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# The Association of C381T Polymorphism in *Notch3* Gene With Cerebral Stroke

**Afsoon Ghoreishizadeh[1], Seyed Mahmoud Tabatabaei[2*], Ashkan Adibi[3], Saba Dastar[1]**

## Abstract

**Objectives:** Cerebral stroke is a common multifactorial trait that does not follow Mendelian pattern of inheritance. The phenomena of gene-gene or gene-environment interactions may be responsible for the multifactorial traits. Any mutation may be associated with silencing implicated in other disorders. This case-control study explored the association between *Notch3* polymorphism and stroke in Iranian-Azeri population.

**Materials and Methods:** In this case-control study, C381T polymorphism in *Notch3* gene was evaluated among 65 patients with ischemic stroke and 65 individuals without any stroke as control group. The samples were recruited from 5 clinical centers during 2014-2015. PCR-SSCP and sequencing methods were used to obtain the data.

**Results:** In this study, the frequencies of C and T alleles in the patient group were 85% and 15%, and in the control group were 94% and 6%, respectively. The frequencies of CC, CT and TT genotypes were 72%, 26% and 2% in the patient group, and 88%, 12% and 0% in the control group, respectively. Both control and patient groups had significant difference considering their both allele and genotype frequencies. The individuals with C381T polymorphism in *Notch3* gene were in a significantly higher risk of thrombotic stroke ($P = 0.02$, CI: 0.128-0.256: 95%, odds ratio [OR]: 2.72).

**Conclusions:** Our results showed that combination of T allele of this gene conferred higher risk for cerebral stroke. The interaction of gene mutation with post-translation modification may serve as a novel field for stroke research.

**Keywords:** Cerebral stroke, C381T polymorphism, *Notch3* gene

## Introduction

When blood supply to brain is compromised by the rupture or blockage of arteries, it results in death of brain cells due to lack of sufficient oxygen and finally a cerebral stroke may occur (1-4). The main factors leading to ischemic stroke include: thrombosis (5,6), embolism (7-9) and systemic hypofusion, and the risk factors for cerebral stroke are: high blood pressure, cardiovascular disorders (10-12), dyslipidemia (13), Homocysteineuria (14,15), and blood disorders such as sickle cell anemia (16-18). Cerebral stroke symptoms usually occur suddenly within a few seconds to several minutes and in most cases these symptoms do not have much progress, and the types of stroke symptoms depend on brain regions involved (19). Thus, the more a certain region of the brain is damaged by stroke, the less functional that region or area will be (2, 20).

*Notch3* gene (also called LMNS) is one of the crucial genes for pharmacogenetical studies. This gene is located on 19p13.12 and includes 33 exons (21). *Notch3* gene encodes a receptor protein on vascular smooth muscle cell on the arterial vessel with both extracellular and intracellular domains (22). When ligands bind to the extracellular domain of the receptor, the cytoplasmic domain gets activated. Following this activation, a series of intracellular signals are initiated, leading to transcription of genes that are recruited to repair damaged tissues. This signaling pathway is called Notch3-dependent signaling pathway (23,24). Notch3 receptor plays an essential role in the survival and function of vascular smooth muscle cells. Thus, these receptors are necessary for healthy muscle cells of the brain arteries (23). More than 150 mutations in the *Notch3* gene have been identified (25), from which the most important mutation is mutation 381TT/TC which is located in exon 1 and is known to lead to stroke (26). Almost all of these mutations lead to a change in the structure and function of signaling proteins, which interfere with the function of the receptor. Malfunction of Notch3 receptor may lead to cell suicide and consequently to vascular damage in the brain and to the resulting cerebral stroke (26,27).

## Objectives

The main aim of this study was to investigate the

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[1]Department of Genetics, Ahar Branch, Islamic Azad University, Ahar, Iran. [2]Department of Physiology, Tabriz Branch, Islamic Azad University, Tabriz, Iran. [3]Department of Genetics, Tabriz Branch, Islamic Azad University, Tabriz, Iran.
*Corresponding Author: Seyed Mahmoud Tabatabaei, Tel: +984134792132, Email: smt@iaut.ac.ir

association of C381T polymorphism in *Notch3* gene with cerebral stroke in East Azerbaijan, Iran.

## Materials and Methods

In this study, 65 patients with ischemic stroke were recruited from 5 clinical centers during 2014-2015. Moreover, 65 cancer-free controls were selected. Informed consent was obtained from the subjects in both patient and control groups. A 5-mL peripheral blood sample was collected from each subject.

DNA was extracted from the peripheral blood using saturated salt method (28). The quality and quantity of the extracted DNA was evaluated using spectrophotometry and electrophoresis on 1% Agarose gel.

For analyzing the 381TT/TC polymorphism in the *Notch3* gene, PCR-single strand conformation polymorphism (PCR-SSCP) was used. This method is determined as a conformational difference of single-stranded nucleotide sequences with identical length as induced by differences in the sequences under certain experimental conditions. The primers used in the PCR were Notch3F: 5′-GAGGACCCCTGTCACTCAGGC -3′ as a forward primer and Notch3R: 5′-TTAGGACTGACCACACCCCCG -3′ as a reverse primer. PCR technique was performed in 25 µL reaction mixture as final volume containing 0.2 mM dNTP, 0.5 U of Taq DNA polymerase, 5 pmoles of each primer, 1.5 mM $MgCl_2$, 1× PCR buffer, and ~1µg of genomic DNA on a Thermal Cycler (Eppendorf, Germany). The program for PCR reaction was as follows: initial denaturation at 95°C for 5 minutes, followed by 32 cycles of denaturation at 95°C for 20 seconds, annealing at 68°C for 20 seconds, and extension at 72°C for 20 seconds, and a final extension at 72°C for 5 minutes.

PCR-SSCP assay was used to detect 381TT/TC polymorphism in the *Notch3* gene. The 5-µL PCR products were mixed with 7 µL formamid loading buffer and then denaturation process was performed at 95°C for 20 minutes. Immediately after termination of the required time, the products were incubated at -20°C for 10 minutes to be dimerized. Products obtained were then loaded on 28% polyacrylamide electrophoresis gel for 2 hours at 4°C under 120 V and the patterns were identified using silver nitrate.

To further validate and estimate the genotyping assay of PCR-SSCP for C381T polymorphism, different PCR products with different genotypes were confirmed by direct sequencing using an automated sequencer technique.

### Statistical Analyses

Chi-square test was used to analyze the frequency of allele and genotype and their difference in the control group and the stroke subjects. The results from statistical analysis were used to study the genotype correlation with the response to the treatment and clinical symptoms. The confidence limit was considered 95% in all tests and $P < 0.05$ was considered statistically significant.

## Results

### General Characteristics of the Subjects

A total of 130 subjects were recruited for this study, including 65 ischemic stroke patients and 65 healthy control subjects. There were no significant differences between the subjects of both groups in terms of gender and age distribution; in other words, the subjects were not matched based on these variables. The characteristics of the stroke ischemic patients and control subjects and the distribution of genotypes of the *Notch3* gene C381T polymorphism are shown in Table 1.

### Detection and Genotyping of *Notch3* SNP

In the present study, one allelic variant (C381T) was investigated by the PCR-SSCP method. Sequence analysis suggested that C381T polymorphism was caused by C to T mutation in the exon1 of the human *Notch3* gene. The PCR product of C381T was 159 bp.

### Genotypic Frequencies

The chi-square test for the C381T variant in the considered subjects suggested that the polymorphic site was in Hardy-Weinberg equilibrium ($P > 0.05$, Table 2). The allelic and genotypic frequencies of the C381T polymorphism is presented in Table 2. Results showed that the frequencies of CC, CT and TT genotypes in the patient group were 72%, 26% and 2%, and in the control group were 94%,

**Table 1.** CI and OR of Genotypes

| | *P* Value | 95% CI | OR |
|---|---|---|---|
| CC vs. CT + TT | 0.02 | 0.733-0.867 | 1.91 |
| CT vs CC + TT | 0.02 | 0.128-0.256 | 2.72 |

OR: odds ratios, CI: confidence interval.

**Table 2.** Demographic, Allelic and Genotype Frequencies of the Study Population

| Characters | Groups | | *P* value |
|---|---|---|---|
| | Case | Control | |
| Gender (M/F) | 33/32 | 37/28 | |
| Age (y) | 52 ±14.3 | 54 ±11.7 | |
| Allelic frequency (%) | | | |
| C | 85 | 94 | 0.01 |
| T | 15 | 6 | 0.06 |
| Genotype frequency (%) | | | |
| CC | 72 | 88 | 0.005 |
| CT | 26 | 12 | 0.01 |
| TT | 2 | 0 | 0.06 |

6%, and 0%, respectively (Table 2). To ensure the results, some TT and CC homozygotes and CT heterozygote were sequenced and the produced results were blasted.

### *Notch3* Polymorphisms and Risk of Ischemic Stroke

Analysis of the association of genotypes/alleles from the C381T SNP with the risk of ischemic stroke suggested that there was a significantly increased risk of ischemic stroke. Statistical analysis of the study groups in the frequency of T allele in the patients with *Notch3* gene C381T polymorphism and following the control group showed significant difference (Table 2).

## Discussion

High risk of cerebral stroke may bring about death or cause serious irreversible damage. Therefore, it is rational to check the risk factors beforehand. *Notch3* gene is an essential component of the vascular system, and any mutation in this gene can compromise the normal function of protein which can ultimately lead to arterial damage. Under such circumstances, the artery would be more susceptible to clot formation and this clot can end up as occlusion in any area of brain, disrupting the blood flow (thus shortage of oxygen) and resulting in cerebral stroke. In this study, a significant difference was observed between the control group and the patient group considering allele and genotype frequencies.

Moreover, in this research, significant difference was observed between the control and patient groups considering their C381T polymorphism. In one study in 2015 performed by Li et al, the results demonstrated that *Notch3* gene mutations and SNPs are important for the totality and function of small vessels. This study was performed as a large case-control study on Chinese patients and investigated the combined effect of MTHFR 677TT, ALOX5AP 2354AA, and *Notch3* C381T on higher risk of thrombotic stroke. In conclusion, the results of the study suggested that *Notch3* SNPs are likely to be associated with lacunar infarction. In particular, they found that rs1043994 was associated with lacunar infarctions (29). Previously, this combination was investigated for thrombotic stroke by Liu et al in 2009. Their results showed that combination of ALOX5AP T2354A, MTHFR C677T and *Notch3* C381T alleles conferred higher risk for cerebral stroke in comparison with single risk allele (30).

In a similar study in 2011, Schmidt et al investigated all common SNPs in *Notch3* gene and showed that 4 of these SNPs, rs10404382, rs1043994, rs10423702 and rs1043997, were significantly associated with both the presence and progression of age-related white matter lesions (27). In one study in 2010, two SNPs on exons 3 and 4 of *Notch3* gene were investigated for migraine by Menon et al. Results of this study indicated that C381T (rs3815188) and G684A (rs1043994) SNPs in *Notch3* gene may play a role in susceptibility that influences both the severity and subtype of migraine (26). *Notch3* gene polymorphisms have been also investigated in other diseases. Shen et al showed that the *Notch3* gene 684G>A polymorphism may be used as a prognostic marker for gliomas. Their results showed that the 684G>A polymorphism was closely associated with a higher tumor grade, poorer tumor differentiation, and performance score in these glioma patients. One important point in this finding was that 684G>A polymorphism was significantly associated with the prognosis of glioma in the patients regardless of their treatment manner (31). Other polymorphisms of *Notch3* gene have been shown to play a role in the development of type 2 diabetes (T2DM). In a recent study performed by Ozbayer et al, a significant association was found between the rs1043994 and rs3815188 variants of the *Notch3* gene and the risk of developing T2DM among Turkish individuals. In conclusion, the rs1043994 variant was found to be associated with T2DM due to a significant ratio with the presence of the A allele, meaning that AA genotype increased the risk of T2DM compared to the GG genotype. Similarly, carriage of the rs3815188 CC genotype and absence of the T allele was observed in T2DM subjects. The genotype distribution of the rs3815188 variant suggested that the CC genotype was associated with an 11.351-fold increase in the risk of T2DM as compared to the CT genotype carriers (32).

## Conclusions

Our results confirmed that the combination of T allele of the exon 1 may impose higher cerebral stroke risk than what C allele may impose. The interaction between gene mutation and post-translation modification may serve as a novel and exquisite area for stroke research.

## Conflict of Interests

Authors have no conflict of interests.

## Ethical Issues

This study was approved by Ahar Branch, Islamic Azad University, Ahar, Iran.

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