frequency of 20%, an odds ratio >1.51. All calculations were considered significant for $P<0.05$.

Results

The clinical characteristics of the population in cases and controls are shown in Table 1. Older age, hypercholesterolemia, hypertension and atrial fibrillation were significantly associated with the presence of IS. Incidence of MI and PAD among cases was 13.9% and 3.9%, respectively.

No significant difference was detected in genotype and allelic frequencies for all *PDE4D* and *ALOX5AP* gene markers among cases and controls. All gene polymorphisms were in Hardy–Weinberg equilibrium (HWE). Tables 2 and 3 report the multivariate analysis for all *PDE4D* and *ALOX5AP* gene polymorphisms. No statistically significant association was found between the polymorphisms tested and the risk of IS, even after stratification by gender (data not shown). Figure 1 shows LD analysis for the two genes.

Next, based on findings from the Icelandic population,[8] we constructed haplotypes using the combination of AC008818-1 and SNP45 markers of *PDE4D* gene (Table 4). There was no evidence of association with risk of IS for any of these haplotypes. In addition, we looked at all possible haplotypes constructed from all SNPs for both *PDE4D* and *ALOX5AP* genes (Tables 5 and 6). Again, no significant association was found with the risk of IS.

Discussion

The present study examined the association between *PDE4D* and *ALOX5AP* genes with the risk of IS in a genetically homogeneous population from Sardinia, Italy. In particular, we characterized for each gene a group of SNPs, selected on the basis of earlier reports, for which significant associations had been reported earlier.[8,16–18,23–27] The results of our study provided no evidence of association between both genes and the risk of IS in Sardinians.

Table 2 Single-marker and microsatellite AC008818-1 allelic associations in the *PDE4D* gene

SNP	*Variant allele*	*% Controls*	*% Cases*	*Per-allele OR (95% CI)*[a]
SNP87	T	41.9	42.7	1.08 (0.82–1.41)
SNP83	C	53.6	53.9	1.04 (0.79–1.37)
SNP45	A	19.1	19.2	1.04 (0.73–1.46)
SNP32	C	37.4	35.5	0.88 (0.66–1.17)
AC008818	−8	19.3	18.6	1.00 (0.70–1.42)
	−4	19.5	20.8	1.18 (0.85–1.63)
	0	23.0	21.3	0.86 (0.62–1.19)
	4	23.6	24.4	0.97 (0.71–1.32)
	8	13.9	14.5	1.08 (0.74–1.58)
	12	0.43	0.17	—

[a]Adjusted by age, sex, smoking status and presence of history of diabetes, hypercholesterolemia, hypertension, and atrial fibrillation.

Table 3 Single-marker allelic associations in the *ALOX5AP* gene

SNP	*Variant allele*	*% Controls*	*% Cases*	*Per-allele OR (95% CI)*[a]
SG13S32	A	48.7	46.8	0.85 (0.64–1.13)
SG13S89	A	5.5	3.4	0.79 (0.43–1.44)
ALO2A	C	20.0	19.5	1.05 (0.74–1.48)

[a]Adjusted by age, sex, smoking status and presence of history of diabetes, hypercholesterolemia, hypertension, and atrial fibrillation.

Table 1 Baseline characteristics of ischemic stroke cases and controls

	Controls (n = 235)	*Ischemic strokes (n = 294)*	*Odds ratio (95% CI)*	P-*value*
Median age in years (IQR)[a]	71 (60–79)	75 (68–83)	1.03 (1.02–1.05)	0.001
Males	132 (56.2%)	176 (59.9%)	1.16 (0.82–1.65)	0.392
Smoking	83 (36.9%)	110 (37.4%)	1.17 (0.81–1.68)	0.400
Alcohol	31 (13.2%)	31 (10.7%)	0.79 (0.46–1.34)	0.375
Diabetes	60 (26.0%)	69 (23.9%)	0.89 (0.60–1.33)	0.582
Hypercholesterolemia[a]	28 (12.1%)	64 (22.2%)	2.07 (1.3–3.36)	0.003
Hypertension[a]	108 (47.2%)	187 (65.2%)	2.10 (1.47–2.99)	<0.001
Atrial fibrillation[a]	33 (14.5%)	74 (25.6%)	2.03 (1.29–3.02)	0.002

IQR, inter-quartile range.
[a]Indicates variables that are significantly different between case and control groups.

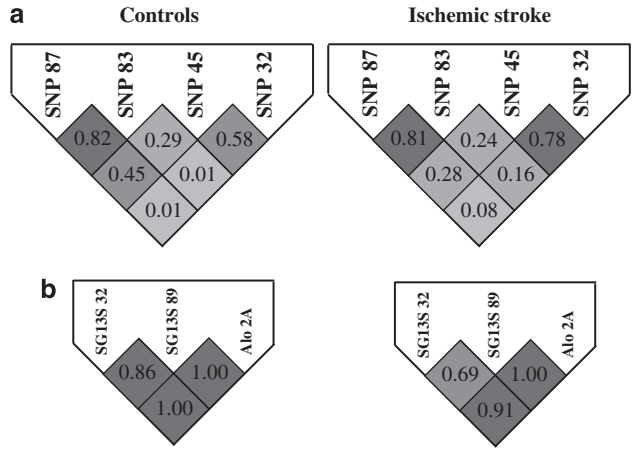

Figure 1 Analysis of linkage disequilibrium (LD) among the SNPs of *PDE4D* gene (**a**), and among the SNPs of *ALOX5AP* gene (**b**) in controls and in cases. The pairwise correlation between the SNPs was measured as *D*′, and is shown in each diamond.

Table 4 Association of PDE4D haplotype reported earlier (allele 0 of microsatellite AC008818-1 and SNP45)

Haplotype	*% Controls*	*% Cases*	*OR (95% CI)*[a]
GX	59.4	59.3	Reference
AX	18.6	18.6	1.05 (0.76–1.46)
G0	21.3	21.4	0.99 (0.72–1.36)

A0 was present only in 0.7% of the overall population and then excluded from the analysis.
X designates the joint set of alleles, excluding the original at-risk allele 0 of microsatellite AC008818-1.
GX haplotype is the composite of all haplotypes including the *G* allele of SNP45, except for the G0 haplotype and AX haplotype, which include the A allele of SNP45.
[a]Adjusted by age, sex, smoking status and presence of history of diabetes, hypercholesterolemia, hypertension, and atrial fibrillation.

PDE4D gene resides within a 20-cM region on chromosome 5p12 (STRK1), identified through genome-wide linkage analysis.[8] Several SNPs in the intronic and flanking regions of the *PDE4D* gene are known. Most of the significant associations with IS were found for SNPs located within the 5′ end of the gene. Of interest, a risk haplotype of SNP45 and microsatellite AC008818-1 was found to associate with two major subtypes of IS. However, evidence of a functional relevance for any of these SNPs has not been reported so far.

Several analyses were subsequently carried out in different populations through the case–control approach, exploring the possible correlation between *PDE4D* gene polymorphisms and IS. However, results were controversial and conflicting, even suggesting the possibility of spurious associations. In particular, whereas no association with PDE4D SNPs and risk of stroke was found in several European populations,[12,28,23] evidence of a significant positive association was reported in studies conducted in other cohorts from Europe,[24] North America,[25] Australia,[26] and Asia.[16] It is of interest that two studies on the US populations [18,27] have shown a potential role of hypertension in directing the effect of *PDE4D* gene. Finally, a recent meta-analysis on 5216 cases and 6615 controls has failed to replicate the original association with IS.[29] Our current results may support the hypothesis that any association that may exist is likely to be weak and possibly restricted to specific populations.[29]

The human ALOX5AP is located on chromosome 13q12-13 and consists of five exons. In the original paper by the deCODE group, few SNPs were found to associate to almost two-fold increased risk of MI and stroke, particularly in males.[9] Furthermore, males carrying mutant SNPs had a significantly greater production of leukotrienes B4, which may result in a more evident pro-inflammatory activity

Table 5 PDE4D gene haplotype frequencies and association

	Allele combination						
Haplotype	*SNP87*	*SNP83*	*SNP45*	*SNP32*	*% Controls*	*% Cases*	*OR (95% CI)*[a]
Hap1	C	T	G	G	1.5	3.1	Reference
Hap2	C	T	G	C	3.5	2.7	0.41 (0.08–2.18)
Hap3	C	T	A	G	3.5	1.8	0.19 (0.03–1.11)
Hap4	C	T	A	C	0	0.4	—
Hap5	C	C	G	G	23.0	24.4	0.51 (0.13–1.94)
Hap6	C	C	G	C	15.9	14.7	0.36 (0.10–1.36)
Hap7	C	C	A	G	8.5	9.5	0.45 (0.11–1.73)
Hap8	C	C	A	C	2.1	0.9	0.47 (0.07–3.08)
Hap9	T	T	G	G	20.6	17.9	0.37 (0.10–1.43)
Hap10	T	T	G	C	14.1	15.9	0.50 (0.13–1.91)
Hap11	T	T	A	G	2.3	4.4	0.98 (0.20–4.88)
Hap12	T	T	A	C	0.8	0	—
Hap13	T	C	G	G	1.0	2.2	0.92 (0.15–5.64)
Hap14	T	C	G	C	1.0	0	—
Hap15	T	C	A	G	2.0	2.3	0.51 (0.10–2.56)
Hap16	T	C	A	C	0	0	—

[a]Adjusted by age, sex, smoking status and presence of history of diabetes, hypercholesterolemia, hypertension, and atrial fibrillation.

Table 6 ALOX5AP gene haplotype frequencies and association

	Allele combination					
Haplotype	*SG13S32*	*SG13S89*	*ALO2A*	*% Controls*	*% Cases*	*OR (95% CI)*[a]
Hap1	C	G	T	30.4	33.7	Reference group
Hap2	C	G	C	20.5	18.9	0.84 (0.59–1.20)
Hap3	C	A	T	0.41	0.53	1.24 (0.18–8.69)
Hap4	C	A	C	0	0	—
Hap5	A	G	T	43.6	43.5	0.90 (0.67–1.20)
Hap6	A	G	C	0	0.01	—
Hap7	A	A	T	5.1	2.9	0.50 (0.25–1.01)
Hap8	A	A	C	0	0	—

[a]Adjusted by age, sex, smoking status and presence of history of diabetes, hypercholesterolemia, hypertension, and atrial fibrillation.

and progression of atherosclerosis.[9] Replication of these findings in other populations has yielded conflicting results. A significant association between ALOX5AP polymorphisms and risk of cardiovascular disease (either MI or IS) was found in Scottish,[30] white US,[17,31] Chinese,[32] Japanese,[33] and German[23] populations, whereas no evidence of a significant association was reported in different US and German cohorts.[12,34]

It is of note that allelic and genotypic distributions of *PDE4D* and *ALOX5AP* gene SNPs and of PDE4D microsatellite in Sardinians were similar to those reported for other European and US populations.

Owing to their relatively homogenous genetic background, Sardinians comprise an efficient population for small-scale genetic association studies. In addition, by including subjects with a mean age of 75 years, belonging to an old islander generation that did not certainly undergo any further genetic admixture, we assured an even higher degree of genetic homogeneity to our sample. On the other hand, incidence of cardiovascular risk factors among controls could not be avoided and it was comparable to that observed in several healthy cohorts of the same age.

The statistical power of our case–control sample was also calculated. Based on the result, we may not have been able to detect only modest contributions of *PDE4D* and *ALOX5AP* genes to the risk of IS.

In summary, our study suggests no association between genes and risk of IS in Sardinians. In addition, haplotype analysis, and particularly that constructed with the markers AC 008818-1 and SNP45 of *PDE4D* gene, resulted negative.

In an attempt to confirm the validity of the earlier-reported associations of *PDE4D* and *ALOX5AP* genes with IS in different stroke populations, the present study, conducted on a Caucasian population well known for its high suitability for genetic analyses, may provide a useful contribution.

Acknowledgements

This work was supported by grants from the Italian Ministry of Health (IRCCS to MV and SR) and from the InGenious HyperCare European Program (to MV).

Conflict of interest

The authors declare no conflict of interest.

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