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# A Novel Functional Polymorphism in the *NINJ2* Promoter Predicts Risk of Large Artery Atherosclerotic Stroke

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**Abstract** A recent genome-wide association study (GWAS) identified two common polymorphisms (rs12425791 and rs11833579) on chromosome 12p13 that confer risk to stroke, particularly for large artery atherosclerotic (LAA) stroke. However, these two polymorphisms are located ~11 kb upstream of the *NINJ2* gene and their effects on *NINJ2* expression have not been well characterized. Through linkage disequilibrium and fine-mapping analysis, we identified a novel functional polymorphism in the *NINJ2* promoter (rs3809263 G > A) and examined its association with risk of LAA stroke in Chinese population. Rs3809263 was genotyped using the improved multiple ligase detection reaction in 414 patients with LAA stroke and 423 healthy controls. A significant decreased risk of LAA stroke was found for the rs3809263 GA (adjusted odd ratio [OR] = 0.63, 95 % confidence interval [CI] = 0.46–0.88) and AA (OR = 0.54, 95 % CI = 0.35–0.84) genotypes. Moreover, genotype-phenotype correlation analysis indicated that the AA genotype carriers had significantly increased *NINJ2* mRNA expression levels in the Chinese population, suggesting that the rs3809263 G > A polymorphism is a functional SNP and a biomarker for risk of LAA stroke. Further validation of the functionality of the *NINJ2* rs3809263 G > A polymorphism and its association with risk of LAA stroke in other ethnic populations is warranted.

**Keywords** Chromosome 12p13 · Polymorphism · Stroke

## Introduction

Traditional risk factors for ischemic stroke include older age, smoking, hypertension, and diabetes mellitus. In recent years, accumulating evidence indicates that genetic variation plays an important role in the development and prognosis of ischemic stroke [1]. Large artery atherosclerotic (LAA) stroke, a common type of ischemic stroke, is a complex disease that is caused by environmental factors, genetic factors, as well as their interactions [2].

In 2009, Ikram et al. [3] reported that two common polymorphisms (rs12425791 and rs11833579) on Chromosome 12p13 were associated with LAA stroke risk. However, these two SNPs (rs12425791 and rs11833579) are located ~11 kb upstream of the *NINJ2* gene (Fig. 1) and their effects on *NINJ2* expression have not been well characterized. Several studies have investigated the associations between these two polymorphisms and risk of stroke, but the results are still inconsistent [4–10], suggesting that additional functional studies are warranted to provide some mechanistic support.

In this study, we identified a novel functional polymorphism in the *NINJ2* promoter (rs3809263 G > A) through linkage disequilibrium and fine-mapping analysis and examined its association with risk of LAA stroke in the Chinese population.

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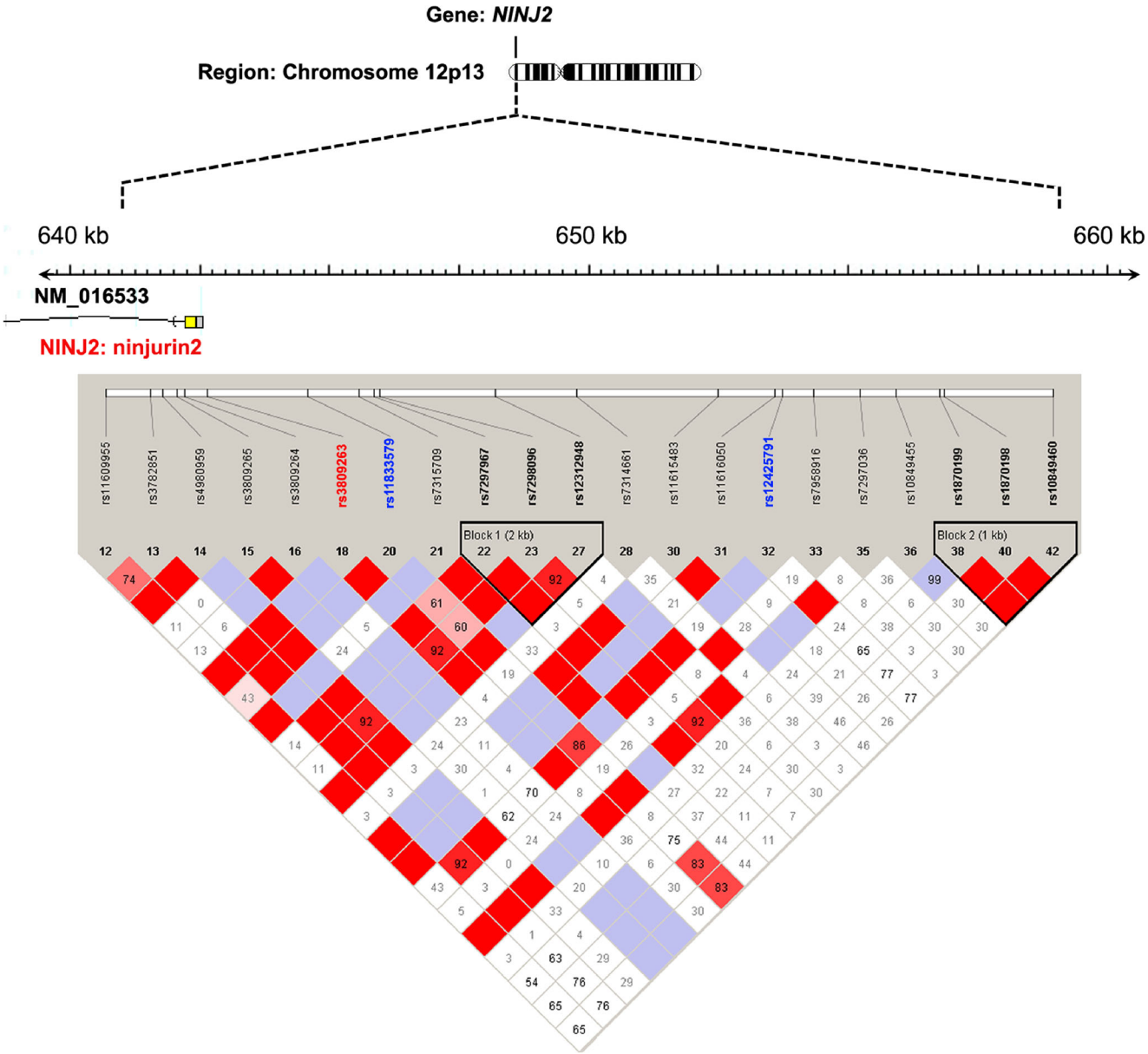


**Fig. 1** Linkage disequilibrium (LD) between SNPs on chromosome 12p13

## Methods

### Study Subjects

This on-going study was approved by the Institutional Review Board of Jinling Hospital (Nanjing, China). The detailed methods of recruiting subjects of Han Chinese have been described previously [11]. The subtypes of ischemic stroke were classified according to the TOAST criteria [12]. The stroke-free controls were genetically unrelated to the case subjects, had no history of stroke, and were recruited from those who were seeking health care in the sample hospital. Individual information was obtained by interviews. We frequency matched the control group to the case subjects by the age and gender.

### SNP Selection, Identification, and Genotyping

The rs12425791 and rs11833579 are located at ~11 kb from the 5′ untranslated region (UTR) of the *NINJ2* gene. Based on the HapMap data, these SNPs are in strong linkage disequilibrium with functional SNPs in the promoter region of *NINJ2* (Fig. 1). To identify the real functional variants, we selected the SNPs with a minor allele frequency (MAF) of >0.10 in the promoter region of *NINJ2* (2 kb upstream the 5′ UTR) from the HapMap database. Among the five SNPs in the promoter

region of *NINJ2* (2 kb upstream the 5′ UTR), two (rs12820097 and rs17754970) were not chosen because of its null heterozygosity in Han Chinese population. Two other SNPs (rs3809265 and rs3809264) were also eliminated because of low frequency (MAF < 0.10). As a result, rs3809263 G > A (MAF = 0.40) was included in our study.

We conducted genotyping by Improved Multiple Ligase Detection Reaction (iMLDR) [13], with technical support from Center for Human Genetics Research, Shanghai Genesky Biotechnology Company. Genotyping was conducted without knowing the status of subjects (case or control). Moreover, negative controls were also included to ensure accuracy of the genotyping experiment. About five percent of the samples were selected randomly for repeated assays, and the reproducibility was 100 %.

### Genotype-Phenotype Correlation Analysis

To further evaluate the biological plausibility of our findings, we adopted the data on *NINJ2* genotypes and *NINJ2* messenger RNA (mRNA) levels available online (http://app3.titan.uio.no/biotools/help.php?app=snpexp) to analyze the genotype-phenotype correlation [14].

### Statistical Analysis

Logistic regression analyses were done to obtain crude and adjusted odds ratios (ORs) for risk of LAA stroke and their 95 % confidence intervals (CIs). A chi-squared ($\chi^2$) test was adopted to assess differences in frequency distributions of categorical variables between cases and controls. We used student's *t* test to evaluate the differences in the relative mRNA expression levels among different genotypes. All statistical analyses were two-sided and were implemented with SAS software (version 9.1.3).

## Results

### Genotyping Results and its Associations With Stroke Onset

First, we evaluated whether the rs3809263 polymorphism affected the age of LAA stroke onset. As a result, the mean age of onset for the GG, GA and AA groups were 56.72 ± 9.82, 56.46 ± 9.86 and 54.96 ± 9.59, respectively ($P = 0.503$). This result suggested that the rs3809263 polymorphism was not related to age of disease onset.

### Characteristics of the Study Subjects

The characteristics of the study subjects are shown in Table 1. The cases and controls appeared to be adequately matched on age and gender ($P = 0.228$ and 0.207, respectively). Nevertheless, there were more subjects with hypertension (64.5 %) and diabetes (13.5 %) among the cases than among the controls (38.8 and 7.8 %, respectively). Therefore, these variables were further adjusted for in the multivariate logistic regression analysis.

**Table 1** Distribution of selected variables between cases and control subjects

| Variables | Cases ($n = 414$) | | Controls ($n = 423$) | | $P^a$ |
|---|---|---|---|---|---|
| | *n* | % | *n* | % | |
| Age (years) | | | | | 0.228 |
| <50 | 109 | 26.3 | 132 | 31.2 | |
| 50–70 | 285 | 68.8 | 276 | 65.2 | |
| >70 | 20 | 4.8 | 15 | 3.6 | |
| Gender | | | | | 0.207 |
| Male | 305 | 73.7 | 295 | 69.7 | |
| Female | 109 | 26.3 | 128 | 30.3 | |
| Hypertension | | | | | <0.001 |
| No | 147 | 35.5 | 259 | 61.2 | |
| Yes | 267 | 64.5 | 164 | 38.8 | |
| Hyperlipidemia | | | | | 0.660 |
| No | 274 | 66.2 | 286 | 67.6 | |
| Yes | 140 | 33.8 | 137 | 32.4 | |
| Diabetes | | | | | 0.007 |
| No | 358 | 86.5 | 390 | 92.2 | |
| Yes | 56 | 13.5 | 33 | 7.8 | |

[a] Two-sided $\chi^2$ test between the cases and controls

### Genotyping Results and its Associations With Stroke Risk

As shown in Table 2, the frequencies of the GG, GA, and AA genotypes were 36.5, 49.7, and 13.8 %, respectively, among

**Table 2** Association of the rs3809263 polymorphism with risk of LAA stroke

| Genotypes | Cases ($n = 414$) | | Controls ($n = 423$) | | $P^a$ | OR (95 % CI)[b] |
|---|---|---|---|---|---|---|
| | *n* | % | *n* | % | | |
| Rs3809263 | | | | | | |
| GG | 151 | 36.5 | 113 | 26.7 | 0.004 | 1.00 |
| GA | 206 | 49.7 | 227 | 53.7 | | 0.63 (0.46–0.88) |
| AA | 57 | 13.8 | 83 | 19.6 | | 0.54 (0.35–0.84) |
| GG | 151 | 36.5 | 113 | 26.7 | 0.002 | 1.00 |
| GA/AA | 263 | 63.5 | 310 | 73.3 | | 0.61 (0.45–0.83) |
| A allele | 0.386 | | 0.465 | | | |
| $P_{trend}$ | | | | | 0.001 | |

**Table 3** Stratification analyses between the rs3809263 and risk of LAA stroke

| Variables | N | Percentage (cases/controls) | | Adjusted OR (95 % CI)[a] | |
|---|---|---|---|---|---|
| | (cases/controls) | GG | GA/AA | GG | GA/AA |
| Total | 414/423 | 36.5/26.7 | 63.5/73.3 | 1.00 | 0.61 (0.45–0.83) |
| Age (years) | | | | | |
| ≤55 | 175/267 | 38.3/26.6 | 61.7/73.4 | 1.00 | 0.60 (0.39–0.90) |
| >55 | 239/156 | 35.2/26.9 | 64.8/73.1 | 1.00 | 0.59 (0.37–0.95) |
| Sex | | | | | |
| Male | 305/295 | 36.4/25.4 | 63.6/74.6 | 1.00 | 0.57 (0.40–0.82) |
| Female | 109/128 | 36.7/29.7 | 63.3/70.3 | 1.00 | 0.71 (0.39–1.29) |
| Hypertension | | | | | |
| No | 147/259 | 40.8/26.2 | 59.2/73.8 | 1.00 | 0.48 (0.31–0.75) |
| Yes | 267/164 | 34.1/27.4 | 65.9/72.6 | 1.00 | 0.74 (0.48–1.14) |
| Hyperlipidemia | | | | | |
| No | 274/286 | 38.3/25.2 | 61.7/74.8 | 1.00 | 0.50 (0.34–0.73) |
| Yes | 140/137 | 32.9/29.9 | 67.1/70.1 | 1.00 | 0.89 (0.52–1.49) |
| Diabetes | | | | | |
| No | 358/390 | 36.3/26.9 | 63.7/73.1 | 1.00 | 0.61 (0.44–0.85) |
| Yes | 56/33 | 37.5/24.2 | 62.5/75.8 | 1.00 | 0.56 (0.21–1.52) |

[a] Adjusted for age, sex, hypertension, hyperlipidemia, and diabetes

the cases, and 26.7, 53.7, and 19.6 %, respectively, among the controls ($P = 0.004$). The observed genotype frequencies among the controls were in agreement with the Hardy-Weinberg equilibrium ($P = 0.105$).

When we used the rs3809263 GG genotype as the reference, we found that both GA and AA genotypes were associated with a statistically significantly decreased risk of LAA stroke (adjusted OR = 0.63, 95 % CI = 0.46–0.88 for GA and OR = 0.54, 95 % CI = 0.35–0.84 for AA; Table 2), and the A allele was associated with the decreased LAA stroke risk in a dose–response manner ($P_{trend} = 0.001$). Moreover, a significant decreased risk of stroke was observed in the combined variant genotypes GA/AA, compared with the GG genotype (adjusted OR = 0.61, 95 % CI = 0.45–0.83; Table 2). In further stratification analysis, this decreased risk was more obvious among subgroups of male (0.57, 0.40–0.82), and those without hypertension (0.48, 0.31–0.75) and hyperlipidemia (0.50, 0.34–0.73; Table 3).

**Table 4** *NINJ2* mRNA expression by the genotype, using data from the HapMap

| Genotype | mRNA expression No. | Mean ± sd | $P^a$ | $P^b$ |
|---|---|---|---|---|
| All populations[c] | | | | |
| rs3809263 | | | | |
| GG | 136 | 7.73 ± 0.47 | – | 0.920 |
| GA | 104 | 7.69 ± 0.41 | 0.491 | – |
| AA | 28 | 7.75 ± 0.37 | 0.854 | – |
| CHB | | | | |
| rs3809263 | – | – | – | – |
| GG | 17 | 7.40 ± 0.26 | – | 0.019 |
| GA | 20 | 7.48 ± 0.26 | 0.357 | – |
| AA | 8 | 7.73 ± 0.42 | 0.024 | |

[a] Two-side student's *t* test within the stratum

[b] *P* values for the trend test of *NINJ2* mRNA expression among three genotypes for each SNP from a general linear model

[c] All populations include CEU (Utah residents with Northern and Western European ancestry from the CEPH collection), CHB (Han Chinese in Beijing, China), JBT (Japanese in Tokyo, Japan) and YRI (Yoruban in Ibadan, Nigeria)

## Correlation Between Rs3809263 Genotype and *NINJ2* mRNA Expression Levels

Table 4 shows *NINJ2* mRNA expression according to rs3809263 genotype. In all populations, the *NINJ2* mRNA expression levels for GA and AA genotypes were similar with that of GG genotype (trend test for the A allele effect: $P = 0.920$; Fig. 2). For the Chinese population (CHB, $n = 45$),

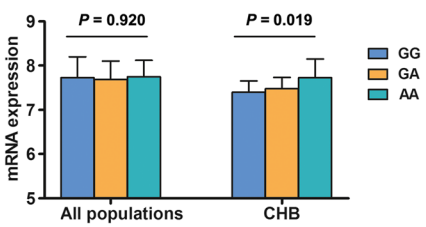


**Fig. 2** *NINJ2* mRNA expression by the rs3809263 genotype, using data from the HapMap

cell lines with the variant AA genotype had statistically higher *NINJ2* mRNA expression levels, compared with the GG genotype (trend test for the A allele effect: $P = 0.019$; Fig. 2).

## Discussion

In the present study, we identified a novel functional polymorphism (rs3809263 G > A) in the *NINJ2* promoter and examined its association with LAA stroke risk in a Chinese population. We observed a statistically significant association between the rs3809263 polymorphism and risk of LAA stroke. Moreover, genotype-phenotype correlation analysis indicated that the AA genotype carriers had significantly increased *NINJ2* mRNA expression levels in the Chinese population, suggesting that the rs3809263 G > A polymorphism is a functional SNP and a biomarker for risk of LAA stroke.

Ikram et al. [3] carried out a large GWAS (19,602 white persons) and found that 2 SNPs (rs12425791 and rs11833579) on chromosome 12p13 were associated with stroke and, in particular, atherothrombotic stroke risk. Both rs12425791 and rs11833579 were in close proximity to *NINJ2*, which encodes ninjurin2. Ninjurin2 is a surface–adhesion molecule, which is expressed in mature sensory and enteric neurons. It is reported that ninjurin2 level was upregulated after nerve injury [15]. Moreover, the ninjurin2 level influences how the brain tolerates ischemic insults. Therefore, *NINJ2* may be a candidate gene for stroke risk.

Thereafter, lots of studies were conducted to validate the association between these two SNPs (rs12425791 and rs11833579) and stroke risk/prognosis in different populations worldwide. Our previous results indicated that these two SNPs were associated with both risk and prognosis of large artery atherosclerotic stroke in the Chinese population [11, 16]. However, these two SNPs (rs12425791 and rs11833579) are located ~11 kb upstream of the *NINJ2* gene (Fig. 1), and the exact mechanisms of how these two SNPs regulate NINJ2 expression are still unknown. Through linkage disequilibrium and fine-mapping analysis, we identified a novel functional polymorphism in the *NINJ2* promoter (rs3809263 G > A) and examined its association with risk of LAA stroke in the Chinese population. As a result, we found that both GA and AA genotypes were associated with a statistically significantly decreased risk of LAA stroke, compared with the GG genotype. Given that the rs3809263, which are located in promoter of *NINJ2*, showed a significant association with stroke risk, we then used the SNPexp online tool to further evaluate biological plausibility underlying the observed association. As expected, the AA genotype carriers had significantly increased *NINJ2* mRNA expression levels in the Chinese population.

Our study has several limitations. First, because of the hospital-based study design, we cannot eliminate the possibility of selection bias of subjects. However, the distributions of genotype in our population were similar to that reported in HapMap for Chinese populations. For instance, the MAF of rs3809263 among our 423 southern Chinese controls were 0.46, compared with 0.40 in northern Chinese populations in the HapMap (45 unrelated Han Chinese in Beijing). Second, we used the SNPexp online tool to evaluate the genotype-phenotype correlation. The expression levels of the *NINJ2* mRNA associated with the promoter rs3809263 polymorphism should be confirmed in biological samples from patients and controls in future studies. Another limitation was our relatively small sample size. However, our power calculation indicated that we have 84 % power at a 0.05 significance level to detect a minimal OR of 0.65 with an exposure frequency of 40 % under the current sample size.

In conclusion, we have identified a novel functional polymorphism (rs3809263 G > A) in the *NINJ2* promoter and our results suggested that this functional promoter polymorphism may modulate the risk of large artery atherosclerotic stroke. Larger functional studies are needed to explore the fundamental role of NINJ2 and describe the inherent mechanisms in the association of this functional polymorphism with large artery atherosclerotic stroke risk.

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**Compliance with Ethical Standards** This on-going study was approved by the Institutional Review Board of Jinling Hospital (Nanjing, China).

**Conflict of Interest** The authors declare that they have no competing interests.

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