

A Novel Mutation Outside of the EGFr Encoding Exons of NOTCH3 Gene in a Chinese with CADASIL

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Background: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a hereditary cerebral small vascular disease caused by the mutations of the NOTCH3 gene. The NOTCH3 gene consists of 33 exons. The pathogenic mutations of the NOTCH3 gene in CADASIL are located in 2–24 exons coding for the 34 EGFr (epidermal growth factor-like repeat) domains. The classical clinical manifestations are recurrent TIA or ischaemic stroke, migraine, cognitive disorder and affective disorder. The deposition of granular osmiophilic material (GOM) in the vascular wall is considered as a hallmark of the disease. **Methods:** Here, we report a rare pathogenic mutation on exon 29 of the NOTCH3 gene in a Chinese family. Clinical data for the proband and available relatives is collected. Mutation analysis of the NOTCH3 gene was performed by screening the entire 33 exons in this family and 200 normal controls. A complete imaging evaluation and skin biopsy were performed on the proband. **Results:** We identified a novel R1761H (c.5282G>A) mutation. The same mutation was not founded in 200 normal controls. The proband had recurrent stroke, depression, cognitive decline and cerebral lobe hemorrhage. Cranial MRI showed white matter lesions and multiple infarction. Susceptibility weighted imaging revealed numerous microbleeds. Most importantly, the deposition of GOM was found in the proband. **Conclusion:** 33 exons of NOTCH3 gene should be performed for individuals with a convincing CADASIL phenotype and positive family history.

Key Words: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy—NOTCH3—novel mutation

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Introduction

The NOTCH3 gene encodes for a single-pass transmembrane receptor protein, with a large extracellular domain (ECD), including 34 EGFr (epidermal growth factor-like repeat) domains and 3 Notch/Lin-12 repeats (LNR) repeats, a single transmembrane (TM) portion and an intracellular domain (ICD) containing RAM (RBP-J associated molecule) domain, ankyrin-repeat motifs and a

PEST domain (Fig. 1).¹ The signal transduction in the NOTCH3 pathway is mainly achieved through the binding of the receptor and the ligand between adjacent cells. After binding of a ligand, the TM region experiences a range of proteolytic cleavage steps, resulting protein cleavage and the production of a transcriptionally active ICD. After ICD is released, it can be directly translocated into the nucleus, combined with RAM region and ankyrin region, subsequently activate downstream target genes.^{2,3}

The deposition of granular osmiophilic material (GOM) in the small vessels is credible evidence for the diagnosis of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL).⁴ The majority of CADASIL mutations result in gain or loss of a cysteine residue within the 34 EGFr domains. The unpaired cysteine residues which is predicted to disrupt typical disulfide bridging may contribute to the abnormal accumulation of NOTCH3 ectodomain.^{5,6} NOTCH3

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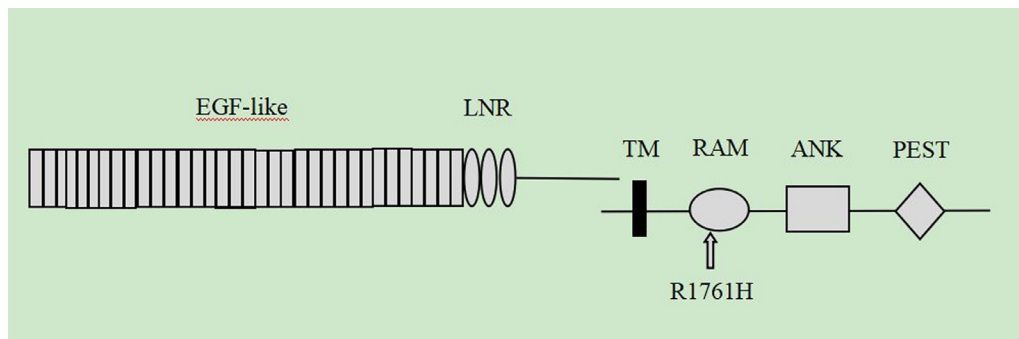


Fig. 1. Schematic representation of the NOTCH3 receptor indicating the p.R1761H mutation with respect to the different domains (EGF-like, epidermal growth factor-like; LNR, Lin12 Notch Repeats; TM, Transmembrane Domain; ANK, ankyrin repeats)

ectodomain forms microscopic aggregates around vascular smooth-muscle cells. More recent investigation showed the NOTCH3 ectodomain to be a major component of GOM.^{7,8}

Herein, a cysteine-sparing mutation outside of the EGFR encoding exons was identified to be associated with GOM positive patient.

Subjects and method

Case report

The proband (Fig. 3 III:4) is a 40-year-old man who was referred to our hospital for progressive memory disorders since the age of 39. He had suffered from a cerebral hemorrhage in left occipital at the age of 38. The patient experienced recurrent small strokes since the age of 35. Relatives complained that the patient was a narrowing of the field of interest and unwilling to take the initiative with other. Arterial hypertension was diagnosed at the age 33. We performed a comprehensive neurological evaluation for him. The results on the Mini-Mental Status Examination (MMSE) and Montreal Cognitive Assessment (MoCA) was impaired (a score of 19 and 12). These tests revealed that he had visual space executive dysfunction, decrease in memory and attention. The Hamilton Depression Scale (HAMD) suggested that he had mild depression. Brain MRI indicated multiple subcortical infarction in basal ganglia and the periventricular. Confluent white matter hyperintensities in the periventricular white matter, basal ganglia, extended to external capsule, and centrum semiovale. Multiple microbleeds were located simultaneously in various parts of the brain with a preference for cortical-subcortical regions, thalamus. Cranial MR angiography were normal. There was no evidence for head trauma, arteriovenous malformations, aneurysms or neoplasia (Fig. 2).

The mother of the proband (Fig. 3 II:4) initially presented dizziness, then she experienced cerebral infarction episode characterized by left arm weakness at the age of 70. His uncle (Fig. 3 II: 2) was diagnosed with dementia at the age 40. At present, he is bedridden. The son of his uncle (Fig. 3 III: 1) had presented personality changes

characterized by depression since the age of 20, then suicided at the age of 21. The other uncle (Fig. 3 II:3) of the proband had a history of recurrent stroke. The proband was the youngest of three siblings. He has two sons. One is 8-year-old and the other is 15-year-old. The eldest brother of the proband (Fig. 3 III:2) was diagnosed as TIA associated with balance disorder. The other brothers (Fig. 3 III:3) and two sons of proband (Fig. 3 IV:1 and IV:2) is asymptomatic. The pedigree of this CADASIL family was shown in Fig. 3. The clinical data of these subjects are summarized in Table 1.

Methods

Genomic DNA from peripheral blood mononuclear cells of the proband and six family members. Three symptomatic members and three asymptomatic members were tested. 200 unrelated neurologically unaffected Chinese were recruited as controls. Human NOTCH3 cDNA and protein sequences were obtained from GenBank (accession NG-009819.1). All 33 exons and their flanking intronic sequences of NOTCH3 gene were amplified by polymerase chain reaction (PCR). PCR amplification was performed with specific primer which were designed using the online design software Primer 5.0. the specificity of primer was tested using the BLAST software in NCBI. PCR products were assessed via electrophoresis on a 2% low-melting-point agarose gel followed by phenol-chloroform extraction and ethanol precipitation. Purified PCR products were directly sequenced using an ABI3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing reads and lists were compared using the Chromas software and NCBI blast. The biological function of amino acid changing the protein structure and function of NOTCH3 were evaluated by software PolyPhen-2 (available from <http://genetics.bwh.harvard.edu/pph2>) and SIFT (available from <http://sift.jcvi.org>). skin biopsies was performed on the proband. The specimens were initially fixed in 2.5% glutaraldehyde, then in 1% buffered osmium tetroxide, dehydrated in ethanol and embedded in Epon. To select arterioles for Semithin sections were cut and stained with toluidine blue. Thin sections were double-stained

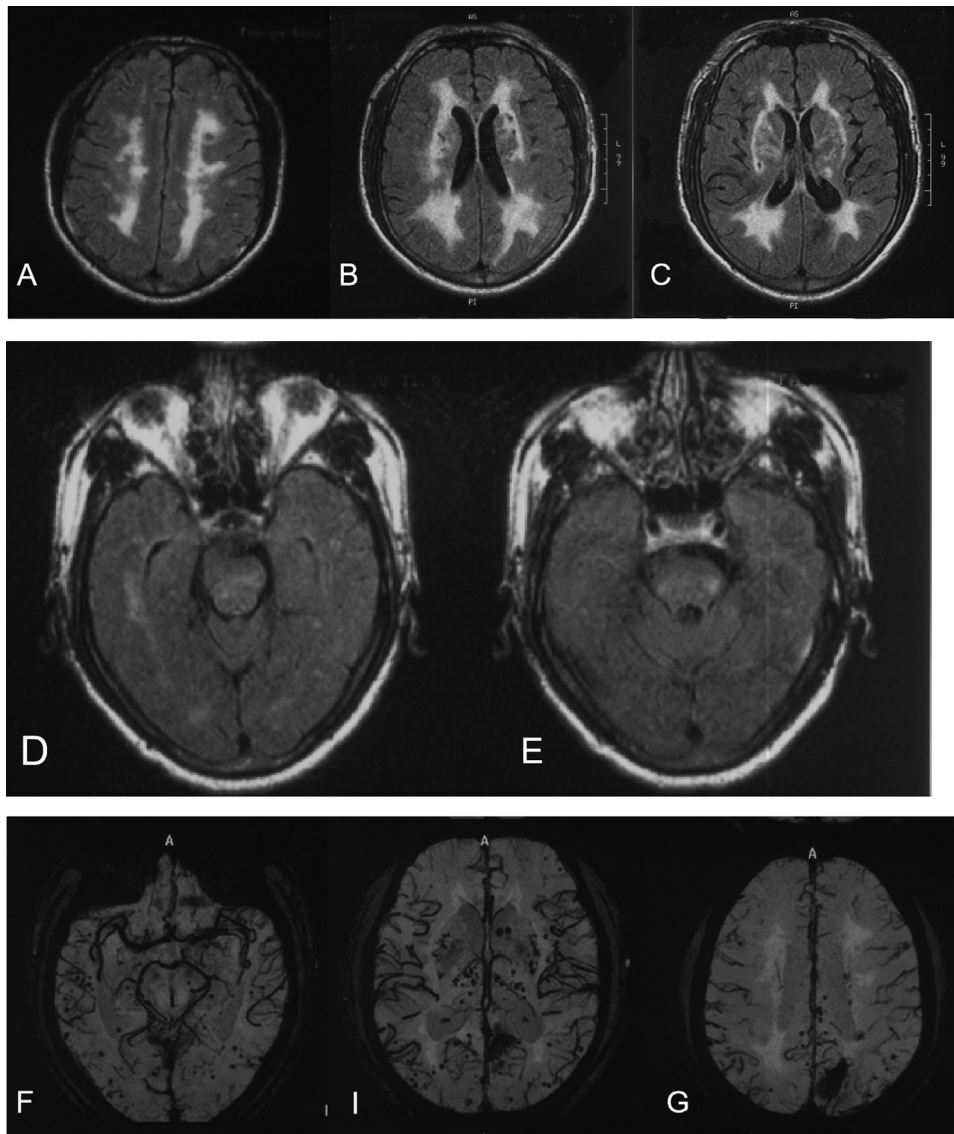


Fig. 2. A-E Brain MRI findings of the proband. FLAIR image demonstrates hyperintensity lesions in the centrum semiovale, periventricular white matter, basal ganglia, and brainstem, characteristically in the external capsule. F-G Susceptibility weighted imaging shows cerebral hemorrhage in the left occipital lobe and multiple cerebral microbleeds in the cortical-subcortical regions, thalamus, bilateral basal ganglia.

with Uranyl acetate and lead citrate improved the detection rate of GOM. At last, the samples were examined under an electron microscope.

Written informed consent forms were provided from the family and 200 healthy subjects. The study was supported by the Institutional Review Board of Zhengzhou University.

Results

Genetic analysis

A novel NOTCH3 mutation C.5282G>A (p.R1761H) in exon 29 was identified (Fig. 4). The same variant

was detected in his mother and his eldest brothers. The same mutation were not found in 200 normal controls. The new mutation were not being reported in HGMD (<http://www.hgmd.cf.ac.uk/ac/index.php>) and NOTCH3 Gene Mutation Database (<http://www.LOVD.nl/NOTCH3>).SIFT and PolyPhen-2 analyses predicted the damaging effect of the variants.

Electron microscopy

In our proband, electron microscopy of skin biopsies indicates vascular smooth muscle cells with atrophy. Skin biopsy showed CADASIL-specific deposits of GOM around vascular smooth-muscle cells (Fig. 5).

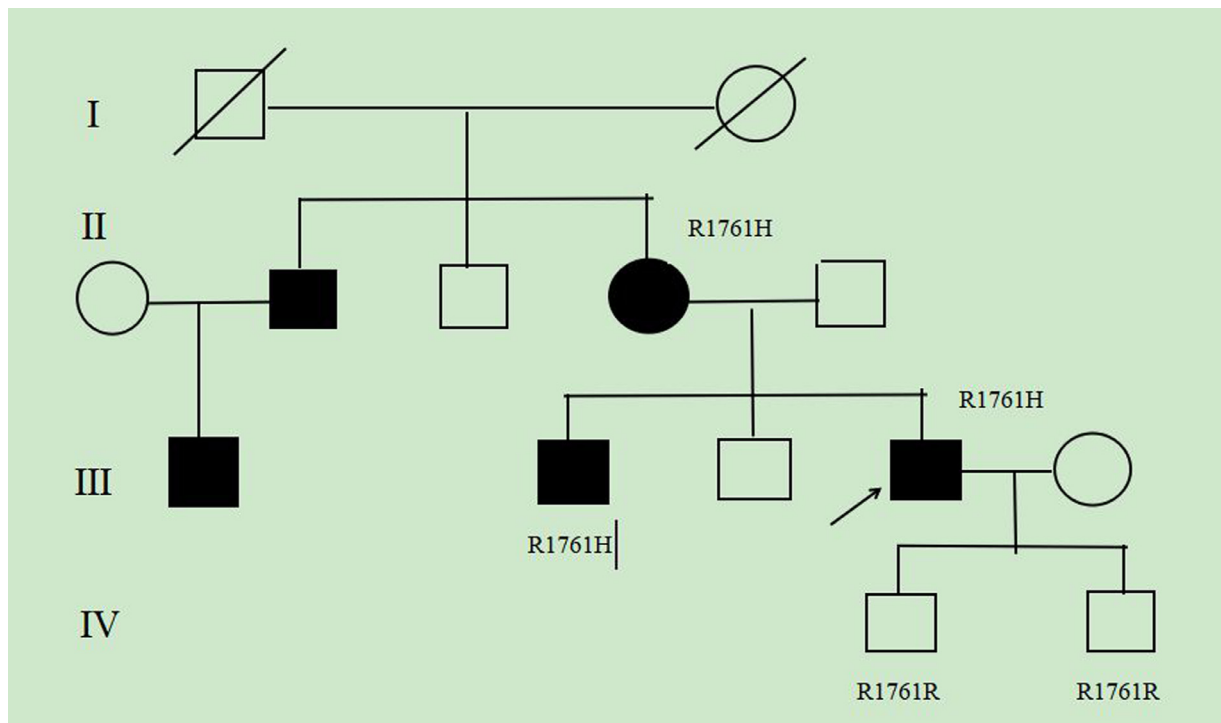


Fig. 3. The genealogy of this Chinese CADASIL family was shown. The arrow points to the proband. Square and circles indicate males and females, respectively. A bar across the symbol means a deceased individual. Symptomatic family members are shown in black-filled. NOTCH3 genetic status is indicated for each tested subject.

Discussion

We described a novel heterozygous missense mutation (c.5282 G>A) in exon 29 of NOTCH3 gene. The other mutation was not found in the remaining exons. Clinical presentation of the proband was found to be consistent with CADASIL. Multiple subcortical infarcts and extensive white matter abnormalities including the external capsule was seen in the proband. However, white matter abnormalities in the anterior temporal pole which are regarded as characteristic in CADASIL were absent in the index case. We think absence of anterior temporal pole

changes on T₂-weighted and FLAIR images may be related to the following factors. Firstly, anterior temporal pole changes are less frequent in Asian populations.⁹ Secondly, the absence of anterior temporal pole involvement maybe a radiographic characteristics of CADASIL patients with cysteine-sparing mutations.¹⁰ We evaluated its segregation within the family, identifying the same mutation in his mother and brother. PolyPhen-2 and SIFT shows that the mutation may cause structure destruction of NOTCH3 protein. The same mutation was not found in 200 healthy collators. To assess the significance of the novel NOTCH3 variant, we performed ultrastructural

Table 1. Main clinical, genetic and MRI features of family members Key: C, current age; D, age at death; NP, not performed.

Patient	Age (y)	Clinical syndrome	MRI data	C 5282 G>A mutation	Skin biopses
III:4	C:40	Mood disturbance, cognitive disorder, stroke, ICH	Severe leukoencephalopathy subcortical infarction, CMBs	+	GOM
II:4	C:72	Dizziness, stroke	Leukoencephalopathy, subcortical infarction	+	NP
II:2	C:74	Cognitive disorder	NP	ND	NP
III:1	D:21	Mood disturbance	Leukoencephalopathy, subcortical infarction	ND	NP
II:3	C:65	Stroke	NP	ND	NP
III:2	C:43	TIA	NP	+	NP
III:3	C:41	Asympomatic	NP	-	NP
IV:1	C:15	Asympomatic	NP	-	NP
IV:2	C:8	Asympomatic	NP	-	NP

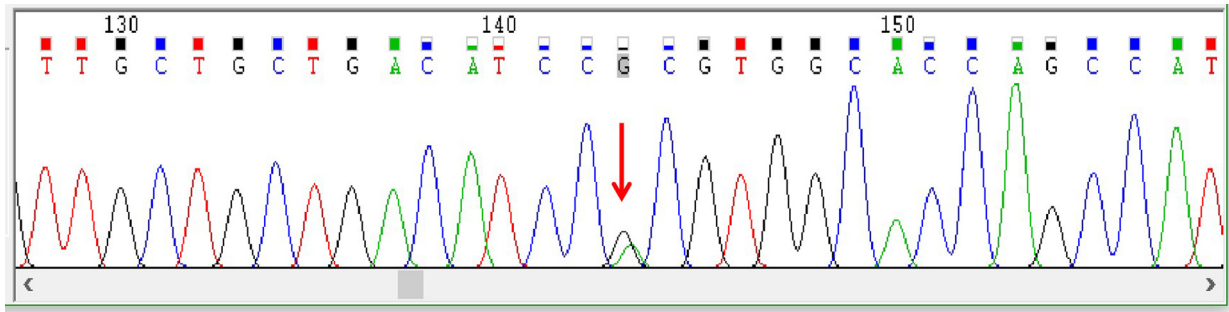


Fig. 4. The gene map above shows heterozygous 5282G>A mutation of NOTCH3 analysis in a CADASIL Chinese patient.

studies of skin biopsies obtained from the proband, the presence of GOM supports the CADASIL diagnosis. In conclusion, we considered p.R1761H was potentially pathogenic mutation.

Herein, We reported a cysteine-sparing mutation located in the RAM region adjacent to the TM domain. Cysteine-sparing mutations in EGFr domain of NOTCH3 gene were reported.^{11,12} Wollenweber et al. described enhanced aggregation behavior of the D80G mutation was resemble that cysteine-affecting mutations in vitro, drawing a view cysteine-sparing mutations might cause CADASIL.¹¹ Mizuno et al. indicated p.R75P mutation maybe is pathogenic by conformational change in the first EGFr domain of NOTCH3 protein.¹²

In recent years, NOTCH3 mutations outside of the EGFr encoding exons were reported. Fouillad et al. reported a p.L1515P substitution in a patient with medical records were suggestive for an inherited small vessel-disease, but lacking GOM deposits. The majority of CADASIL mutations appear to retain normal level of signaling activity, while very few mutations show reduced activity. However, The p.L1515P mutant exhibits increased canonical NOTCH3 signaling. It was suggested that the p.L1515P mutation have a neomorphic effect of toxicity.

NOTCH3 activating mutations should be further considered for molecular analysis of patients with cerebral small-vessel-disease.¹³ Bersano et al. described a 49-year-old woman suffered from migraine, progressive memory impairment, affective disturbance, loss of consciousness, but the history of stroke is absence. Brain MRI revealed severe diffuse leukoencephalopathy. The family was identified the amino acid substitution p.V1762M in exon 29 of the NOTCH3 gene. However, the presence of GOM was not found in all mutation carriers. The author considers the p.V1762M variant of NOTCH3 gene is a rare nucleotide polymorphism.¹⁴ The RAM region is indispensable for transcriptional activation.^{2,3} The p. R1761H substitution in the RAM domain may affect the transcription of the NOTCH3 signal. The mutations near the TM area disrupt the structural integrity of NOTCH receptors. In the Nocth1 gene, the mutations near the TM region weaken the interaction between the extracellular domain and the intracellular domain, causing abnormal activation of signal transduction.^{15,16} Although there is no similar study in the NOTCH3 gene, considering the similarity in the structure of the Notch gene, the novel mutation near the TM region in NOTCH3 gene may cause disease in the same way, which requires further study.

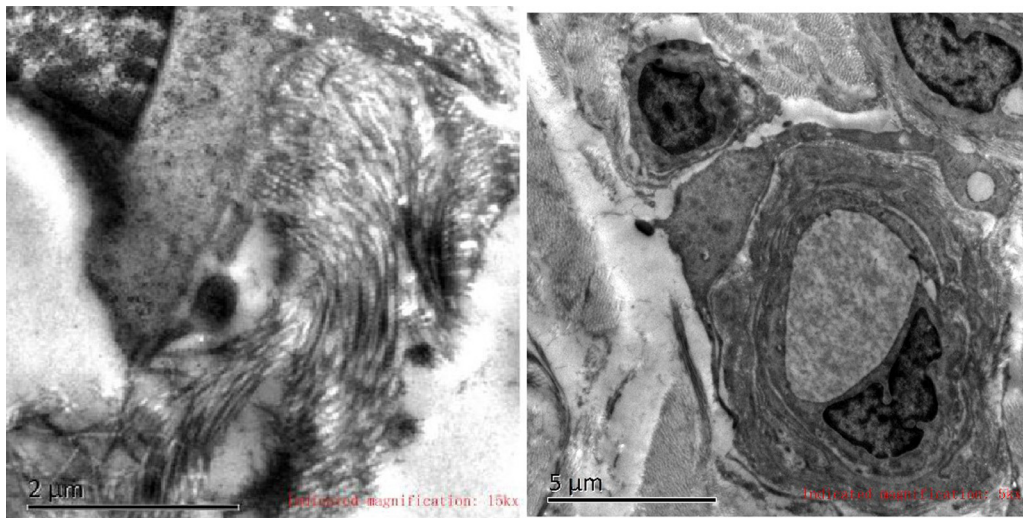


Fig. 5. Electron microscopy of skin biopsies from the proband with deposits of GOM are shown, which was the characteristic of CADASIL.

Conclusion

Our study broadens the mutational spectrum of NOTCH3 gene. We advocate comprehensive NOTCH3 screening for suspected cases though the way is time consuming and expensive. If NOTCH3 screening reveal a unclassified missense mutation in a patient with a convincing CADASIL phenotype, then taking a skin biopsy is recommended.

Declaration of Competing Interest

None.

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References

1. Larsson C, Lardelli M, White I, Lendahl U. The human NOTCH1, 2, and 3 genes are located at chromosome positions 9Q34, 1P13-P11, and 19P13.2-P13.1 in regions of neoplasia-associated translocation. *Genomics* 1994;2:253-258.
2. Lubman OY, Ilagan MX, Kopan R, Barrick D. Quantitative dissection of the Notch: CSL interaction: insights into the notch-mediated transcriptional switch. *J Mol Biol* 2007;3:577-589.
3. Friedmann DR, Wilson JJ, Kovall RA. RAM-induced allostery facilitates assembly of a Notch pathway active transcription complex. *J Biol Chem* 2008;21:14781-14791.
4. Markus HS, Martin RJ, Simpson MA, et al. Diagnostic strategies in CADASIL. *Neurology* 2002;8:1134-1138.
5. Dichgans M, Ludwig H, Muller-Hocker J, Messerschmidt A, Gasser T. Small in-frame deletions and missense mutations in CADASIL: 3D models predict misfolding of Notch3 EGF-like repeat domains. *Eur J Hum Genet* 2000;4:280-285.
6. Duering M, Karpinska A, Rosner S, et al. Co-aggregate formation of CADASIL-mutant NOTCH3: a single-particle analysis. *Hum Mol Genet* 2011;16:3256-3265.
7. Joutel A, Andreux F, Gaulis S, et al. The ectodomain of the Notch3 receptor accumulates within the cerebrovasculature of CADASIL patients. *J Clin Invest* 2000;5:597-605.
8. Ishiko A, Shimizu A, Nagata E, Takahashi K, Tabira T, Suzuki N. Notch3 ectodomain is a major component of granular osmiophilic material (GOM) in CADASIL. *Acta Neuropathol* 2006;3:333-339.
9. Liao YC, Hsiao CT, Fuh JL, et al. Characterization of CADASIL among the Han Chinese in Taiwan: distinct genotypic and phenotypic profiles. *Plos One* 2015;8:e136501.
10. Muino E, Gallego-Fabrega C, Cullell N, et al. Systematic review of cysteine-sparing NOTCH3 missense mutations in patients with clinical suspicion of CADASIL. *Int J Mol Sci* 2017:9.
11. Wollenweber FA, Hanecker P, Bayer-Karpinska A, et al. Cysteine-sparing CADASIL mutations in NOTCH3 show proaggregatory properties in vitro. *Stroke* 2015;3:786-792.
12. Mizuno T, Muranishi M, Torugun T, et al. Two Japanese CADASIL families exhibiting Notch3 mutation R75P not involving cysteine residue. *Intern Med* 2008;23:2067-2072.
13. Fouillade C, Chabriat H, Riant F, et al. Activating NOTCH3 mutation in a patient with small-vessel-disease of the brain. *Hum Mutat* 2008;3:452.
14. Bersano A, Ranieri M, Ciammola A, et al. Considerations on a mutation in the NOTCH3 gene sparing a cysteine residue: a rare polymorphism rather than a CADASIL variant. *Funct Neurol* 2012;4:247-252.
15. Sanchez-Irizarry C, Carpenter AC, Weng AP, Pear WS, Aster JC, Blacklow SC. Notch subunit heterodimerization and prevention of ligand-independent proteolytic activation depend, respectively, on a novel domain and the LNR repeats. *Mol Cell Biol* 2004;21:9265-9273.
16. Malecki MJ, Sanchez-Irizarry C, Mitchell JL, et al. Leukemia-associated mutations within the NOTCH1 heterodimerization domain fall into at least two distinct mechanistic classes. *Mol Cell Biol* 2006;12:4642-4651.