Novel mutations in HTRA1-related cerebral small vessel disease and comparison with CADASIL

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Received: 7 May 2022; Revised: 29 July 2022; Accepted: 10 August 2022

Annals of Clinical and Translational Neurology 2022; 9(10): 1586–1595
doi: 10.1002/acn3.51654

Abstract

Objective: There is evidence showing both heterozygous HTRA1 and homozygous HTRA1 mutations as causal for familial cerebral small vessel disease (CSVD). The clinical and neuroimaging signs of heterozygous HTRA1-related CSVD can mimic cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). We aimed to characterize the genotypic and phenotypic features of HTRA1-related CSVD, and we compared the features of heterozygous HTRA1-related CSVD and CADASIL.

Methods: We carried out genetic sequencing in a series of unrelated patients with suspected familial CSVD from China. Clinical and imaging characteristics of heterozygous HTRA1-related CSVD and CADASIL were compared.

Results: We identified nine heterozygous HTRA1 mutations and one homozygous HTRA1 mutation, seven of which are novel. Compared with CADASIL, patients with heterozygous HTRA1-related CSVD had a higher proportion of spine disorders and a lower proportion of white matter hyperintensities involving the anterior temporal lobe (p < 0.001).

Interpretation: This study shows that most HTRA1-related CSVD patients in China carry heterozygous HTRA1 mutations. The specific extra-neurological features and neuroimaging features reveal informative differences between heterozygous HTRA1-related CSVD and CADASIL. We expand the mutational spectrum of HTRA1.

Introduction

Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) was previously considered a unique nonhypertensive recessive form of cerebral small vessel disorder hallmarked by stroke, cognitive defects, lumbago, and alopecia.1 Neuroimaging features are similar to cerebral small vessel diseases (CSVD), such as the presence of cerebral white matter hyperintensities (WMH), lacunes, visible perivascular spaces (PVS), cerebral microbleeds (CMBs), and cerebral atrophy.2,3 Characteristic pathological changes in the vasculature include thickening of small arteries, loss of smooth muscle cells (SMCs), and splitting of the elastic lamina.1–7

There is growing data showing heterozygous HTRA1 mutation as a cause of familial CSVD with a dominant inheritance pattern.8–10 The clinical characteristics are different from classic CARASIL, including, for example, later age of onset and the absence of typical extra-neurological features of CARASIL, such as alopecia.10 The dominant inheritance pattern, as well as the clinical and neuroimaging characteristics, resemble autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL). There have been only three high-quality cohorts from Europe (10 probands),10 Japan (6 index cases and 2 affected siblings),9 and Taiwan (7 index cases and 2 affected siblings)8 have characterized the disease features and the influence of the mutations on HTRA1 protease activities. The inheritance pattern, clinical, and
genetic features of HTRA1-related CSVD in the Chinese population remain unclear.

In the present study, we aimed to determine the genotypic and phenotypic features of HTRA1-related CSVD in a series of suspected familial CSVD from China. We also compared heterozygous HTRA1-related CSVD and CADASIL to help better differentiate these two types of autosomal-dominant CSVD.

**Methods**

**Study population**

One hundred and eighty-one unrelated Chinese patients were recruited from the Neurology Department of Beijing Tiantan Hospital between 2014 and 2019. The inclusion criteria were as follows: (1) the presence of moderate to severe white matter hyperintensities; (2) the presence of more than one of the following features: stroke-like episodes, cognitive disorders, gait disturbance, psychiatric disorder; and (3) a family history of stroke, cognitive decline, or white matter hyperintensities. Leukodystrophy and acquired causes (infections, autoimmune syndromes, etc.) were excluded. This study was approved and performed following the guidelines of the Ethics Committee of Beijing Tiantan Hospital (KY 2018-002-02) and informed consent was obtained from all patients or their families.

**Clinical and Imaging data acquisition and analysis**

Clinical and imaging data from all index patients and their available affected family members were collected and analyzed. Brain neuroimages were obtained using a 3.0T magnetic resonance imaging (MRI) scanner (Magnetom Trio Tim; Siemens) including the following scanning sequences: whole-brain axial T2-weighted images, fluid-attenuated inversion recovery images, sagittal T1-weighted images, and susceptibility-weighted images. All CSVD-related MRI analyses (WMH, PVS, lacunes, CMBs, and atrophy) were conducted in line with the Standards for Reporting Vascular Changes on Neuroimaging recommendations. The total CSVD score was calculated as previously described (an ordinal score of total CSVD burden, range 0–4). The experienced investigators were blinded to all clinical and genetic information.

**Genetic sequencing, analysis, and interpretation**

We performed whole-exome Illumina sequencing or gene-panel sequencing (genes included in the panel were NOTCH3, HTRA1, COL4A1, TREX1, KRIT1, CCM2, PDCD10, GSN, APP, CST3, COL4A2, TGFBR1, TGFBR2, COL3A1, ABCG6, MTHFR, CBS, ITM2B, and PDE4D) on samples obtained from the patients. Genomic DNA was isolated from peripheral leukocytes using a DNA Isolation Kit from Bioteke (catalog no. AU1802). The DNA libraries were sequenced on the HiSeq X10 platform (Illumina, San Diego, USA). The called variants were annotated with public databases (1000 genomes project, Genome Aggregation Database [gnomAD], Human Gene Mutation Database [HGMD], and an in-house database). Sanger sequencing was conducted in all probands and the available family members, including the affected members and the spouse, to confirm the detected variants and find out whether the variant cosegregated with the affected status. The functional impact of the candidate variants was predicted by Mutation Taster, PolyPhen-2, and SIFT.

**HTRA1 protease activity**

Mutant constructs of human HTRA1 (p.A20V, p.V175M, p.F278L, p.P285L, p.Q318H, p.V339M, and p.G350E) were prepared from wild-type pcDNA3.1/myc-His. The primers and their complementary primers are shown in Table S1. Transfection of HEK293 cells with mutant and wild-type HTRA1 was done as described previously. Protease activity for wild-type and mutant HTRA1 were measured using the Pierce® Fluorescent Protease assay kit following previous reports.

**Statistical analysis**

SPSS (version 22.0) and GraphPad Prism 7.0 were used for analyses.

**Results**

**Mutations of the HTRA1 gene**

Among the 181 unrelated patients with suspected familial CSVD, a total of 10 HTRA1 mutations were identified in 11 index patients (6.08%), including nine heterozygous mutations (eight missense mutations and one splicing mutation) and one homozygous missense mutation (Tables 1 and 2). Seven were novel HTRA1 mutations, one of them was a homozygous missense mutation (c.59C>T), and the other six mutations were heterozygous (c.832T>G, c.834C>G, c.954G>C, c.973-2A>G, c.1015G>A, and c.1049G>A). Eight of the 10 detected HTRA1 variants were not present in public data resources including 1000 Genomes, HGMD, or an in-house database comprising 200 healthy control individuals in
China, with a low allele frequency in gnomAD \(^{18}\) (Table 2); they were predicted as pathogenic by Mutation Taster\(^{13}\), PolyPhen-2\(^{14}\), and/or SIFT\(^{15}\) (Table 2). The A protein structure of HTRA1 with the mutant positions is displayed in Figure 1A,B.

### HTRA1 protease activity

To investigate the functional influence of these novel HTRA1 missense mutations, the protease activity of wild-type and mutant HTRA1 proteins to hydrolyze fluorescein isothiocyanate-labeled casein was measured. All the HTRA1 mutants had dramatically decreased proteolytic activity than cells with a plasmid for wild-type HTRA1 (Fig. 1C; \(p < 0.001\)). To investigate whether these HTRA1 mutations had a dominant negative effect on the HTRA1 enzymatic function, we transfected human embryonic kidney cells 293 with 1/2 dosage of wild-type HTRA1, then we measured the HTRA1 protease activities purified from the cells. The catalytic capacity of the HTRA1 mixture of wild-type and each mutant HTRA1 was lower compared with wild-type HTRA1 (Fig. 1D; WT/V175M, WT/Q318H, WT/V339M, and WT/G350E, \(p < 0.001\); WT/A20V, WT/F278L, and WT/P285L, \(p < 0.01\)).

### Clinical and pathological characteristics

Twenty-two of the patients (11 index patients and 11 affected family members) carrying the HTRA1 mutations had a variety of clinical symptoms and MRI features (Table 2). The characteristics of patients with HTRA1 mutations in this study are shown in Table 1, and the detailed clinical and pathological characteristics are described below.

### Table 1. Characteristics of patients with HTRA1 mutations in this study.

| Family | Member | cDNA change | Protein change | Mutation type | Sex/age | Vascular risk factors | Main symptoms | MRI features |
|--------|--------|-------------|----------------|---------------|---------|-----------------------|---------------|--------------|
| 1      | 1      | c.59C>T    | p.A20V         | Homozygous    | M/53    | Smoking and HTN       | CH, recurrent IS, GD, and SD | WMH*, PVS, CMBs, lacunes, and atrophy |
| 2      | 5      | c.59C>T    | p.A20V         | Heterozygous  | M/60    | HTN                   | CH, CI, GD, and SD | WMH*, CMBs, and PVS |
| 3      | 6      | c.59C>T    | p.A20V         | Heterozygous  | F/31    | Abs                   | IS and SD     | WMH* |
| 4      | 7      | c.59C>T    | p.A20V         | Heterozygous  | F/57    | Abs                   | IS, CI, and SD | WMH and PVS |
| 5      | 8      | c.G523A    | p.V175M        | Heterozygous  | M/44    | HTN                   | IS, CI, GD, and SD | WMH*, PVS, CMBs, lacunes, and atrophy |
| 6      | 9      | c.832 T-C  | p.F278L        | Heterozygous  | M/42    | HTN                   | IS, CI, and SD | WMH, PVS, and lacunes |
| 7      | 10     | c.832 T-C  | p.F278L        | Heterozygous  | M/41    | HTN                   | IS, CI, and SD | WMH* and PVS |
| 8      | 11     | c.834 C-G  | p.F278L        | Heterozygous  | M/54    | Abs                   | IS, CI, GD, and SD | WMH*, PVS, CMBs, lacunes, and atrophy |
| 9      | 12     | c.854 C-T  | p.P285L        | Heterozygous  | M/42    | Abs                   | IS, CI, GD, and SD | WMH*, PVS, and lacunes |
| 10     | 13     | c.954 G-C  | p.Q318H        | Heterozygous  | M/46    | Abs                   | IS, CI, and SD | WMH* and atrophy |
| 11     | 14     | c.954 G-C  | p.Q318H        | Heterozygous  | M/46    | Abs                   | IS, CI, GD, and SD | WMH*, PVS, and lacunes |
| 12     | 15     | c.954 G-C  | p.Q318H        | Heterozygous  | M/39    | Abs                   | IS and SD     | WMH*, PVS, and lacunes |
| 13     | 16     | c.954 G-C  | p.Q318H        | Heterozygous  | M/39    | Abs                   | IS and SD     | WMH*, PVS, and lacunes |
| 14     | 17     | c.954 G-C  | p.Q318H        | Heterozygous  | M/36    | Abs                   | IS, CH, migraine, GD, and SD | WMH* and PVS |
| 15     | 18     | c.1015 G>A | p.V339M        | Heterozygous  | M/44    | HTN                   | IS, CH, CI, migraine, GD, and SD | WMH*, PVS, CMBs, lacunes, and atrophy |
| 16     | 19     | c.973-2A>G | p.F55          | Heterozygous  | M/55    | HTN                   | IS, CI, migraine, and GD | WMH*, PVS, CMBs, lacunes, and atrophy |
| 17     | 20     | c.973-2A>G | p.M69          | Heterozygous  | M/69    | DM                    | IS, CI, migraine, and GD | WMH*, PVS, lacunes, and atrophy |
| 18     | 21     | c.973-2A>G | p.F58          | Heterozygous  | M/58    | HTN                   | CI, migraine, and GD | WMH* and PVS |
| 19     | 22     | c.1049 G>A | p.G350E        | Heterozygous  | M/39    | Smoking               | IS, CI, alopecia, and SD | WMH*, PVS, and lacunes |

Abbreviations: Abs, absence; CH, cerebral hemorrhage; CI, cognitive impairment; CMBs, cerebral microbleeds; DM, Diabetes mellitus; GD, gait disturbance; HTN, hypertension; IS, ischemic stroke; MRI, magnetic resonance imaging; PVS, perivascular spaces; SD, spine disorders; WMH, white matter hyperintensities.

*White matter hyperintensities not involving the anterior temporal lobe.
Table 2. HTRA1 variants identified in this study.

| cDNA change | Protein change | Exon | Protein domain | Mutation type | Mutation Taster | PolyPen-2 | SIFT   | 1000 Genomes | HGMD | gnomAD |
|-------------|----------------|------|----------------|---------------|----------------|-----------|--------|--------------|-------|---------|
| c.59C>T     | p.A20V         | 1    | Signal peptide | Homozygous    | Polymorphism   | Benign    | Tolerated | 5.07188 x 10^-2 | Abs   | 1.29992 x 10^-2 |
| c.59C>T     | p.A20V         | 1    | Signal peptide | Heterozygous  | Polymorphism   | Benign    | Tolerated | 5.07188 x 10^-2 | Abs   | 1.29992 x 10^-2 |
| c.G523A     | p.V175M        | 2    | —              | Disease causing | Probably damaging | Deleterious | Abs     | Abs          | 4.06055 x 10^-6 | Abs   | 4.06861 x 10^-6 |
| c.832 T>C   | p.F278L        | 4    | Serine protease | Disease causing | Probably damaging | Deleterious | Abs     | Abs          | 4.06445 x 10^-6 | Abs   | 4.07153 x 10^-6 |
| c.834C>G    | p.F278L        | 4    | Serine protease | Disease causing | Probably damaging | Deleterious | Abs     | Abs          | 4.06445 x 10^-6 | Abs   | 4.06861 x 10^-6 |
| c.854C>T    | p.P285L        | 4    | Serine protease | Disease causing | Probably damaging | Deleterious | Abs     | Abs          | 4.06445 x 10^-6 | Abs   | 4.06861 x 10^-6 |
| c.954G>C    | p.Q318H        | 4    | Serine protease | Disease causing | Probably damaging | Deleterious | Abs     | Abs          | 4.06445 x 10^-6 | Abs   | 4.06861 x 10^-6 |
| c.973-2A>G  | splicing       | 5    | Serine protease | Disease causing | NA             | NA        | Abs     | Abs          | 4.06062 x 10^-6 | Abs   | 4.06861 x 10^-6 |
| c.1015G>A   | p.V339M        | 6    | Serine protease | Disease causing | Probably damaging | Deleterious | Abs     | Abs          | Abs   | Abs     |
| c.1049G>A   | p.G350E        | 6    | Serine protease | Disease causing | Probably damaging | Deleterious | Abs     | Abs          | Abs   | Abs     |

Abbreviations: Abs, absence; NA, not available.
with complete demographic, clinical, and MRI information were included; these data are presented in Table 1. The mean age (±SD) was 48.9 ± 11.3 years; 11 (50%) patients were male. Hypertension was the most frequent risk factor (50%). Ischemic stroke and cognitive impairment were common neurological symptoms. Eighteen (95.5%) patients had spine involvement, and 1 (4.5%) had alopecia. Histopathologic analysis of the skin from the proband of family 7 showed an abnormal thickness of artery walls and aberrant loss of smooth muscle cells (Fig. 2).

**Imaging characteristics**

MRI data are presented in Table 1. Neuroimaging evaluation revealed that all the patients had WMH. Only two
9.1% of patients had WMH involving the anterior temporal lobe, and 19/22 (86.4%) patients showed PVS, followed by lacunes 14/22 (63.6%), CMBs 7/19 (36.8%), and brain atrophy 8/22 (36.4%). It was noteworthy that patient III-4 with heterozygous \textit{HTRA1} mutation (c.954G > C) from family 8 had an arachnoid cyst in the right temporal and frontal lobe, which is a phenotype that has not been previously reported for heterozygous \textit{HTRA1}-related CSVD (Fig. 3). Lumbar and cervical MRI revealed variable levels of retrogression of vertebrae and intervertebral discs, prolapse of intervertebral discs, and canal stenosis (Fig. 3).

**Figure 2.** Histopathologic findings in this study. (A–D) Skin artery biopsy of the proband from family 7 with heterozygous P285L mutation. (A) Hematoxylin–eosin staining shows obviously fibrotic and thickened arteries. (B) CD34 immunohistochemical staining shows thickening of the vascular walls. (C) \textgreek{a}-actin staining shows the wall of arterial wall irregularly decreased and the degeneration of smooth muscle cells. (D) Electron micrographs shows marked luminal narrowing of an artery.

Genotypic and phenotypic features

We found that individuals carrying both homozygous and heterozygous \textit{HTRA1} mutations could manifest CSVD-related symptoms, while the healthy family members had no \textit{HTRA1} mutations. Although patients from three different families (Family 1 to Family 3) carried the same heterozygous \textit{HTRA1} mutation (c.59C>T, p.A20V), the clinical manifestations were different, some presented with cerebral hemorrhage, whereas others manifested as ischemic stroke or cognitive decline. A similar phenotypic heterogeneity phenomenon was observed among the hemorrhagic and ischemic individuals harboring the homozygous mutation (c.59C>T). Note that there was no obvious difference in the severity of phenotypes between the heterozygous carriers and homozygous carriers.

Comparison between heterozygous \textit{HTRA1}-related CSVD and CADASIL

Among the 181 patients of suspected familial CSVD, there were 34 patients with (1) complete clinical and MRI data who were also (2) diagnosed with CADASIL and (3) had...
Figure 3. MRI findings in patients with heterozygous HTRA1 mutation. MRI from family 8 with heterozygous HTRA1 mutation. Fluid-attenuated inversion recovery images of the brain show symmetrical hyperintensities in the periventricular area, whereas anterior temporal poles were not obviously involved (II-3, III-2, and III-3); lacunar infarcts in the deep brain white matter and basal ganglia (II-3, III-2, and III-3); T2-weighted images of the brain revealing visible perivascular spaces in the basal ganglia and deep brain white matter (III-1). Susceptibility-weighted images demonstrate microbleeds in the basal ganglia (III-3); the arachnoid cyst is noted in the right temporal and frontal lobe of III-4; T2-weighted cervical and lumbar MRI revealed multilevel degenerative disease, including disc herniation, canal stenosis, and degeneration of vertebral bodies (II-3, III-1, and III-2).
confirmed NOTCH3 pathogenic mutations (Table S2). These patients were examined with clinical history and neuroimaging to enable comparative analysis with heterozygous HTRA1-related CSVD individuals. Based on the demographic and clinical characteristics between heterozygous HTRA1-related CSVD and CADASIL, we detected no differences in age, gender, vascular risk factors, neurological symptoms, or alopecia. However, patients with heterozygous HTRA1-related CSVD had a higher proportion of spine disorders compared with CADASIL \((p = 0.001)\). For the CSVD neuroimaging features, patients with heterozygous HTRA1-related CSVD showed a lower proportion of WMH involving the anterior temporal lobe compared with CADASIL (both \(p < 0.001\)) (Table 3); there were no other CSVD neuroimaging feature differences between the two groups.

### Discussion

Our study shows that most HTRA1-related CSVD patients in China carry heterozygous HTRA1 mutations. We broaden the genotypic spectrum of HTRA1-related CSVDs. We found that ischemic stroke and cognitive impairment are frequent neurological presentations of HTRA1-related CSVD, which is similar to CADASIL. However, spinal disorders are the most common extra-neurological manifestation in HTRA1-related CSVD, with alopecia seen less frequently. Spine disorders, when combined with neuroimaging features, may indicate differential trends between the two similar familial CSVDs.

To date, only four cohorts have reported the heterozygous HTRA1-related CSVD. Verdura et al.\(^6\) first reported 11 probands harboring deleterious heterozygous HTRA1 mutations from French familial CSVD in 2015 and found all of them resulted in impaired protease activity. In 2016, Nozaki et al.\(^8\) identified four heterozygous HTRA1 mutations upon screening 113 unrelated Japanese CSVD cases; they investigated potential molecular mechanisms and found that heterozygous HTRA1 exerted a dominant negative effect. In 2017, Donato et al.\(^8\) identified five heterozygous HTRA1 mutations from five Italian families. In 2018, Lee et al.\(^8\) identified seven heterozygous HTRA1 mutations among 337 unrelated Taiwanese CSVD patients and found that these mutations led adding to impaired HTRA1 protease activity. These findings support that heterozygous HTRA1 mutations can cause familial CSVD. Our results further expand the HTRA1 genetic spectrum and validate a causative role for heterozygous HTRA1 mutation in familial CSVD.

Regarding the heterozygous HTRA1 clinical phenotypes in our study, the main clinical features include ischemic stroke, cognitive decline, and spine disorders, finding in line with the previous cohort study.\(^8\) It has been reported that the frequency of extra-neurological manifestations in dominant CSVD is lower in patients with heterozygous missense mutations than in CARASIL.\(^9\) In our study, patients with heterozygous HTRA1 mutations had a higher frequency of spine disorders and a low frequency of alopecia, which is congruent with previous studies.\(^8,9\) Intracranial arachnoid cyst is commonly considered a congenital lesion; it occurs most commonly in the temporal fossa and is relatively more prevalent in male patients.\(^21\) The pathogenic mechanisms for cyst remain unclear,\(^21\) and whether this manifestation is associated with HTRA1 needs to be further elucidated.

Multiple features detected in patients with heterozygous HTRA1 resemble CADASIL, including, for example, the dominant inheritance pattern, main neurological symptoms including stroke and cognitive decline, and typical CSVD-related neuroimaging markers including white

## Table 3. Comparison of the heterozygous HTRA1-related CSVD and CADASIL.

| Characteristics                      | Heterozygous HTRA1-related CSVD (n = 20) | CADASIL (n = 34) | \(p\) value |
|---------------------------------------|----------------------------------------|-----------------|-------------|
| Age, years, mean ± SD                 | 47.9 ± 11.3                            | 47.2 ± 9.3      | 0.807       |
| Male, n (%)                           | 10 (50)                                | 16 (47.1)       | 0.835       |
| Smoking, n (%)                        | 5 (25.0)                               | 7 (20.6)        | 0.970       |
| Medical history, n (%)                | 10.0                                   | 11.2            | 0.199       |
| Hypertension                          | 2 (10.0)                               | 2 (5.9)         | 0.583       |
| Diabetes mellitus                     | 2 (10.0)                               | 30 (88.2)       | <0.001      |
| Clinical features, n (%)              |                                        |                 |             |
| Migraine                              | 4 (20.0)                               | 7 (20.6)        | 1.000       |
| Ischemic stroke                       | 14 (70.0)                              | 28 (82.4)       | 0.474       |
| Cerebral hemorrhage                   | 2 (10.0)                               | 4 (11.8)        | 0.841       |
| Gait disturbance                      | 11 (55.0)                              | 12 (35.3)       | 0.157       |
| Cognitive impairment                  | 14 (70.0)                              | 26 (76.5)       | 0.600       |
| Alopecia                              | 1 (5.0)                                | 0 (0.0)         | 0.370       |
| Spine disorders                       | 19 (95.0)                              | 16 (47.1)       | <0.001      |
| WMH involves the anterior temporal lobe | 2 (10.0)                              | 30 (88.2)       | <0.001      |
| Presence of lacunes                   | 12 (60.0)                              | 25 (73.5)       | 0.301       |
| Presence of visible PVS               | 17 (85.0)                              | 31 (91.2)       | 0.492       |
| Presence of cerebral microbleeds      | 6 (35.3)                               | 19 (63.3)       | 0.064       |
| Presence of cerebral atrophy          | 6 (30.0)                               | 17 (50.0)       | 0.151       |
| Total CSVD score, median (IQR)        | 2 (1–4)                                | 3 (2–4)         | 0.075       |

Abbreviations: CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; CSVD, cerebral small vessel disease; IQR, interquartile range; PVS, perivascular spaces; SD, standard deviation; WMH, white matter hyperintensities.

*Three heterozygous HTRA1-related CSVD and four CADASIL patients missing the susceptibility-weighted images data.
matter hyperintensities, lacunes, and cerebral microbleeds. Although migraine has been considered a hallmark of CADASIL, patients with heterozygous HTRA1-related CSVD can also present with this symptom, which can make clinical diagnosis challenging. It is true that genetic screening and skin biopsy can support a definite diagnosis and can distinguish the two diseases, but these methods are high cost and invasive. In our study, we found patients with heterozygous HTRA1-related CSVD had a higher proportion of skeletal disorders and less involvement of the anterior temporal lobe compared with CADASIL, the combined features indicate informative differences between these two similar forms of inherited CSVD. Nevertheless, only genetic screening can definitely discriminate the inherited CSVD.

HTRA1 functions in protein quality control. Most of the variants identified in our study affect residues located within HTRA1’s serine protease domain. It is noteworthy that we found three families carrying homozygous or heterozygous p.A20V. Although this variant was predicted to be benign using in silico tools, all the affected family members (family 1, family 2, and family 3) presented with neurological symptoms that carried this mutation, although the clinical manifestations are varied. The p.A20V (c.59C>T) mutation affects residues within HTRA1’s signal peptide domain and the function of HTRA1’s N-terminal domain is not completely understood; it has been observed that the autolysis of the N-domain in vivo does not result in decreased protease activity. Future studies to elucidate the pathogenic impact(s) of this mutation are warranted.

In conclusion, our genetic, clinical, and neuroimaging study of a large cohort of CSVD cases in China broadens the genetic spectrum of HTRA1 and sheds light on the substantial heterozygous genetic etiology of this neurological disease. Patients with heterozygous HTRA1-related CSVD had a higher proportion of spine disorders and a lower proportion of WMH involving the anterior temporal lobe compared with CADASIL.

**Author Contributions**

ZQZ and CZ formulated the study concept; ZQZ acquired funding for the study. CZ, SWL, STN, WL, XGW, and ZQZ collected the data. CZ, ZQZ, SWL, HHZ, and XL analyzed the data. CZ wrote the manuscript. ZQZ revised the manuscript.

**Funding Information**

This study was supported by a grant from the Beijing Municipal Science and Technology Commission (No. Z171100001017080).

**Conflict of Interest**

The authors have no potential conflict of interest.

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### Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Primers for site-directed mutagenesis of HTRA1.

**Table S2.** NOTCH3 variants from 34 patients with cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy.