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Original Article

A homozygous *NOTCH3* mutation p.R544C and a heterozygous *TREX1* variant p.C99MfsX3 in a family with hereditary small vessel disease of the brain

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Abstract

Background: Mutations in the *TREX1* and *NOTCH3* genes cause retinal vasculopathy with cerebral leukodystrophy (RVCL) and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), respectively. Both are hereditary small vessel diseases of the brain (HSVDB).
Methods: We performed mutational analyses of *TREX1* in genomic DNA from 39 unrelated patients who were *NOTCH3*-negative in genetic testing, selected out of 72 unrelated consecutive patients with HSVDB.
Results: Only one patient had a *TREX1* sequence variation, a heterozygous *TREX1* c.294dupA, putatively resulting in a truncated protein, p.C99MfsX3. The medical history of the patient's family was scrutinized, which revealed that heterozygous *TREX1* p.C99MfsX3 was not segregating with the HSVDB. Re-examination of the *NOTCH3* sequence data of the proband led to the identification of a homozygous *NOTCH3* c.1630C>T (p.R544C) mutation, which segregated with the HSVDB in the family. The proband had a slightly more severe phenotype in comparison with her heterozygous p.R544C sister.
Conclusion: *TREX1* mutation is not a common cause of HSVDB. *TREX1* p.C99MfsX3 is not a dominant mutation. Homozygosity of the *NOTCH3* p.R544C has a modestly deleterious effect on the CADASIL phenotype. The *NOTCH3* mutation may cause CADASIL through a gain-of-toxic function effect, which can be modified by other genetic or environmental factors and results in the phenotypic variation of CADASIL.

Keywords: CADASIL; homozygous *NOTCH3* mutation; *NOTCH3*; RCVL; *TREX1*

1. Introduction

Hereditary small vessel diseases of the brain (HSVDB) are a clinically and genetically heterogeneous group of diseases sharing common characteristics of multiple lacunar infarctions and diffuse leukoencephalopathy. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and retinal vasculopathy with cerebral leukodystrophy (RVCL) are two different HSVDB with clearly identified genetic causes.[1,2] CADASIL is the most common monogenic HSVDB, caused by *NOTCH3* mutations. It is an adult-onset, dominantly inherited disease with recurrent ischemic strokes, dementia, and less frequently, migraine

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or psychiatric symptoms.[3] *NOTCH3* encodes a cell-surface receptor, NOTCH3, which is expressed on vascular smooth muscle cells and plays an important role in arterial development.[4] It is a single-pass transmembrane protein with a large extracellular domain containing 34 tandem epidermal growth factor-like (EGF-like) repeats. There are six cysteine residues within each EGF-like repeat and almost all mutations identified in CADASIL to date result in either a gain or loss of one cysteine residue within a given EGF-like repeat domain of the NOTCH3 protein.[5] As the *NOTCH3* mutations leave one cysteine residue unpaired, which provides one free sulfhydryl group, the mutant NOTCH3 molecules may interact with adjacent mutant NOTCH3 or other proteins by aberrant disulfide bond formation. The structurally altered proteins may accumulate nearby and become toxic to the vascular smooth muscle cells.[4]

RVCL is a rare autosomal dominant disease. It features a combination of various symptoms such as progressive visual loss, ischemic stroke, dementia, migraine, and Raynaud's phenomenon attributable to retinal, cerebral, and systemic microangiopathy.[2] RVCL encompasses three previously described neurovascular syndromes, including cerebroretinal vasculopathy (CRV),[6] hereditary endotheliopathy, retinopathy, and nephropathy (HERNS)[7,8] and hereditary vascular retinopathy (HVR),[9] and has been found to be caused by C-terminal truncations in human 3′-5′ DNA exonuclease (*TREX1*).[2] *TREX1* serves a DNA proofreading function and is also involved in the regulation of immunity and granzyme A-mediated apoptosis.[10,11] In addition to RVCL, different *TREX1* mutations have been associated with Aicardi-Goutières syndrome (AGS),[12] familial chilblain lupus (FCL),[13] and systemic lupus erythematosus (SLE).[2] AGS is an autosomal recessive infantile encephalopathy with brain atrophy, leukodystrophy, basal ganglia calcification, chronic cerebrospinal fluid (CSF) lymphocytosis, and elevated α-interferon levels in CSF. FCL is a rare cutaneous form of SLE, presenting with painful bluish-red inflammatory acral skin lesions in early childhood. FCL and most SLE are autosomal dominant diseases.

Vascular retinopathy is the most prominent symptom of RVCL, and most patients with RVCL reported to date have been diagnosed with retinopathy prior to genetic testing.[6–9] The genetic etiologies of many patients with isolated HSVDB remain elusive. It is still unknown whether *TREX1* mutations may still be associated with HSVDB in the absence of retinopathy. Therefore, to further understand the genetic basis of HSVDB, we were determined to investigate *TREX1* mutations in HSVDB patients without retinopathy.

We performed mutational analyses of *TREX1* in genomic DNA from 39 unrelated patients without gross *NOTCH3* mutations in genetic testing; they were selected out of 72 unrelated consecutive patients with HSVDB. Only one patient had a *TREX1* sequence variation, a heterozygous *TREX1* c.294dupA, putatively resulting in a truncated protein, p.C99MfsX3. Study of the patient's family revealed that heterozygous *TREX1* p.C99MfsX3 was not segregating with the HSVDB. Re-scrutiny of the *NOTCH3* sequence data of the proband led to the identification of a homozygous *NOTCH3* c.1630C>T (p.R544C) mutation, which segregated well with the HSVDB in the family. Herein, we describe the clinical, genetic, and magnetic resonance imaging (MRI) features of this HSVDB family harboring the homozygous *NOTCH3* p.R544C mutation and the heterozygous *TREX1* p.C99MfsX variant. This study also expands the general base of knowledge about the role of *TREX1* mutations in HSVDB.

2. Methods

The protocols for this study were approved by the institutional review board of Taipei Veterans General Hospital. Written informed consent was obtained from all participants.

2.1. Patients

The study population consisted of 39 patients out of 72 unrelated consecutive patients with HSVDB who were recruited from the Neurology Service, Taipei Veterans General Hospital. These patients had previously tested negative for *NOTCH3* mutations. The mean age (±SD) of the study population was 53.8 ± 13.4 years (range 31−78 years), and 28 of them (71.8%) were male. Twenty patients had hypertension (51.3%), and 11 had diabetes mellitus (DM; 28.2%). The initial clinical manifestations of our patients included ischemic stroke in 24 patients (61.5%), dementia in seven (17.9%), hemorrhagic stroke in four (10.3%), progressive gait disturbance in three (7.7%), and bipolar disease in one (2.6%). No patient had any visual complaints. All patients were of Han Chinese descent and had at least one other affected family member with a history of ischemic stroke or vascular dementia. The presence of cerebral small vessel disease was defined as leukoaraiosis in addition to multiple lacunar infarcts on the brain MRI.

2.2. Mutational analysis

Genomic DNA was extracted from peripheral blood using standard protocols. Mutation analyses of *TREX1* and exons 2 to 24 of *NOTCH3* were performed by polymerase chain reaction (PCR) amplification using intronic primers and direct nucleotide sequencing. Both sense- and antisense-strands of all amplicons were sequenced using the Big Dye 3.1 dideoxy terminator methods (Applied Biosystems, Foster City, CA, USA) and ABI Prism 3700 Genetic Analyzer (Applied Biosystems). The amplicon sequences were compared with the published human gene sequences (*TREX1*, RefSeq NM_033629.2; *NOTCH3*, RefSeq NM_000435.2) in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). After the identification of the sequence variations, subcloning and subsequent sequence analyses of the amplicons were further performed to confirm the sequence changes.

3. Results

3.1. Mutational analysis

Out of the 39 patients, only one (III:4) (Fig. 1) was found to carry a heterozygous *TREX1* c.294dupA mutation (Fig. 2A), which was confirmed by the subcloning of the PCR products and repeated sequencings and was not found in 300 healthy controls. This single nucleotide insertion caused a frameshift that resulted in expression of a putatively truncated TREX1 protein, p.C99MfsX3. *TREX1* c.294dupA was not a typical causative mutation for RVCL, which was most frequently reported as a C-terminal truncation of TREX1. To explore the pathogenic role of *TREX1* c.294dupA in this family with HSVDB, we sequenced two other affected and two healthy members of the family and realized that *TREX1* c.294dupA did not segregate with the HSVDB in this family (Fig. 1).

To look into the genetic etiology of disease in this family, we re-examined the original sequence reads of *NOTCH3* of III:4 and unexpectedly, identified a homozygous *NOTCH3* c.1630C>T (p.R544C) mutation (Fig. 2B), which was overlooked in the first sequence reading. The homozygous *NOTCH3* c.1630C>T mutation was confirmed by repeated PCR amplification and sequencing using two different sets of primers to avoid false homozygosity from asymmetric PCR amplification. The other aforementioned family members were also re-sequenced. *NOTCH3* p.R544C segregated well with the HSVDB in the family, suggesting that *NOTCH3* p.R544C, rather than *TREX1* p.C99MfsX3, caused HSVDB in this particular pedigree. Re-examination of the original sequence of *NOTCH3* of the other 38 patients confirmed the absence of mutation in either *NOTCH3* or *TREX1*.

3.2. Clinical information of the family

The proband III:4, at the age of 63 years, presented with a 3-month history of left hemiparesis and memory impairment. She denied any previous history of stroke, migraine, or psychiatric symptoms, but her father and three other siblings all previously had strokes. She did not have other vascular risk factors, such as hypertension, DM, cigarette smoking, or hyperlipidemia. Physical examinations revealed a mild weakness in the left lower limb (4+/5 according to the Medical Research Council scale) and slightly more brisk deep tendon reflexes in the left knee and ankle. Her score on the Mini-Mental State Examination (MMSE) was 24 out of 30. Brain MRI revealed diffuse white matter abnormalities with anterior temporal lobe involvement and a few lacunar infarcts in the corona radiata and the right putamen (Fig. 3A). Mutational analyses identified a heterozygous *TREX1* p.C99MfsX3 (c.294dupA) mutation (Fig. 2A) and a homozygous *NOTCH3* p.R544C (c.1630C>T) mutation (Fig. 2B). She did not have any clinical manifestation suggesting SLE or Sjögren's syndrome (SS), and her serum antinuclear antibody testing was negative. Additionally, fluorescein angiography revealed no retinal vasculopathy.

The proband's eldest sister (III:1) had a nasopharyngeal carcinoma with radiotherapy at the age of 65 years, a temporal lobe epilepsy originating from the left medial temporal region at the age of 67 years, and a mild stroke resulting in dysarthria at the age of 70 years. She did not have other major vascular risk factors. Brain MRI at the age of 67 years revealed mild but diffuse white matter abnormalities with anterior temporal lobe involvement and a lacunar infarct in the left corona radiata (Fig. 3B). Her score on the MMSE at the age of

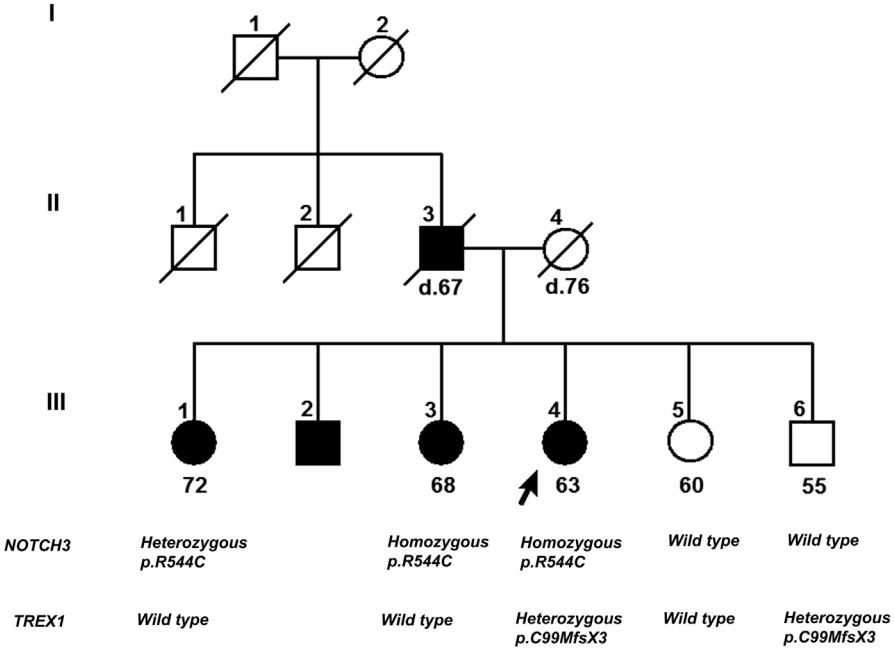

Fig. 1. The pedigree with hereditary small vessel disease of the brain (HSVDB) simultaneously harboring *TREX1* and *NOTCH3* mutations. The HSVDB segregates with *NOTCH3* p.R544C, rather than *TREX1* p.C99MfsX3. Affected individuals are shown as filled symbols and the arrow points to the proband. A slash indicates deceased individuals. Below the symbol is the age at examination or death.

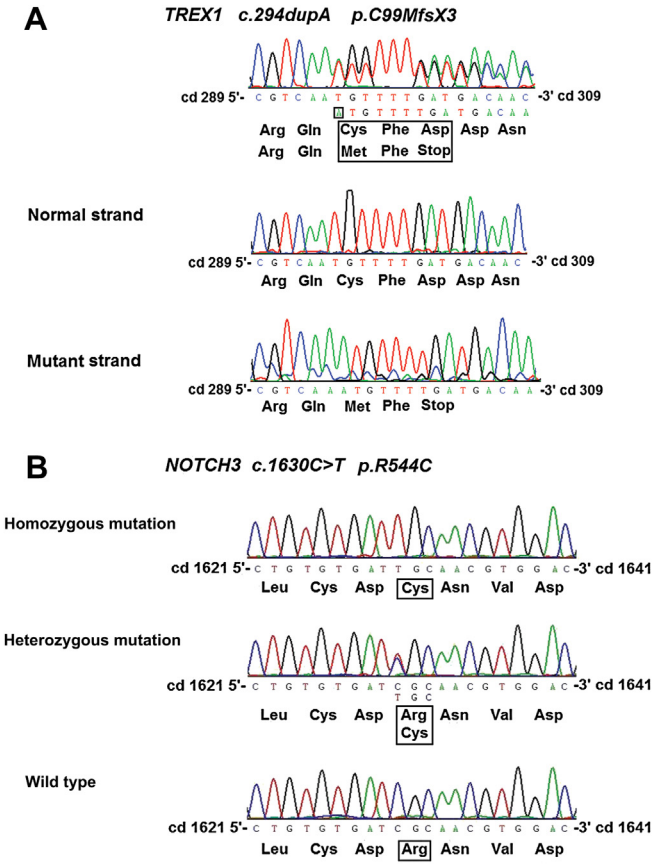

Fig. 2. (A) The electropherograms demonstrating a heterozygous c.294dupA mutation in the *TREX1* gene and a limited reading frame depicting the corresponding amino acid substitutions (p.C99MfsX3). (B) The electropherograms demonstrating a homozygous and a heterozygous c.1630C>T mutation in the *NOTCH3* gene, with a limited reading frame depicting the corresponding amino acid substitutions (p.R544C).

72 years was 26. Mutational analysis revealed a heterozygous *NOTCH3* p.R544C (c.1630C>T) mutation and wild-type *TREX1* genes.

The proband's elder sister (III:3) with DM and hypercholesterolemia had her first ischemic stroke at 58 years of age with residual gait difficulty, which was followed by repeated ischemic events resulting in dysarthria and dysphagia at the age of 62 years, and severe dementia at the age of 64 years. A brain CT at age 66 years featured a marked diffuse white matter abnormality with multiple lacunar infarcts in the corona radiata and basal ganglia. Mutational analysis revealed a *NOTCH3* homozygous p.R544C (c.1630C>T) mutation and wild-type *TREX1* genes.

The proband's younger sister (III:5), at the age of 60 years, and brother (III:6), at the age of 55 years, both had normal brain MRI and appeared normal on physical examinations. Mutational analysis revealed that III:5 had wild-type *NOTCH3* and *TREX1* genes, while III:6 had wild-type *NOTCH3* genes but a heterozygous *TREX1* mutation, p.C99MfsX3. III:6 had no clinical features suggesting either SLE or SS. The proband's elder brother (III:2) allegedly had repeated ischemic strokes. The clinical information about the proband's father (II:3) and mother (II:4) was not available.

4. Discussion

This is the first *TREX1* study in a cohort of HSVDB that did not involve significant patient visual complaints. We demonstrated that *TREX1* mutation was not present in a consecutive series of 72 patients with HSVDB and, unexpectedly, identified a patient harboring both a heterozygous *TREX1* p.C99MfsX3 and a homozygous *NOTCH3* p.R544C. Given the clinical manifestations, neuroimage features, and genotype information of the family members, *NOTCH3* p.R544C, rather than *TREX1* p.C99MfsX3, was likely to be the cause of HSVDB in this family. These findings may have five implications.

First, *TREX1* mutation is not a common cause of HSVDB. Although C-terminal truncating mutation in *TREX1* has been identified as the leading cause of RVCL,[2] only a limited number of RVCL families have been reported, indicating that RVCL is not a common disease. Therefore, in clinical practice, testing for *TREX1* mutations is only warranted in patients with both HSVDB and documented retinopathy or a family history of *TREX1* mutation. It is unclear if our findings are also applicable to families with other ethnic backgrounds. Investigating *TREX1* mutation in HSVDB patients from different ethnic populations may provide more information about the role of *TREX1* in HSVDB.

Second, our data suggests that *TREX1* p.C99MfsX3 is likely nonpathogenic or a recessive mutation based on several observations: (1) the younger brother (III:6) who carried a heterozygous *TREX1* p.C99MfsX3 has thus far remained clinically disease-free (confirmed through neuroimaging studies), and the proband (III:4) and her younger brother (III:6) had neither AGS, FCL, SLE, nor SS; (2) the proband (III:4) harboring this mutation and a homozygous *NOTCH3* mutation had a later disease onset and less disease severity than her elder sister (III:3) who only had the homozygous *NOTCH3* mutation. The TREX1 protein is composed of three conserved motifs, including an active catalytic domain and a highly hydrophobic transmembrane C-terminal domain.[2,14] All of the patients with RVCL reported so far have C-terminal frameshift mutations in the *TREX1* gene, leading to the production of proteins with truncated transmembrane domain and altered intracellular localization but preserved enzymatic function.[2] The p.C99MfsX3 TREX1 mutant protein is expected to lose two-thirds of its amino acid residues, including exonuclease regions, and is likely a recessive nonfunctional mutation. The single-copy wild-type allele may produce sufficient TREX1 protein to maintain the physiological function. This may well explain why heterozygous carriers of *TREX1* p.C99MfsX3 remain clinically "invisible".

Third, homozygosity of the *NOTCH3* p.R544C mutation only mildly aggravated the CADASIL phenotype. *NOTCH3* R544C mutation is very common and accounts for approximately one half of CADASIL among the Chinese population in Taiwan.[15] This pedigree, with two patients (III3 and III4) harboring homozygous p.R544C, offers a rare and valuable opportunity to assess the effects of homozygous *NOTCH3* mutation on the phenotype of CADASIL. Compared with the

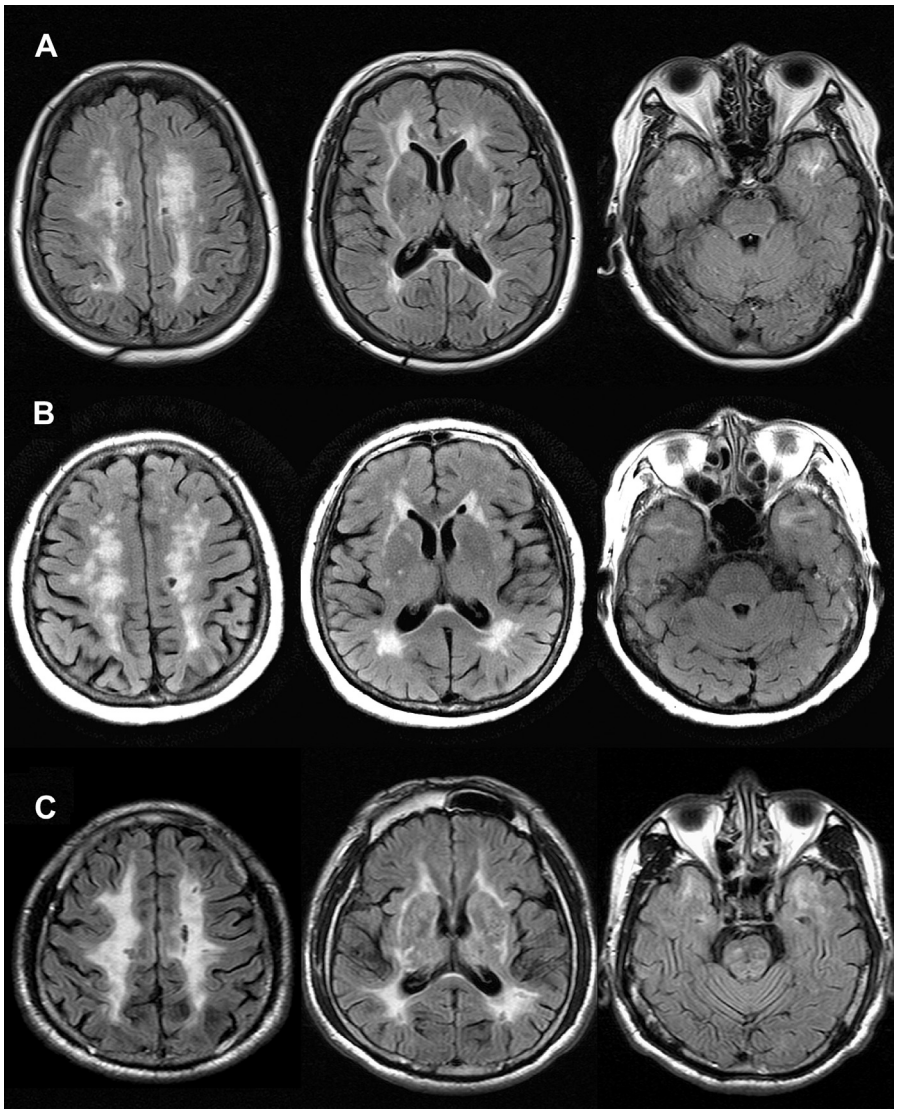

Fig. 3. Fluid attenuated inversion recovery (FLAIR) images depicting multiple lacunar infarcts and diffuse leukoencephalopathy with involvement of corona radiata, external capsules, and anterior temporal poles in III-4 with a homozygous *NOTCH3* p.R544C mutation (A), her sister (III-1) with a heterozygous *NOTCH3* mutation (B), and another unrelated 61-year-old female with a heterozygous *NOTCH3* p.R544C mutation (C).

heterozygous sister (III:1), the homozygous proband III:4 had a slightly earlier age of onset and mildly increased clinical severity, suggesting that under a similar genetic background, homozygosity of the *NOTCH3* mutation might have a dosage-dependent, albeit small, effect on the phenotype of CADASIL.

Fourth, the phenotype of CADASIL may be modified by other genetic or environment factors in addition to *NOTCH3* mutation. The mean age of onset of CADASIL in our population was 47.6 years.[15] However, the proband and her sister (III-3) harboring homozygous R544C mutation had ages of onset (63 and 58 years) later than the average, suggesting the existence of other modifying genetic and/or environmental factors that alleviate the phenotype of CADASIL. Compared with the proband, her homozygous sister (III-3) with DM and hyperlipidemia had a more severe phenotype, raising the possibility that other vascular risk factors may worsen the severity of CADASIL.

Lastly, the *NOTCH3* mutations causing CADASIL may work through a gain-of-toxic function mechanism. *NOTCH3* is predominantly expressed in small arterial smooth-muscle cells and is vital for their differentiation and maturation.[5] Thus, if CADASIL-associated *NOTCH3* mutations worked through a haploinsufficiency or dominant-negative effect, homozygous mutations would have been devastating and lead to a more severe or lethal phenotype. In the literature, two patients with disparate homozygous *NOTCH3* mutations have been separately reported before. One patient with a homozygous p.R133C had a severe phenotype of CADASIL,[16] and the other one with a homozygous p.R578C had a very mild phenotype, which was not significantly different from that of the heterozygous sibling.[17] Two sisters with homozygous p.R544C in our study had a typical CADASIL with later age of onset. The highly variable clinical features of the patients with homozygous *NOTCH3* mutations suggest that the phenotypic presentations of

CADASIL are not solely dictated by the *NOTCH3* mutations, but may also be modified by other factors.

In the electropherogram (Fig. 2B), heterozygous mutations, which would appear as double peaks in two different colors at the same nucleotide location, are very conspicuous and therefore hard to miss. However, homozygous mutations, which would appear as a single overlapping peak in one single color, are easily overlooked by visual inspection. As CADASIL is an autosomal dominant disease, homozygous *NOTCH3* mutations in CADASIL rarely occur. Therefore, in our earlier experience, we focused on heterozygous variants on the electropherogram of *NOTCH3* sequencing and, therefore, missed one single *NOTCH3* R544C homozygous mutation. After realizing this oversight, we now routinely double-check all our electropherograms involved with *NOTCH3* sequencing in all patients to make sure this will never happen again.

In conclusion, this study demonstrates that *TREX1* mutation is not a common cause of HSVDB. *TREX1* p.C99MfsX3 is not a dominant mutation. A single copy of wild-type *TREX1* may be functionally sufficient. Homozygous *NOTCH3* p.R544C mutation had slightly enhanced deleterious effect on CADASIL phenotype. A gain-of-toxic function effect of the *NOTCH3* mutation may be responsible for the pathogenesis of CADASIL, which could be modified by other genetic or environmental factors and results in the phenotypic variation of CADASIL.

Acknowledgments

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