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LETTERS TO THE EDITOR

No association between chromosome 12p13 single nucleotide polymorphisms and early-onset ischemic stroke

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Ischemic stroke, a major cause of death and disability worldwide, has a strong genetic basis, particularly when occurring at a relatively young age (< 65 years) [1]. However, there is little information on whether or not early-onset ischemic stroke shares similar disease pathways and genetic predisposition with late-onset disease. A recent genome-wide association study, conducted in the frame of the Cohorts in Heart and Aging Research Consortium (CHARGE), identified two single nucleotide polymorphisms (SNPs) at chromosome 12p13 associated with stroke at genome-wide significance [2]. The SNPs, which lay within 11 kilobases of the *NINJ2* gene (encoding ninjurin 2, a transmembrane protein that acts as a cell-cell adhesion molecule [3]), were associated with incident stroke (hazard ratio, HR, of 1.26 and 1.30 for rs11833579 and rs12425791, respectively), ischemic stroke (HR of 1.33 for both SNPs) and atherothrombotic stroke (HR of 1.35 and 1.37, for rs11833579 and rs12425791). Because the CHARGE consortium consists of several population-based prospective studies with age of onset of stroke ranging from 66 to 81 years in the different substudies [2], the variants identified on chromosome 12p13 must be considered an expression of the genetic risk for late-onset stroke. In this study, the association of rs11833579 and rs12425791 with ischemic stroke was assessed in an Italian case–control cohort of early-onset (< 65 years) ischemic stroke. The present attempt to assess the association of these SNPs with early-onset ischemic stroke should help to establish whether or not there is a common genetic background in early- and late-onset ischemic stroke.

Cases were 501 unrelated individuals referred to the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center of the University of Milan (n = 386, 78%) and to the Department of Neurology, University La Sapienza, Sant'Andrea Hospital, Rome (n = 115, 22%), for thrombophilia screening after a first ischemic stroke occurring at a young age. The diagnosis of ischemic stroke was confirmed by a neurologist, after review of the clinical documentation. Stroke subtypes were defined on the basis of the trial of ORG 10172 in acute stroke treatment (TOAST) criteria [4]. A total of 1211 unrelated Caucasians, enrolled in the study as healthy controls, were chosen from friends and non-consanguineous relatives, comparable for age and gender with the cases, who accompanied patients to the centres. Previous arterial or venous thrombosis in the controls was excluded using a validated questionnaire [5]. After quality-control (QC) procedures, including assessment of DNA purity and concentration and exclusion of individuals with non-Caucasian ethnicity, 419 cases and 1077 controls were available for the study. The presence, at the time of stroke for patients and at the time of blood sampling for controls, of hypertension, hypercholesterolemia, diabetes and smoking (at least five cigarettes daily) was recorded. The study was approved by the Institutional Review Boards of the University of Milan, University of Rome and University of Florence and all subjects gave their informed consent.

Genomic DNA was isolated from peripheral blood by the FlexiGene kit (Qiagen, Hilden, Germany). A TaqMan assay (Applied Biosystems, Foster City, CA, USA) was used to directly genotype the two SNPs: C_12094896_10 assay for rs12425791 and C_1665834_10 assay for rs11833579. Statistical analysis was performed using the SPSS package v11.5. Haplotype reconstruction and frequency estimation were independently performed using the PHASE v2.1 software (SPSS, Chicago, IL, USA) [6] and R package haplo.stats by expectation-maximization strategy (EM algorithm). The type of statistical test used for each analysis is specified in Table 1.

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Table 1 Demographic and clinical characteristics and genotype distribution of rs12425791 and rs11833579 in controls and patients with ischemic stroke

	Stroke patients (n = 419)	Controls (n = 1077)	P
Age*	43.0 (1–65)	41.0 (12–65)	0.304[†]
Sex (male) n (%)	186 (44.4)	478 (44.4)	0.998[‡]
Smoking habit n (%)	162 (38.7)	290 (26.9)	< 0.0001[‡]
Diabetes n (%)	10 (2.4)	3 (0.3)	< 0.0001[‡]
Hypertension n (%)	111 (26.5)	74 (6.9)	< 0.0001[‡]
Dyslipidemia n (%)	81 (19.3)	33 (3.1)	< 0.0001[‡]
BMI (kg m^{-2})*	24.2 (16.6–56.1)	23.7 (15.6–45.2)	0.071[†]
Stroke subtypes n (%)			
Large-artery	57 (13)		
Possible	25 (6)		
Probable	32 (7)		
Cardioembolic	46 (11)		
Possible	29 (7)		
Probable	17 (4)		
Lacunar	41 (10)		
Possible	22 (5)		
Probable	19 (5)		
Undetermined etiology	226 (54)		
Multiple possible causes	8 (2)		
Negative complete evaluation	108 (26)		
Incomplete evaluation	110 (26)		
Other determined etiology	49 (12)		
Chromosome 12p13 SNPs			
rs12425791			
GG	626 (58.1)	240 (57.3)	0.830
GA	391 (36.3)	158 (37.7)	
AA	60 (5.6)	21 (5.0)	
A frequency	0.237	0.239	
H-W[§]	0.011	0.633	
rs11833579			
GG	525 (48.7)	208 (49.6)	0.927
GA	454 (42.2)	172 (41.1)	
AA	98 (9.1)	39 (9.3)	
A frequency	0.302	0.298	
H-W[§]	0.0001	0.157	

n, number of patients; BMI, body mass index. *Expressed as median (range). [†]Mann–Whitney U-test. [‡]Chi-square test. [§]Hardy–Weimberg equilibrium, expressed as chi-square test value.

The power of this replication study to detect an association of each of the two SNPs was calculated using the CaTS power calculator [7] assuming an incidence of stroke of 3% [8], an alpha of 0.05, a minor allele frequency (MAF) of 0.30 and an effect size of 1.33 for rs11833579 [2], and a MAF of 0.24 and an effect size of 1.33 for rs12425791 [2]. MAFs used in the calculations were the allele frequencies found in the controls of this study. The general features of cases and controls are presented in Table 1. Established risk factors for ischemic stroke had a higher incidence in cases of early-onset stroke than in controls. The association of rs11833579 and rs12425791 with disease status was assessed in an unadjusted analysis. The estimated statistical power to detect a significant ($P < 0.05$) association was 92% and 89% for rs11833579 and rs12425791, respectively. However, for both SNPs no association with early-onset ischemic stroke was found, either in single SNP analysis (Table 1) or after haplotype reconstruction (not shown). For rs11833579 the risk allele frequency was even slightly higher in controls than in cases. An overestimation of the effect size in the original report (winner's curse bias) might affect our study power calculation. Given the multiple studies that contributed to CHARGE, in all of which chromosome 12p13 SNPs were associated with stroke, it is unlikely that the effect size of the variants is overestimated. In addition, it is in principle expected that early-onset cases of stroke have a stronger genetic contribution [1]. Finally, the complete lack of a trend of association suggests that study power, if indeed winner's curse bias is present, is not likely to be the (only) explanation for the results. The following differences between the population of the original report and that of this study might explain lack of replication: (a) ethnicity (American Caucasian and European Caucasian versus European Caucasian), (b) study design (population-based prospective vs. case–control), (c) age of onset (late-onset versus early-onset), and (d)

incidence of stroke subtypes. It is unlikely that different ethnicity is responsible for lack of replication. European and American Caucasians have a high degree of similarity in the patterns of linkage disequilibrium (LD) of common SNPs [9]. In addition, in the Rotterdam Study, the European substudy of CHARGE, the chromosome 12p13 SNPs were significantly associated with ischemic stroke. An association of the SNPs with fatal ischemic stroke could explain the lack of association in our hospital-based case population that includes only survivors of ischemic stroke (survival bias). However, because young patients are more likely to survive a stroke than older ones [8,10], this early-onset population should be less prone to this bias. The early age of onset and the pattern of the incidence of stroke subtypes in the population of this study, which was characterized as expected for early-onset stroke by a high incidence of cryptogenetic cases, might be valid explanations for the negative results. In CHARGE ischemic strokes were classified in two subtypes: cardioembolic and atherothrombotic (defined as non-cardioembolic ischemic strokes). Using this subgroup definition, chromosome 12p13 SNPs showed a slightly stronger association with atherothrombotic stroke in CHARGE [2]. We chose to perform a subgroup analysis for patients with atherothrombotic disease according to the TOAST criteria (either possible or probable large-artery or lacunar ischemic stroke; $n = 98$). Subgroup analyses were also performed according to age and gender. No association, nor trend of association, was found for patients with atherothrombotic stroke (chi-squared test; $P = 0.301$). Subgroup analyses according to gender and age were also negative (not shown). The results of this study most likely indicate that chromosome 12p13 variants affect (or tag variants affect) the risk of ischemic stroke in elderly patients with disease mechanisms that are not shared by early-onset forms of the disease. Consistent with this view, rs12425791 was found to have the weakest association within ARIC, the study cohort with the earliest age of onset (mean, 66 years) in the frame of CHARGE [2]. Further studies are needed to identify shared or specific genetic risk factors for early-onset ischemic stroke.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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