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ORIGINAL ARTICLE

Genetic variants within *Ninjurin 2* gene are associated with risk of ischemic stroke in Iranian population

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Abstract

Previous genetic and epidemiological studies have shown the contribution of genetic factors in conferring the risk of ischemic stroke. Among the acknowledged risk factors of stroke are the single nucleotide polymorphisms (SNPs) near *Ninjurin 2* (*NINJ2*) gene which encodes a surface adhesion protein. In the current study, we investigated the role of two SNPs near this gene in ischemic stroke in Iranian population. The frequency of the A allele of the rs11833579 was significantly lower in cases compared with controls (OR (95% CI) = 0.68 (0.54–0.86), adjusted *P* value = 0.002). The rs11833579 was significantly associated with risk of stroke in co-dominant (AA vs. GG: OR (95% CI) = 0.39 (0.23–0.66), adjusted *P* value = 0.003) and recessive (OR (95% CI) = 0.44 (0.27–0.72), adjusted *P* value = 0.001) models. The rs3809263 was associated with risk of stroke in dominant model (OR (95% CI) = 1.5 (1.09–2.06), adjusted *P* value = 0.02). The A C haplotype (rs11833579 and rs3809263) decreased the risk of stroke (OR (95% CI) = 0.72 (0.57–0.91), adjusted *P* value = 0.03), while the G T haplotype conferred susceptibility to stroke (OR (95% CI) = 1.42 (1.11–1.82), adjusted *P* value = 0.02). Consequently, the present case-control study supports the role of *NINJ2* as a risk locus for ischemic stroke in Iranian population.

Keywords Ninjurin 2 · *NINJ2* · Ischemic stroke · Cerebrovascular diseases · Ninjurin 2 gene variations · Polymerase chain reaction

Introduction

Cerebrovascular disease (CVD) is among the most frequent causes of neurological emergencies and imposes huge health burden. It has been ranked as the second most frequent origin of death and the foremost source of disability [1, 2]. Stroke is a kind of CVD which has been defined as an acute disturbance of the cerebral perfusion or circulation or cerebrovascular accident. From a pathological point of view, stroke can be caused by either ischemic or hemorrhagic disorders of the cerebral blood circulation [3]. Ischemic stroke (IS) is a chief neurologic source of severe incapacity and mortality [4] which comprises about 80% of total cases [5]. Although stroke has a complex background, based on the both genetic and epidemiological investigations, genetic factors are regarded as putative determinants of this neurological condition [6]. Epidemiological studies have shown higher probability of stroke among individuals with a positive familial history of this condition in accordance with the presence of genetically determined risk factors in addition to environmental parameters [7]. Among the acknowledged risk factors of stroke are the single nucleotide polymorphisms (SNPs) near *Ninjurin 2* (*NINJ2*) gene which encodes a surface adhesion protein whose expression is induced following nerve injury [6]. Located at 12p13, *NINJ2* participates in endothelial inflammation and activation thus controlling atherosclerosis evolution [8]. Genome-wide association studies (GWAS) have revealed that the rs11833579 near this gene is a susceptibility

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locus for ischemic stroke in both Black persons and Dutch individuals [6]. Replication studies in Chinese population have verified the results [9]. In addition to the previously appreciated rs11833579 SNP, an intronic *NINJ2* variant namely rs34166160 has been associated with incident ischemic stroke in European-Americans [10]. Totally, the authors have resequenced a 196-kb region nearby the *NINJ2* gene and reported associations between single common variants of this gene and higher risk of ischemic stroke. On the contrary, they demonstrated that rare variants were collectively associated with reduced stroke risk [10]. As the rs12425791 and rs11833579 risk SNPs which were identified through GWAS reside about 11 kb upstream of the *NINJ2* gene, their influence on the expression of the gene was not evident. Consequently, Zhang et al. have performed linkage disequilibrium and fine-mapping assessments and recognized a putative functional variant in the *NINJ2* promoter (rs3809263). Their analyses demonstrated associations between this variant and risk of large artery atherosclerotic stroke in Chinese population [11].

Although several independent studies have shown associations between *NINJ2* SNPs and risk of ischemic stroke, extra reproduced researches are required to be conducted in diverse ethnic groups so as to deduce the causal effect of these SNPs in stroke. Therefore, we performed the current investigation to assess associations between two *NINJ2* SNPs (rs11833579 and rs3809263) and stroke in Iranian population. This study is a case-control study which aimed at identifying the contribution of *NINJ2* in risk of stroke. The results of the current study would recommend manipulation of lifestyle risk factors in individuals having the predisposing variants.

Material and methods

Enrolled individuals

In the present project, a total of 303 patients with history of ischemic stroke and 310 age- and sex-matched healthy subjects were enrolled. The occurrence of ischemic stroke was verified in patients by a neurologist according to the proposed guidelines [12]. Exclusion criteria were other types of stroke rather than ischemic stroke and the presence of comorbidities such as liver or renal failure, malignancy, autoimmune, or systemic disorders. Persons enrolled in the control group had no previous occurrence of ischemic conditions or cardiovascular disorders. Moreover, they did not have hypertension, hyperlipidemia, diabetes mellitus, atherosclerotic disorders, and malignancy. The study protocol was approved by the ethical committee of Ardabil University of Medical Sciences. Written informed consent forms were obtained from all study participants.

Genotyping

Genotyping was performed on genomic DNA extracted from peripheral blood samples of enrolled individuals. Salting out method was used for DNA extraction. Tetra-primer amplification-refractory mutation system (ARMS)-PCR method was used for SNP genotyping. Ten percent of the reactions were subjected to Sanger sequencing for verification of tetra-primer-ARMS-PCR method. Table 1 summarizes the nucleotide sequences of primers, PCR conditions, and the expected PCR product sizes. The prepared reactions were subjected to a

Table 1 Nucleotide sequences of primers used for tetra-primer ARMS-PCR

SNP	Primer sequence	Tm	Annealing temperature	PCR product size (bp)
rs11833579	Forward inner primer (A allele): CTTTCTGGAAAACCTTAATTCGGCTA	63 °C	59 °C	170 bp (A allele)
	Reverse inner primer (G allele): GGATAAATAGTTAATATGTTGCTTCTTGC	57 °C		236 bp (G allele)
	Forward outer primer: AATTTTTTTTTAATTGAGCTAGATGTGGC	60 °C		351 bp (two outer primers)
	Reverse outer primer: ATATTCGAGTACTGTTCTCTTTTGCATT	60 °C		
rs3809263	Forward inner primer (A allele): CTTCAAGCCCTGAATTGGATTACTGG	69 °C	62 °C	262 bp (G allele)
	Reverse inner primer (G allele): GTAGACGTGCTTGGCAGAGTGTTCAT	70 °C		227 bp (A allele)
	Forward outer primer: GACTAAAATATGGCACCCATCCTATC ATC	69 °C		437 bp (two outer primers)
	Reverse outer primer: ATGGAGCATGGAGTAGTTGTACCTTCGA	69 °C		

Table 2 The general data of enrolled individuals

Variable	Cases (%)	Controls (%)
Male/female [no. (%)]	188 (62.1)/115 (37.9)	190 (61.3)/120 (38.7)
Age (mean ± SD, years)	49 ± 1.2	48 ± 0.3
Age range (years)	24–66	22–60
Age at onset (mean ± SD, years)	47 ± 0.9	–

preliminary denaturing step at 95 °C for 5 min; 35 cycles at 95 °C for 30 s, specific annealing temperature for 30 s, 72 °C for 60 s, and a final extension phase at 72 °C for 5 min.

Statistical analyses

The anticipated and detected rates of rs11833579 and rs3809263 genotypes were compared in each study group using the Chi-square test to appraise their agreement with Hardy-Weinberg equilibrium (HWE). Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated for the assessment of associations between SNPs and disease status in allelic, recessive, dominant, and co-dominant models. *P* values were corrected using Bonferroni correction test. *D′* and *r* values were calculated to judge the linkage disequilibrium (LD) between rs11833579 and rs3809263. SNPStats online programme [13] was used for data analysis. The level of significance was set at $P < 0.05$.

Results

General information of study participants

Table 2 shows the general data of enrolled individuals.

Accordance with HWE

The accordance of mentioned SNPs with HWE was approved in both study groups. Table 3 shows the results of comparison between expected and observed frequencies of SNPs in cases and controls.

Table 3 Exact test for HWE assessment

SNP	rs11833579			*P* value	rs3809263			*P* value
	GG	AG	AA		CC	CT	TT	
Patients	130	145	28	0.17	124	150	29	0.09
Healthy control	106	146	58	0.54	158	127	25	0.94

Associations between *NINJ2* SNPs and risk of stroke

The frequency of the A allele of the rs11833579 was significantly lower in cases compared with controls (OR (95% CI) = 0.68 (0.54–0.86), adjusted *P* value = 0.002). The rs11833579 was significantly associated with risk of stroke in co-dominant (AA vs. GG: OR (95% CI) = 0.39 (0.23–0.66), adjusted *P* value = 0.003) and recessive (OR (95% CI) = 0.44 (0.27–0.72), adjusted *P* value = 0.001) models. The rs3809263 was associated with risk of stroke in dominant model (OR (95% CI) = 1.5 (1.09–2.06), adjusted *P* value = 0.02). Table 4 shows the results of association analysis between mentioned SNPs and risk of stroke.

Based on the calculated *D′* and *r* values ($D' = 0.8$, $r = 0.18$), the mentioned SNPs were in strong LD but cannot substitute each other. Assessment of frequencies of estimated haplotypes showed that the A C haplotype (rs11833579 and rs3809263) decreases the risk of stroke (OR (95% CI) = 0.72 (0.57–0.91), adjusted *P* value = 0.03), while the G T haplotype confers susceptibility to stroke (OR (95% CI) = 1.42 (1.11–1.82), adjusted *P* value = 0.02). Table 5 shows the frequencies of estimated haplotypes in the study groups.

Discussion

In the current investigation, we demonstrated associations between two SNPs and risk of stroke in Iranian population. The rs11833579 has been previously recognized as a risk locus for ischemic stroke in diverse populations [6, 9]. However, researchers have stated that this intergenic SNP is possibly not the causative variants. Instead, it is probably linked with another variant that confers risk of stroke. Based on the results of a previous study indicating the promoter-located SNP rs3809263 in modulation of *NINJ2* expression [11], we also assessed association between this SNP and risk of stroke. As expected, LD analysis revealed strong linkage between rs11833579 and rs3809263.

We reported that the rs3809263 was associated with increased risk of stroke in dominant model (CT+TT vs. CC).

Table 4 The results of association analysis between mentioned SNPs and risk of stroke

SNP	Model		Cases number (%)	Controls number (%)	OR (95% CI)	*P* value	Adjusted *P* value
rs11833579	Allele	A vs. G	201 (33) 405 (67)	262 (42) 358 (58)	0.68 (0.54–0.86)	0.001	0.002
	Co-dominant	AA vs. GG AG vs. GG	28 (9) 145 (48)	58 (19) 146 (47)	0.39 (0.23–0.66) 0.81 (0.57–1.14)	0.002	0.003
	Dominant	AG+AA vs. GG	173 (57.1) 130 (43)	204 (65.8) 106 (34)	0.69 (0.5–0.96)	0.03	0.06
	Recessive	AA vs. AG+GG	28 (9) 275 (90.8)	58 (19) 252 (81.3)	0.44 (0.27–0.72)	7.4e−4	0.001
rs3809263	Allele	T vs. C	208 (34) 398 (66)	177 (29) 443 (71)	1.31 (1.03–1.67)	0.03	0.06
	Co-dominant	TT vs. CC CT vs. CC	29 (9.6) 150 (49.5)	25 (8.1) 127 (41)	1.47 (0.83–2.63) 1.52 (1.07–2.08)	0.04	0.09
	Dominant	CT+TT vs. CC	179 (59.1) 124 (40.9)	152 (49) 158 (51)	1.5 (1.09–2.06)	0.01	0.02
	Recessive	TT vs. CT+CC	29 (9.6) 274 (90.4)	25 (8.1) 285 (91.9)	1.21 (0.69–2.11)	0.51	1.00

Zhang et al. have reported CT and TT increase risk of stroke in Chinese population [11]. They also detected higher levels of *NINJ2* in TT genotype carriers [11]. Such higher expression of NINJ2 in association with increased risk of stroke is in line with the recently proposed role for NINJ2 in regulation of expression of genes involved in inflammation and atherosclerosis in vascular endothelial cells. Participation of NINJ2 in the inflammatory responses has been further verified through the observed upregulation of this gene in lipopolysaccharide-stimulated cells and its role in induction of monocytes attachment with endothelial cells [8].

Moreover, we reported lower frequency of the A allele of the rs11833579 in patients compared with controls and associations between this SNP and risk of stroke in co-dominant and recessive inheritance models. Contrary to our study, Ikram et al. have reported that minor allele of this SNP increases the risk of stroke [6]. A family-based case-control investigation in Chinese population reported the same trend [9]. Meta-analyses of available data in Asian population have not shown any association between the rs11833579 and risk of ischemic stroke under allelic, dominant, or recessive inheritance model [14, 15]. In addition to GWAS, associations between the rs11833579 and stroke have been assessed in Chinese population more recently when Zhang et al. reported higher risk of stroke in AA genotypes carriers compared with GG genotype. However, they demonstrated decreased levels of *NINJ2* transcripts in rs11833579 AA carriers compared with other genotypes [16]. Ding et al. found no indication for association between rs11833579 and ischemic stroke in Chinese Han population in their meta-analysis of three independent cohorts [17]. Our results regarding the frequency of AA genotype in patients contradict Zhang et al.'s study but are in accordance with the proposed association between AA genotype and *NINJ2* expression as well as the role of *NINJ2* in inflammation and atherosclerosis. The observed discrepancies in genotype frequencies of this SNP in patients from different populations further imply that the rs11833579 is not the causative variant in the stroke but is linked with another functional variant (probably rs3809263 or other functional variants).

Consistent with the genotype/allele frequencies, we demonstrated that the A C haplotype (rs11833579 and rs3809263) decreases the risk of stroke, while the G T haplotype confers susceptibility to stroke. Therefore, we propose these haplotypes as protective/susceptibility haplotypes in Iranian

Table 5 The frequencies of estimated haplotypes in the study groups

rs11833579	rs3809263	Frequency in cases	Frequency in controls	Total frequency	OR (95% CI)	*P* value	Adjusted *P* value
A	C	0.32	0.39	0.35	0.72 (0.57–0.91)	0.006	0.03
G	C	0.34	0.33	0.33	1.08 (0.85–1.37)	0.52	1.00
G	T	0.33	0.25	0.29	1.42 (1.11–1.82)	0.005	0.02
A	T	0.01	0.03	0.02	0.29 (0.09–0.88)	0.02	0.08

population. Further assessment of other variants within NINJ2 would facilitate recognition of causative variants within this gene.

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Compliance with ethical standards The study protocol was approved by the ethical committee of Ardabil University of Medical Sciences.

Conflict of interest The authors declare that they have no conflict of interest.

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