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The Association Between the Genetic Variants of the *NOTCH3* Gene and Ischemic Stroke Risk

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABG 1 Xiaoling Yuan
CDEF 2 Zifeng Dong

1 Department of Neurology, People's Hospital of Liaocheng, Liaocheng, Shandong, P.R. China
2 Department of Anesthesiology, People's Hospital of Liaocheng, Liaocheng, Shandong, P.R. China

Corresponding Author: Xiaoling Yuan, e-mail: xiaolingddy@163.com
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Background: Ischemic stroke (IS) is a leading cause of disability and death and *NOTCH3* as a gene related with cardiac-cerebral vascular disease plays a vital role in IS development. However, the reports about the effect of genetic variants in *NOTCH3* gene on IS are still few.

Material/Methods: In order to explore the association between *NOTCH3* polymorphisms and IS, 134 patients with IS and 115 controls were enrolled in this case-control study. Polymerase chain reaction was used to do the genotyping of polymorphisms. The χ^2 test was performed to evaluate Hardy-Weinberg equilibrium (HWE) in the control group and calculate odds ratio (OR) with corresponding 95% confidence interval (CI) which represented the association intensity of *NOTCH3* gene polymorphisms and IS risk.

Results: The genotype frequencies in the control group all confirmed to HWE. TT genotype of 381C>T was associated significantly with IS risk (OR=2.441, 95%CI=1.021–5.837). TC, CC mutant genotypes of 1735T>C had higher frequencies in cases than controls and the difference was significant (*P*=0.013, 0.041); further, its C allele also increased 0.722 times risk in the case group than controls (OR=1.722, 95%CI=1.166–2.541).

Conclusions: *NOTCH3* 381C>T and 1735T>C polymorphisms were associated with IS and might be the risk factors for IS development, but not *NOTCH3* 605C>T polymorphism.

MeSH Keywords: Association • Optic Neuropathy, Ischemic • Polymorphism, Genetic

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Background

Stroke is caused by cell death owing to abnormal blood supply in brain with high mortality and disability [1–3].The symptoms of stroke are sudden weakness, an inability to move or feel, especially one side of the body, aphasia, hearing disorders, and loss of consciousness [4]. It is divided into hemorrhagic and ischemic stroke (IS) mainly, the latter results in lack of blood flow and finally gives rise to losing part of the brain functions [5,6]. IS, a common type, accounts for 85% of stroke deaths and is effected by multiple factors, such as gene, hypertension, tobacco smoking, diabetes [7]. In 1996, the genetic mutations of *NOTCH3* were found to be associated with stroke for the first time [8].

NOTCH reports play key roles in regulating nervous system development and facilitating neural stem cells renewal and proliferation [9,10]. It is encoded by *NOTCH* gene including four members [11]. The expression of defects and inactivation in NOTCH result in severe nervous and cardiovascular system diseases, sometimes they are fatal [12–14]. *NOTCH3* is a member of the *NOTCH* family and can accelerate cell proliferation and gliogenesis [15]. It is also reported to participate in brain development and some tumors occurrence [16,17]. The mutations of *NOTCH3* have been identified and cause cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) [18,19]. Similarly, mutations in *NOTCH3* gene are found to be associated with Alzheimer's disease in a Turkish family [20]. Further, usually patients with the clinical symptoms of CADASIL present with IS. However, so far, only a few reports explain the relationship between *NOTCH3* gene polymorphisms and IS risk in several populations; the etiology of IS is also unclear.

In the current study, we selected 3 polymorphisms in *NOTCH3* (381C>T, 1735T>C, 605C>T) to examine the association with IS susceptibility in 134 patients with IS and 115 control subjects who came from the Chinese Han population. We hoped that it provided evidence for explaining the pathology and etiology of IS.

Material and Methods

Study subjects

In the case-control study, 134 patients with IS were selected from the neurology department of People's Hospital of Liaocheng diagnosed by clinical pathology in April 2013 to October 2014 as the case group. The patients were aged between 35 and 74 years, and 61.9% of the patients were male. A total of 115 control subjects were frequency-matched with cases for age and sex. The control group comprised patients with mild symptoms from ophthalmology, otorhinolaryngologic, and digestive departments of the same hospital, as well as community residents, who had not had a stroke. The age range was from 33 to 75 years, with an average age of 59.26±9.04. This study design was supported by the Research Ethics Committee of People's Hospital of Liaocheng. All subjects were Chinese Han population without a blood relationship.

We collected the detailed clinical information of every subject after the patient or family member signed the written consent form. Some indexes were examined, such as smoking, alcohol consumption, body mass index, hypertension, hyperglycemia, diabetes. A person who smokes 1 or more cigarettes a day, on average, for at least half a year is a smoker. A person is a drinker who drinks >1 time a week (for men) or ≥1 time a week (for women). Average blood pressure through measuring 3 times ≥140/90 mm Hg or taking antihypertensive drugs is defined as hypertension. Fasting blood glucose (FBG) level ≥6.1 mmol/L is defined as hyperglycemia. FBG level ≥7.8 mmol/L, or 2 hours after taking oral glucose ≥11.1 mmol/L, is diagnosed as diabetes.

DNA extraction and PCR primer design

From every selected person, 2 mL fasting venous blood was collected using a vacuum collective tube with EDTA anticoagulant. Then genome DNA was extracted by conventional chloroform/isoamyl alcohol method and finally stored at –20°C refrigerator.

Table 1. The PCR primer sequences and position of *NOTCH3* gene polymorphisms.

Polymorphisms		Primer sequences	Position	Length (bp)
381 C>T	Forward	5'-GCGTGTTTCTTGCCTGTCTTGTGT-3'	Exon3	248
	Reverse	5'-AGGACAGGGTGAGTTTAGGACTGA-3'		
1735 T>C	Forward	5'-ATTGGTCCGAGGCCTCACTT-3'	Exon11	327
	Reverse	5'-CCATTCCCAACCCCTCTGTG-3'		
605 C>T	Forward	5'-TAGTCGGGGGTGTGGTCAGT-3'	Exon4	450
	Reverse	5'-TCAAACCCTAGCAGGGAA-3'		

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Table 2. The detailed clinical characteristics of all subjects.

Index		Case, n (%)	Control, n (%)	*P*
Age	Mean age	59.26±9.04	61.46±10.23	–
Gender	Males	83 (61.94)	66 (57.39)	0.465
	Females	51 (38.06)	49 (42.61)	
BMI	<25	93 (69.40)	82 (71.30)	0.743
	≥25	41 (30.60)	33 (28.70)	
Smoking	Yes	68 (50.75)	42 (36.52)	0.024
	No	66 (49.25)	73 (63.48)	
Alcohol consumption	Yes	52 (38.81)	35 (30.43)	0.167
	No	82 (61.19)	80 (69.57)	
Hypertension	Yes	86 (64.18)	31 (26.96)	0
	No	48 (35.82)	84 (73.04)	
Hyperglycemia	Yes	54 (40.30)	16 (13.91)	0
	No	80 (59.70)	99 (86.09)	
Diabetes	Yes	21 (15.67)	6 (5.22)	0.008
	No	113 (84.33)	109 (94.78)	

PCR primers were designed in Primer 5.0 software based on GeneBank and the detailed primer sequences were listed in Table 1.

The genotyping of *NOTCH3* gene polymorphisms

Polymerase chain reaction (PCR) was used to conduct the genotyping. PCR reaction solution was a total of 20 μL, containing 0.5 μL genome DNA template (20 ng/μL), 1 μL forward and reverse primers (each 0.5 μL, 10 μmol/L), and 10 μL PCR Mix, and finally added to 8.5 μL deionized water. Subsequently PCR system was performed according to the following steps, firstly initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at (65°C for 381 C>T and 1735 T>C, 60°C for 605 C>T) for 30s, extension at 72°C for 1 min and final extension at 72°C for 8 min.

The PCR products were checked in 2% agarose gel electrophores. Sequencing was performed on an ABI 3130 Genetic Analyser after the samples were purified.

Data analysis

All data were represented by $\bar{\chi}$±s or%. The genotype distributions of gene polymorphisms in the control group was checked to make sure it was consistent with Hardy-Weinberg equilibrium (HWE) by χ^2 test which was also used to calculate odds ratio (OR) and 95% CI evaluating the association intensity between *NOTCH3* gene polymorphisms and IS susceptibility. Statistical analysis was conducted in SPSS 18.0 software and $P<0.05$ was considered as significant difference.

Results

The characteristics of all subjects

There was no significant difference in cases and controls based on age and gender ($P>0.05$). As was shown in Table 2, we saw that BMI and alcohol consumption were not the direct risk factors, however, the number of smoker, suffering from hypertension, hyperglycemia, diabetes was obviously large in cases compared with controls and they might be associated with the development of IS ($P<0.05$).

The comparison of genotype distribution in *NOTCH3* gene polymorphisms between the cases and controls

The results were shown in Table 3, TT genotype frequency of 381C>T was higher in the case group than the control group and might be associated with IS risk (OR=2.441, 95%CI=1.021–5.837). Similarly, the polymorphism 1735T>C was also an independent risk factor for IS. TC, CC genotypes had higher frequency in cases compared with control subjects

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Table 3. The association strength of *NOTCH3* polymorphisms with ischemic stroke.

Genotype\allele		Cases, n (%)		Controls, n (%)		χ^2	*P*	OR (95%CI)	
381 C>T	CC	37	(27.61)	43	(37.39)	–	–	1.000	(Ref.)
	CT	76	(56.72)	62	(53.91)	1.579	0.209	1.425	(0.820–2.476)
	TT	21	(15.67)	10	(8.70)	4.136	0.042	2.441	(1.021–5.837)
	C	150	(55.97)	148	(64.35)	–	–	1.000	(Ref.)
	T	118	(44.03)	82	(35.65)	3.165	0.057	1.420	(0.989–2.039)
1735 T>C	TT	52	(38.81)	65	(56.52)	–	–	1.000	(Ref.)
	TC	67	(50.00)	43	(37.39)	6.162	0.013	1.948	(1.148–3.305)
	CC	15	(11.19)	7	(6.09)	4.179	0.041	2.679	(1.017–7.055)
	T	171	(63.81)	173	(75.22)	–	–	1.000	(Ref.)
	C	97	(36.19)	57	(24.78)	7.546	0.006	1.722	(1.166–2.541)
605 C>T	CC	73	(54.48)	63	(54.78)	–	–	1.000	(Ref.)
	CT	42	(31.34)	39	(33.91)	0.068	0.794	0.929	(0.536–1.612)
	TT	19	(14.18)	13	(11.31)	0.340	0.560	1.261	(0.577–2.757)
	C	188	(70.15)	165	(71.74)	–	–	1.000	(Ref.)
	T	80	(29.85)	65	(28.26)	0.152	0.697	1.080	(0.733–1.593)

and the difference was significant (*P*=0.013, 0.041, respectively). In addition, allele C of 1735T>C was remarkably associated with the increased susceptibility to IS (OR=1.722, 95%CI=1.166-2.541); however, *NOTCH3* 605C>T polymorphism did not show any significant relevance with IS.

Discussion

Stroke is the second leading cause of death and disability and occurs mostly in the middle-aged population, but onset age is inclined to be young nowadays [21]. Most strokes are IS [22]. Multiple elements participate in the development and progression of IS, such as genetic variant, hypertension, high cholesterol, and diabetes. In addition, the people exposed to external factors suffer from IS, so the possible result is abnormal expression of relative genes caused by these factors. To explain the pathogenesis of IS, scholars study the effect of gene mutations on IS occurrence.

Lv et al. explore the relationship between *MTHFR* gene polymorphisms and IS in the eastern China Han population. They indicate that A1298C polymorphism and haplotypes consisting of A1298C, C677T polymorphisms may be associated with the risk of IS [23]. Gao et al. perform a meta-analysis about whether genetic variants of *AGT* gene are related to IS on the basis of previous studies, the results show the significant relevance of *AGT* M235T, T174M polymorphisms with the susceptibility to IS in the Chinese Han population [24]. Wu et al. also prove that β-fibrinogen gene polymorphisms possibly contribute to the risk of IS development in the Chinese Han population [25]. In addition, *COX*, *MMP*, *CCM*, and IL relative genes are identified to involve in the pathology of IS. However, the cause of IS is still not completely clear.

As we know, the mutations of *NOTCH3* are the essential cause of CADASIL. The statistical results of Ungaro et al. show that more than 130 different mutations of *NOTCH3* gene have been identified in CADASIL patients. Its nosogenesis may be a gain or loss of cysteine residue caused by mutation in 1 of the 34 epidermal growth factor-like repeats located in the extracellular domain of NOTCH3 protein [26]. Uyama et al. study a *NOTCH3* mutation (Arg133Cys) in 2 unrelated Japanese families suffering from CADASIL. The conclusion indicates a heterozygous genotype of Arg133Cys present in CADASIL patients, and, furthermore, this locus is in the exon 4 of *NOTCH3* gene, which is a hot spot domain in Caucasian families with CADASIL. This confirms that the occurrence of CADASIL caused by a *NOTCH3* mutation is not restricted by geographic position [27]. Because stroke is the clinical and primary symptom of CADASIL, the relevance of *NOTCH3* polymorphisms and IS is suspected. Joutel et al. report for the first time in *Nature* 1996 the genetic variants of *NOTCH3* involved in the occurrence of stroke [8]. Later, when Ross et al. perform a relative study to

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verify the association in Caucasians, the result is certain that the polymorphisms of *NOTCH3* gene may lessen the risk of IS [28]. The genetic variants of *NOTCH3* gene are also usually detected in patients with acute IS in Korea, which indicates *NOTCH3* polymorphisms can influence IS [29].

In the current study, subjects were from the Chinese Han population, and 3 polymorphisms of *NOTCH3* gene were selected to explore the roles in IS. The homozygous mutant genotype frequency of 381C>T in cases was 2.441 times more than in the control group. The mutant genotypes and allele of 1735T>C in frequency had the significant differences between the 2 groups. These 2 polymorphisms each showed the relevance with IS development in study population, but 605C>T might not be an independent factor in IS.

Conclusions

The accuracy of our results is restricted by some conditions. The study subjects only include the Han population in Shandong Province, China, and the sample size is small. However, we explain the role of only a single polymorphism in IS; the interactions are not considered. Therefore, in exploring the precise pathogenesis of IS, we still have a lot of work to do. We need well-designed studies with sufficient sample size and environmental factors to be performed in the future.

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