High frequency of HTRA1 AND ABCC6 mutations in Japanese patients with adult-onset cerebral small vessel disease

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ABSTRACT

Background This study aimed to clarify the frequency and clinical features of monogenic cerebral small vessel disease (mgCSVD) among patients with adult-onset severe CSVD in Japan.

Methods This study included patients with adult-onset severe CSVD with an age of onset ≤55 years (group 1) or >55 years and with a positive family history (group 2). After conducting conventional genetic tests for NOTCH3 and HTRA1, whole-exome sequencing was performed on undiagnosed patients. Patients were divided into two groups according to the results of the genetic tests: monogenic and undetermined. The clinical and imaging features were compared between the two groups.

Results Group 1 and group 2 included 75 and 31 patients, respectively. In total, 30 patients had NOTCH3 mutations, 11 patients had HTRA1 mutations, 6 patients had ABCC6 mutations, 1 patient had a TREP1 mutation, 1 patient had a COL4A1 mutation and 1 patient had a COL4A2 mutation. The total frequency of mutations in NOTCH3, HTRA1 and ABCC6 was 94.0% in patients with mgCSVD. In group 1, the frequency of a family history of first relatives, hypertension and multiple lacunar infarctions (LIs) differed significantly between the two groups (monogenic vs undetermined; family history of first relatives, 61.0% vs 25.0%, p=0.0015; hypertension, 34.1% vs 63.9%, p=0.0092; multiple LIs, 87.8% vs 63.9%, p=0.0134).

Conclusions More than 90% of mgCSVDs were diagnosed by screening for NOTCH3, HTRA1 and ABCC6. The target sequences for these three genes may efficiently diagnose mgCSVD in Japanese patients.

WHAT IS ALREADY KNOWN ON THIS TOPIC

- Monogenic cerebral small vessel disease (mgCSVD) is a major cause of young-onset stroke, dementia and leukoencephalopathy.
- Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy is the most common condition. However, the frequency of other mgCSVDs remains unknown.

WHAT THIS STUDY ADDS

- Our study revealed that the frequencies of HTRA1 (20.0%) and ABCC6 (12.0%) mutations were high among patients with severe CSVD. NOTCH3, HTRA1 or ABCC6 mutations caused 94% of mgCSVDs.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- Our results showed that screening only three genes can efficiently diagnose mgCSVD in Japan.

INTRODUCTION

Cerebral small vessel disease (CSVD), characterised by lacunar infarction (LI), dilated perivascular spaces (DPVS), microbleeds (MBs) or white matter hyperintensity (WMH) in brain MRI, causes dementia or gait disturbance (GD). Although ageing and hypertension (HT) are CSVD risk factors, the pathogenesis of CSVD remains unknown. CSVD is common in the elderly, and most cases are nonfamilial. Currently, more than 10 genes are known to cause monogenic CSVD (mgCSVD) in familial CSVD, including cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and high-temperature requirement A serine peptidase 1 (HTRA1)-related CSVD. Recently, mgCSVD caused by HTRA1 mutations has been increasingly reported. It was initially described as a rare recessive disease with characteristic clinical features, but HTRA1 mutations can cause CSVD, even in heterozygotes. However, the frequency of CSVD caused by HTRA1 mutations remains unclear.

Diagnosing mgCSVD is challenging in the following respects. First, patients without a family history of CSVD may have mgCSVD. Second, characteristic clinical features are often absent or slight among mgCSVD patients. Therefore, genetic screening is important for diagnosing mgCSVD in patients with CSVD, even in those without a family history. However, it is unclear how and in which cases genetic testing is most effective. In addition,
Table 1 Clinical features of patients with novel mutations in NOTCH3

| Mutation | C.1249T>A, p.C417S | C.1501G>T, p.G501C | C.2861A>G, p.Y954C | C.4111C>T, p.R1371C |
|----------|---------------------|-------------------|-------------------|-------------------|
| EGFr domains | 10                  | 12                | 24                | 34                |
| Sex | Male | Male | Female | Male |
| Family history* | Positive | Positive | Positive | Positive |
| First relatives | Parents | Positive | Positive | Positive | Positive |
| Second relatives | Grandparents | NA | NA | None | None |
| Neurological symptoms/signs | Stroke (years old) | 47 | None | 70 | None |
| CI/Dementia (years old) | 47 | 48 | 79 | 52 |
| GD (years old) | None | None | 40 | None |
| Risk factors | HT | Negative | Positive | Positive | Positive |
| MRI findings | WMHs (Fazekas grade) | 3 and III | 3 and III | 3 and III | 3 and III |
| Pathological findings | GOM | NA | Negative | Negative | NA |

*Family history is defined as an episode of dementia, stroke, or leukoencephalopathy.
†The severity of ECL is classified according to the length of the WMH on the EC. Moderate: >1/2 of the EC. Severe: >1/2 of the EC.‡
ATL, anterior temporal lesion; CI, cognitive impairment; EC, external capsule; ECL, external capsular lesions; EGFr domains, epidermal growth factor-like repeat domains; GD, gait disturbance; GOM, granular osmiophilic material; HT, hypertension; LI, lacunar infarction; NA, not available; WMH, white matter hyperintensity.

Table 2 Summary of diagnosis

| Diagnosis | Total n=75 | Positive n=41 | Negative n=34 |
|-----------|-----------|--------------|--------------|
| CADASIL | 23 (30.7) | 17 (41.5) | 6 (17.6) |
| Heterozygous HTRA1 | 8 (10.7) | 6 (14.6) | 2 (5.9) |
| CARASIL | 2 (2.7) | 0 (0) | 2 (5.9) |
| PXE | 3 (4.0) | 2 (4.9) | 1 (2.9) |
| Heterozygous ABC6 | 2 (2.7) | 0 (0) | 2 (5.9) |
| COLA41 | 1 (1.3) | 0 (0) | 1 (2.9) |
| COLA42 | 1 (1.3) | 1 (2.4) | 0 (0) |
| RVCL | 1 (1.3) | 1 (2.4) | 0 (0) |
| Undetermined | 34 (45.3) | 14 (34.1) | 20 (58.8) |

CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; CARASIL, cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy; COLA41, COLA41-related CSVD; COLA42, COLA42-related CSVD; HTRA1, HTRA1 mutation in ATP Binding Cassette Subfamily C Member 6 (ABCC6); ABC6, ABC6-related CSVD; PXE, pseudoxanthoma elasticum; RVCL, retinal vasculopathy with cerebral leukoencephalopathy.

Genetic tests and measuring HTRA1 protease activity

Genomic DNA was extracted from the blood samples. Conventional genetic tests of exons 2–24 of NOTCH3 and all the exons of HTRA1 were performed using a commercially available kit. The CADASIL diagnoses were based on missense mutations with a change in the number of cysteine residues or previously verified by granular osmiophilic material (GOM) deposition. If retinal vasculopathy with cerebral leukoencephalopathy (RVCL) was suspected according to the clinical or imaging features, a genetic test for exon 2 of three primer exonuclease 1 (TREXI) was also performed. The primer set for these three genes has been previously reported elsewhere.

If novel mutations in the HTRA1 gene were identified, we also measured the protease activity of the mutant HTRA1 protein (online supplemental methods and results).

Whole exome sequencing

We performed whole exome sequencing (WES) on the patients after excluding individuals with NOTCH3, HTRA1 or TREXI mutations. Exome analysis was conducted using an outsourcing service (Macrogen, Korea) (online supplemental methods and results), and data analysis of variants was performed using the Macrogen pipeline.

We initially removed synonymous variants, intronic variants or variants with a minor allele frequency of more than 0.01 using the 1000 Genome Phase 3 or Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org). We then investigated the following mgCSVD-associated genes reported until 2018: FOXC1, PITX2, COLA41, COLA42, CTSF, GLA, CECR1, ABC6, NF1, CBS, IKBKG, TREXI and COLGALT1. In addition, the c-termius region of LAMB1 was investigated (online supplemental figure 1). We evaluated the pathogenicity of the identified mutations using the ClinVar website (https://www.ncbi.nlm.nih.gov/clinvar/) or previous reports. All pathogenic mutations identified using WES were confirmed using conventional Sanger’s methods. The primer set for ABC6 has been previously reported.

In addition, we also investigated COLA41, COLA42, ABC6 and HTRA1 copy number variants (CNVs) in undiagnosed patients and patients with heterozygous mutations in ABC6 and COLA42 using copy number estimation by a Mixture of hereditary diseases usually differ among populations. Therefore, it is necessary to optimise the set of genetic tests for each population. Hence, we investigated the frequency and disease spectrum of mgCSVD among adult-onset severe CSVD patients in Japan to answer these questions.

MATERIAL AND METHODS

Participants

We recruited two groups from patients with adult-onset severe symmetrical WMHs corresponding to Fazekas grade 3/III and at least one of the following conditions, including LIs, pPVS, external capsular lesions (ECLs) or MBs on brain MRIs. Group included patients with an age of onset of neurological symptoms/signs, including stroke, GD and/or CI/dementia, ≤55 years irrespective of family history. Group 2 included patients with an age of onset of neurological symptoms/signs, including stroke, GD and/or CI/dementia, >55 and ≤70 years with a family history. Family history was defined as a clear episode of dementia, stroke or leukoencephalopathy in first or second relatives. CI was defined as a score of the Japanese edition Montreal Cognitive Assessment Battery (MoCA-J)<26.
Cerebrovascular disease

Figure 1  Classifying CSVD patients in group 1 using a decision tree. The bar graph shows the percentage of each mgCSVD and undetermined patient in group 1, classified according to a positive family history of first relatives, HT and the age of onset of neurological symptoms/signs. CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; CARASIL, cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy; COL4A1, COL4A1-related CSVD; COL4A2, COL4A2-related CSVD; heterozygous ABCG6, heterozygous mutation in ATP binding cassette subfamily C member 6 (ABCC6); HT, hypertension; heterozygous HTRA1, heterozygous high-temperature requirement A serine peptidase 1-related CSVD; PXE, pseudoxanthoma elasticum; RVCL, retinal vasculopathy with cerebral leukoencephalopathy; (-), negative; (+), positive.

PoCNVs were verified through a droplet digital PCR (ddPCR) in R software (V.4.1.0). The identified CNVs were classified according to the American College of Medical Genetics and Genomics (ACMG) standards and guidelines.18

Classifying the identified mutations

Computational analysis of the identified mutations was performed using PolyPhen2, SIFT and Provean on the VaProS website (http://p4d-info.mig.ac.jp/vapros/). The PHRED score of each mutation’s combined annotation-dependent depletion (https://cadd.gs.washington.edu/) was calculated. The pathogenicity of each mutation was then classified according to the American College of Medical Genetics and Genomics (ACMG) standards and guidelines.19

Clinical and imaging analysis

Clinical information, such as vascular risk factors, neurological symptoms/signs and MoCA-J scores, was collected using a survey sheet. Brain MRI scans of the patients were collected from each centre. The number and locations of the LIs and MBs were investigated. Multiple LIs were defined as more than one.19 Multiple MBs were defined as more than four, and the distribution of MBs was classified as strictly lobar or non-lobar.20 Severe ECLs were defined as the length of hyperintensity in more than half of the external capsule on T2-weighted images (T2WI)/FLAIR images. A severe anterior temporal lesion (ATL) was defined as confluent hyperintensity in the anterior temporal lobe on T2WI/FLAIR images.21

Statistical analysis

We then divided the included patients into two groups (the mgCSVD and undetermined groups) and statistically compared their clinical and imaging features. Furthermore, we used the genetic test results to divide mgCSVD patients in group 1 into two groups: CADASIL and non-CADASIL mgCSVD. Continuous and categorical variables were compared between the two groups (mgCSVD vs undetermined) using the Wilcoxon rank-sum test and Fisher’s exact test, respectively. Continuous and categorical variables between the three groups (CADASIL vs non-CADASIL vs undetermined) were compared using a one-way analysis of variance and Pearson’s χ² test, respectively. Multiple comparisons using Fisher’s exact tests followed by Hochberg’s correction were performed. We omitted the missing values included in some cases from the statistical analysis. Statistical significance was reached at a p < 0.05. Statistical analysis of clinical and imaging features and logistic regression models using stepwise methods to calculate adjusted ORs with 95% CIs were performed using R (V.4.1.2). CSVD patients were classified with the items with no missing values using a decision tree of statistics

Table 3  Results of multiple comparison of Fisher’s exact tests followed by Hochberg’s correction between CADASIL, non-CADASIL and undetermined in group 1

|                      | Undetermined versus non-CADASIL | Undetermined versus CADASIL | Non-CADASIL versus CADASIL |
|----------------------|---------------------------------|-----------------------------|-----------------------------|
| Family history       |                                 |                             |                             |
| First relatives      | 0.1357                          | 0.0179                      | 0.7477                      |
| Risk factors         |                                 |                             |                             |
| HT                   | 0.1759                          | 0.0468                      | 0.7417                      |
| Neurological symptoms/signs |                    |                             |                             |
| GD                   | 0.746                           | 0.0570                      | 0.0570                      |
| Extraneurological symptoms/signs |                        |                             |                             |
| Alopecia            | 0.3468                          | 0.1943                      | 0.0632                      |
| Spondylosis deformans or lumbago |                |                             |                             |
| Statistical analysis was performed using multiple comparison of Fisher’s exact tests, followed by Hochberg’s correction.

ATL, anterior temporal lesion; CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarct and leukoencephalopathy; DL, dyslipidaemia; GD, gait disturbance; HT, hypertension; LI, lacunar infarction; MBs, microbleeds.
and a machine learning toolbox on MATLAB R2020b Update 3 (9.9.0.153859). The optimisation of the hyperparameters in the decision tree was automatically calculated using MATLAB.

RESULTS
Identifying CADASIL, cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) and heterozygous HTRA1-related CSVD

We recruited 109 patients from 70 neurological centres throughout Japan (online supplemental table 1). Among them, three patients with leukodystrophy including vanishing white matter disease and X-linked adrenoleukodystrophy were excluded from further analysis. The patients were then divided into group 1 (age of onset at 55 years or younger with or without a family history) and group 2 (age of onset at 55 years or older with family history). Group 1 and group 2 included 75 and 31 patients, respectively (online supplemental table 2). Two patients in group 1 were suspected of having a particular disease based on clinical findings and were diagnosed by genetic testing for those genes. A brain tumour-like episode led us to suspect RVCL and identify a mutation in TREX1 (p.L287fs). Another patient was diagnosed with pseudoxanthoma elasticum (PXE) based on skin biopsy and fundus findings, which confirmed a heterozygous compound mutation (p.Q378X and p.L1313fs) in CADASIL.

We then performed genetic tests for exons 2–24 of NOTCH3 and identified 30 patients with CADASIL. All identified mutations had altered numbers of cysteine residues except for p.R75P. One CADASIL patient had two mutations in NOTCH3 (p.R607C and p.R1143C). Four mutations (p.C417S, p.G501C, p.Y954C and p.R1371C) have not yet been reported (online supplemental table 3). Clinical features of the four patients with novel NOTCH3 mutations are summarised in table 1. All four patients had early onset neurological symptoms/signs and extended WMHs on brain MRI, in addition to ECL and LIs. These patients met the diagnostic criteria of CADASIL. Genetic tests for HTRA1 revealed two patients with CADASIL and nine with heterozygous HTRA1 mutations. Among patients with heterozygous mutations in HTRA1, two mutations (p.L253R and p.V279M) have not yet been reported. The protease activity of both HTRA1 mutations was significantly decreased (online supplementals methods and results and figure 2).

WES and identifying other mgCSVD mutations

We performed WES in the 63 undiagnosed patients. We identified seven patients with mgCSVD. One patient was homozygous (p.M848fs), and another had compound heterozygous mutations (p.W14X and p.M848fs) in ABCC6. The p.W14X mutation is novel. A patient with compound heterozygous ABCC6 mutations (p.W14X and p.M848fs) was diagnosed with PXE based on xanthoma and angioid streaks. Another patient with a homozygous ABCC6 mutation (p.M848fs) was lost by follow-up. The other two patients had a heterozygous mutation (p.M848fs) in ABCC6. One patient had a mutation in the 3’-untranslated region of COL4A1 (c.*33T>A), which was previously reported. Another patient had a novel mutation in COL4A2 (p.Gly1176del). p.Gly1176 is located in the region of the triplet glycine sequence in COL4A2.

We used cn.MOPS to examine COL4A1, COL4A2, ABCC6 and HTRA1 CNVs in 52 undiagnosed patients and three patients with heterozygous mutations in ABCC6 or COL4A2. Binary alignment map file of five undiagnosed patients were unavail-

able. In one undiagnosed patient, widespread deletion of the region containing the ABCC6 gene was suspected. ddPCR confirmed the deletion of the ABCC6 gene at one allele (online supplementals methods and results and figure 3).

Per the ACMG criteria, 14 mutations in HTRA1, ABCC6 and TREX1 corresponded to pathogenic/likely pathogenic mutations, and 2 mutations in COL4A1/A2 corresponded to uncertain significance.

Finally, we found that 41/75 patients (54.7%) in group 1 and 9/31 (29.0%) in group 2 had mgCSVD (table 2 and online supplemental table 4). When classified by the presence or absence of a family history, 41.2% of patients in group 1 had mgCSVD, even if there was no family history of the disease. Eight of ten non-CADASIL mgCSVD patients with a family history of first relatives were heterozygous inheritance. Among the mgCSVD types found in group 1, 56.1%, 24.4% and 12.2% of patients had NOTCH3, HTRA1 and ABCC6 mutations, respectively. In contrast, 22.2% of patients with a family history and 28.6% without a family history had HTRA1 mutations. Regardless of the presence or absence of a family history, more than 92% of mgCSVD cases could be diagnosed by searching for the three genes.

Table 4 Summary of genetic mutations identified among patients with leukoencephalopathy

| Subjects | This study | Lynch et al | Chen et al | Mönkäri et al | Kuniti et al |
|----------|------------|-------------|------------|---------------|-------------|
| Total patients with mutation, n (%) | 50 (47.2) | 21 (21.0) | 20 (44.4) | 14 (40.0) | 12 (20.0) |
| Patients with CSVD-related gene mutation, n (%) | 50 (47.2) | 5 (5.0) | 19 (42.2) | 7 (20.0) | 8 (13.3) |
| NOTCH3 | 30 (26.3) | 4 (4.0) | 17 (37.8) | 2 (5.7) | 7 (11.7) |
| HTRA1 | 11 (10.4) | – | 2 (4.4) | 1 (2.9) | – |
| ABCC6 | 6 (5.7) | – | – | – | – |
| COL4A1 | 1 (0.9) | – | – | 2 (5.7) | – |
| COL4A2 | 1 (0.9) | – | – | 1 (2.9) | – |
| TREX1 | 1 (0.9) | – | – | – | – |
| CTSA | 0 (0) | 1 (1.0) | – | – | – |
| GLA | 0 (0) | – | – | – | 1 (1.7) |
| ITM28 | – | – | – | 1 (2.9) | – |

ABCC6, ATP binding cassette subfamily C member 6; CI, cognitive impairment; CSVD, cerebral small vessel disease; CTSA, cathepsin A; GLA, galactosidase alpha; HTRA1, high-temperature requirement A serine peptidase 1; ITM28, integral membrane protein 2B; TREX1, three-prime repair exonuclease-1; WMH, white matter hyperintensity.
Clinical features of mgCSVD patients compared with undetermined patients

We then divided the group 1 patients into two groups (monogenic and undetermined) according to the genetic test or WES results. There were 41 patients with mgCSVD and 34 undetermined patients assigned to each group.

Compared with the undetermined patients, the monogenic group had a significantly higher frequency of a family history of first relatives (61.0% vs 26.5%, \( p = 0.0028 \)), a family history of first and/or second relatives (65.9% vs 41.2%, \( p = 0.0326 \)), positive LIs (92.7% vs 73.5%, \( p = 0.0243 \)), multiple LIs (87.8% vs 67.6%, \( p = 0.0339 \)) and non-lobar MB distributions (22.2% vs 3.4%, \( p = 0.026 \)). The frequency of HT (34.1% vs 64.7%, \( p = 0.0084 \)) was significantly lower in the monogenic group than in the undetermined group (online supplemental table 5).

Among these items, a family history of first relatives (OR 1.3325, 95% CI 1.0914 to 1.6268, \( p = 0.0055 \)), HT (OR 0.7408, 95% CI 0.6031 to 0.9098, \( p = 0.0048 \)) and multiple LIs (OR 1.4184, 95% CI 1.1069 to 1.8177, \( p = 0.0064 \)) remained significant in the logistic regression model using stepwise methods (online supplemental methods and results and table 6).

Then, we classified the items with no missing values using a decision tree to predict mgCSVD. The results of the decision tree divided group 1 into four groups using three nodes: family history of first relatives, HT and age of onset ≤43 years (figure 1 and online supplemental table 7). In CSVD patients without a family history of first relatives, the frequency of mgCSVD was highest among those without HT and with an age of onset of neurological symptoms/signs ≤43 years (75.0%) and lowest among patients with HT (20.0%). Among CSVD patients with a family history of first relatives, the frequency of mgCSVD was 73.5%. CADASIL was identified in all four groups, and more than four mgCSVD cases were identified in the groups with a positive family history of first relatives and a negative family history, negative HT and an age of onset of neurological symptoms/signs ≤43 years.

Furthermore, we divided the patients into CADASIL and non-CADASIL groups and compared the clinical and imaging features of the three groups (online supplemental table 5). The frequency of a family history of first relatives, HT, GD, alopecia, lumbago/spondylosis deformans, positive LIs, severe ATLs, strict lobar distribution of MBs and non-lobar distribution of MBs differed significantly among the three groups (online supplemental table 5). Multiple comparisons of Fisher’s exact tests followed by Hochberg’s correction showed the following results (table 3). First, the frequency of a family history of first relatives (65.2% vs 26.5%, \( p = 0.0179 \)), LIs (100% vs 73.5%, \( p = 0.0233 \)) and non-lobar distributions of MBs (28.6% vs 3.4%, \( p = 0.0452 \)) in CADASIL patients was significantly higher than in the undetermined group. Second, the frequency of spondylosis deformans or lumbago was significantly higher in the non-CADASIL group than in the CADASIL group (66.7% vs 23.8%, \( p = 0.0316 \)).

Lastly, we divided the patients in group 2 into two groups: monogenic (9 patients) and undetermined (22 patients). Compared with the undetermined group, the frequencies of LIs at the semiovale (88.9% vs 45.5%, \( p = 0.0261 \)) and LIs in the cerebellum (22.2% vs 0%, \( p = 0.0223 \)) were significantly higher in the monogenic group (online supplemental table 8).

DISCUSSION

In this study, we found that in a group of patients with severe CSVD developed at 55 years of age or younger, more than 50% of the patients, regardless of family history, had a gene mutation responsible for CSVD. Approximately 40% of patients were diagnosed with mgCSVD without a family history. CADASIL accounted for nearly 60% of the patients, followed by HTRA1-related CSVD in approximately a quarter of the patients. The third most common group was ABCC6-related CSVD, accounting for approximately 10% of cases. These three genes account for more than 90% of the causes of mgCSVD. When compared by family history, the frequency of CADASIL was lower in the group with no family history, but the frequency of patients with HTRA1-related or ABCC6-related CSVD did not change markedly between the groups with or without a family history. These results indicate that the presence or absence of a family history is not useful for inferring mgCSVD and its type.

Figure 2 Proposed flow charts for diagnosis of mgCSVD. A proposed flow chart for diagnosing mgCSVD. First, PXE or RVCL are excluded according to clinical and/or imaging features. Second, genetic testing for NOTCH3 is performed in the remaining patients. Third, HTRA1 genetic testing is performed in the remaining patients without mutations in NOTCH3. Fourth, ABCC6 genetic testing should be applied to patients without mutations in NOTCH3 and HTRA1. Then, genetic tests should terminate if the patient has a family history of first relatives, HT and age of onset ≤43 years. Furthermore, we divided the patients into CADASIL and non-CADASIL groups and compared the clinical and imaging features of the three groups (online supplemental table 5). The frequency of a family history of first relatives, HT, GD, alopecia, lumbago/spondylosis deformans, positive LIs, severe ATLs, strict lobar distribution of MBs and non-lobar distribution of MBs differed significantly among the three groups (online supplemental table 5). Multiple comparisons of Fisher’s exact tests followed by Hochberg’s correction showed the following results (table 3). First, the frequency of a family history of first relatives (65.2% vs 26.5%, \( p = 0.0179 \)), LIs (100% vs 73.5%, \( p = 0.0233 \)) and non-lobar distributions of MBs (28.6% vs 3.4%, \( p = 0.0452 \)) in CADASIL patients was significantly higher than in the undetermined group. Second, the frequency of spondylosis deformans or lumbago was significantly higher in the non-CADASIL group than in the CADASIL group (66.7% vs 23.8%, \( p = 0.0316 \)).

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The frequency of patients with HTRA1-related CSVD was approximately one-third of the CADASIL frequency. However, HTRA1 loss-of-function mutations were found in one of the 450 apparently normal individuals in the UK.32 HTRA1 mutations have also been described as risk factors for sporadic CSVD.28 These findings suggest that the contribution of HTRA1 to CSVD may be higher than that previously thought, especially in Japan.

In addition, ABCC6 is the third most commonly mutated gene in patients with severe CSVD. We identified three cases of PXE and three heterozygous patients with ABCC6 mutations. Compared with the frequency of carriers of ABCC6 mutations in East Asia (0.76%),29 the frequency of heterozygotes found in this study (2.8%) was clearly higher. ABCC6 causes PXE through biallelic mutations, and symptoms related to CSVD were previously reported in PXE.30 Mutations in ABCC6 are more frequent in ischaemic stroke patients, including CSVD.31 Our results indicate that ABCC6 mutations may be strong risk factor of severe CSVD, even in the heterozygous state. ABCC6-related CSVD should be considered a cause of CSVD in Japanese patients.

We summarised recent reports of causative genes of leukoencephalopathy that were determined using next-generation sequencing in table 4.3–35 In these studies, HTRA1 mutations were the second most frequently identified in one of the four studies.35 However, ABCC6 mutations were not identified in these studies, and this type of mutation was observed only in this study. These differences in the frequency of mgCSVD across studies may be due to differences in the included patients or investigated genes.

Next, we examined the clinical mgCSVD features and that the absence of HT, the presence of a family history of first relatives, and multiple LIs may be useful in hypothesising an mgCSVD diagnosis in patients with an age of onset below 55 years (group 1). In addition, we created a decision tree to classify the patients with CSVD into four groups. The mgCSVD frequency was greater than 70% in both groups. The first group had a positive family history of first relatives, and the second group had no family history of first relatives, no HT and an age of onset ≤43 years. The two groups included all cases of CSVD caused by mutations in extremely rare genes (COL4A1, COL4A2 and TREX1) observed in this study.

Based on these results, we propose an efficient strategy for genetically testing adult-onset severe CSVD (figure 2). First, PXE or RVCL should be excluded based on clinical or imaging features.32 36 Next, we recommend NOTCH3, HTRA1 and ABCC6 genetic testing. At this step, 96% of mgCSVD cases were diagnosed in the present analysis group. Finally, include COL4A1/2 genetic testing or WES for patients with an age of onset ≤43 years or an age of onset ≤55 years with a first-degree relative with CSVD. By following this genetic testing strategy, the number of cases requiring WES can be narrowed to 13.2% of the total (14 of 106 patients), using the present analysis as an example.

Our study had some limitations. First, the pathogenicity of several mutations remains unclear. We identified patients with novel COL4A2 and NOTCH3 mutations. Mutations in COL4A2 found in patients with CSVD are usually characterised by the substitution of glycine for another amino acid in the triple repeat sequence.37 38 In addition, we did not evaluate the pathogenicity of COL4A2 mutation such as using skin biopsy.39 Hence, it is unclear whether deletion of a single amino acid (glycine) can cause CSVD. While, patients with novel NOTCH3 mutations met the diagnostic criteria of CADASIL.9 24 However, the pathological findings of GOM were negative in two of the four patients with NOTCH3 mutations. Further studies are required to elucidate the pathogenicity of these mutations. Second, we did not survey the number of relatives with CSVD. Therefore, our study was unable to investigate the association between the number of relatives with or without CSVD and diagnosis of mgCSVD. Thus, our results may be insufficient to clarify the relationship between a diagnosis of mgCSVD and family history. In addition, patients with negative family history included patients for whom information on family history was not available. These points should be clarified in future studies. Third, it is unclear how representative the included patients in this study because there was a bias towards requesting physicians or the requesting institutions were primarily neurology or neurosurgery teaching affiliate institutions. Fourth, we considered that most undetermined patients in this study were caused by vascular risk factors such as HT. Our study indicated that HT was associated with undetermined groups, however, we did not collect detailed information on HT, such as the degree of blood pressure or duration of HT. Therefore, we did not determine how HT contributed to CSVD. In addition, the other possibility is that some of the patients may have genetic mutations that have yet to be elucidated.

CONCLUSION

We have shown that HTRA1 and ABCC6 mutations are not negligible genetic factors of severe CSVD in Japanese patients. Approximately 40% of the cases were due to mutations in these genes in mgCSVD that developed at 55 years of age or younger. Notably, even heterozygotes can develop severe CSVD. Since these cases are often difficult to diagnose based on clinical features alone, gene testing is necessary. Approximately 90% or more of mgCSVD cases can be diagnosed by screening for these three genes, including NOTCH3. All cases are likely to have mutations in these genes because these diseases are widely distributed regardless of family history or age of onset. On the other hand, other rare diseases were identified either in cases with a family history or in cases without a family history or HT and with an age of onset ≤43 years. We believe that targeting this group effectively designates WES as a genetic test for mgCSVD.

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Cerebrovascular disease

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Contributors MU: conceptualisation; data curation; formal analysis; investigation; methodology; software analysis; writing—original draft; writing—review and editing. YH: software analysis. HH: genetic testing; obtaining patient information; writing—review and editing.

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SUPPLEMENTARY METHODS

Measuring the protease activity of novel mutant HTRA1 proteins

The detailed protocols for measuring HTRA1 protease activity have been previously reported. Briefly, an expression plasmid for each HTRA1 complementary DNA (cDNA) variant was generated. The plasmid vectors were transfected into FreeStyle 293 cells (Thermo Fisher Scientific). After incubation, secreted HTRA1 proteins were purified from the culture medium. After pre-incubating 1 µg of the recombinant HTRA1 protein, the protease activities of these HTRA1 proteins were evaluated using fluorescein isothiocyanate (FITC)-labeled casein as a substrate (Fluorescent Protease Assay Kit; Pierce, Rockford, IL, USA). Wild-type (WT) and S328A were used as positive and negative controls, respectively. S328A is a mutant HTRA1 protein that inhibits protease activity. The protease activities of mutant HTRA1 proteins and the positive and negative controls were calculated from the slope of the linear portion of normalized fluorescence vs. time plots at 30 min, 60 min, and 90 min. The amount of HTRA1 protein was analyzed by sodium dodecyl sulfate-polyacrylamide...
gel electrophoresis (SDS-PAGE) and stained with SYPROR©Ruby Protein Gel Stain (Thermo Fisher Scientific, MA, USA).

4 **WES protocols**

Exome analysis was conducted using an outsourcing service (Macrogen, Korea).

The SureSelect Human All Exon v6 Kit (Agilent Technologies, Santa Clara, CA, USA) was used as a capture kit. The next-generation sequencer was run on an Illumina HiSeq 4000 or NovaSeq6000 (Illumina, San Diego, CA) using 150 bp paired-end reads. The raw sequence was mapped to the human genome using the Burrows-Wheeler alignment (BWA) tool. A SAM file was generated using Picard. Picard was used to detect but not remove duplicate reads. Variant calling and annotation were performed using GATK (http://www.broadinstitute.org, org/gatk/) and SnpEff (http://snpeff.sourceforge.net/download.html). The hg19 data from the UCSC and GRCh38 data from the NCBI were used as the reference genome data for 53 and 10 samples, respectively.
Detecting the CNV of causative CSVD genes

We investigated the binary alignment map (BAM) files of WES through 
cn.MOPS using R software (ver. 4.1.0) to identify HTRA1, COL4A1/A2, and ABCC6

CNVs among patients without mutations in causative CSVD genes or patients with
heterozygous mutations in ABCC6 or COL4A2. cn.MOPS is an algorithm for detecting
CNVs from BAM files of next-generation sequencing.² The R package cn.MOPS is
available on the website (http://www.bioinf.jku.at/software/cnmops/). The minWidth
and minReadCount parameters were set to 1 and 100, respectively, and the other
cn.MOPS parameters did not change. The read count ratio and estimation of the copy
number of each HTRA1, COL4A1/A2, and ABCC6 exon were calculated according to
the cn.MOPS algorithm.

A ddPCR was performed to verify the cn.MOPS-identified CNVs. We used
forward and reverse primers and fluorescent probes for exon 25 of ABCC6 and
BCKDHA on genomic DNA as a reference.³ Probes for exon 25 of ABCC6 and
BCKDHA in genomic DNA were labeled with FAM and HEX, respectively. The
forward primer, reverse primer, and probe sequences were 5'-
GCAAGCCCACCTCAGT-3', 5'-AGTCTCTGCTCTCTCTGTGT-3', and 5'-56-
FAM/CTCGTGGGC/Zen/TTCTCTCTGCTCTCTGCT/3IABkFQ, respectively. After the
primer and probe of exon 25 of ABCC6 and BCKDHA were mixed with ddPCR
Supermix for Probes (No dUTP) (Bio-Rad, CA, USA), droplets were generated using a
Bio-Rad QX200 Droplet Generator (Bio-Rad, CA, USA). PCR was then performed.
The ddPCR cycling parameters were as follows: initial denaturation for 10 min at 95°C,
40 cycles of 30 s at 94°C, 2 min at 55°C, and final elongation for 10 min at 98°C. We
calculated the fluorescence value of FAM/HEX and the copy number of ABCC6 exon
25 using a Bio-Rad QX200 Droplet Reader (Bio-Rad, CA, USA).

SUPPLEMENTARY STATISTICAL ANALYSIS

Protease activity of each HTRA1 protein

The protease activity of the HTRA1 proteins was statistically analyzed using
MATLAB R2020b Update 3 (9.9.0.1538559). Groups were compared using an
ANOVA for independent samples, followed by Bonferroni’s correction when the overall p-value was < 0.05.

Logistic regression analysis to investigate the association of Group1 mgCSVD with clinical and imaging features using stepwise selection methods

We performed logistic regression analysis using forward-backward stepwise selection methods to verify independent factors significantly associated with mgCSVD in Group1. Exploratory variables included family history (first relative, second relative, or first and/or second relatives), sex, vascular risk factors (HT, diabetes mellitus (DM), dyslipidemia (DL)), clinical symptoms or signs (stroke, CI/dementia, and GD), neuroimaging findings (positive LI, multiple LIs, severe ECLs, and severe ATLs), and age of onset of neurological findings/signs. Optimal exploratory variables were selected by calculating the Akaike information criterion. Statistical analyses were performed using R (version 4.1.2) in RStudio (2021.09.1).

SUPPLEMENTARY RESULTS
Protease activity of novel mutant HTRA1 proteins

Protease activities of L253R and V279M proteins were significantly decreased compared to those of the WT (Supplementary Figure 2).

Detecting ABCC6 CNVs

The cn.MOPS results indicated that one patient without mutations in any causative CSVD gene had one copy of exon 7–10, 16–17, and 21–25 of ABCC6. None of the patients with heterozygous mutations in ABCC6 or COL4A2 had HTRA1, COL4A1/A2, and ABCC6 CNVs.

The results of ddPCR also indicated one copy of exon 25 of ABCC6 in this patient (Supplementary Figure 3).

Logistic regression analysis to investigate the association of Group1 mgCSVD with clinical and imaging features using stepwise methods
After the logistic regression analysis using stepwise methods, the family history of first relatives ($p = 0.004$), HT ($p = 0.005$), and multiple LIs ($p = 0.003$) remained significant (Supplementary Table 3).
**Supplementary Table 1. Hospitals participated in this study**

| Hospital                                                                 | Number of patients |
|--------------------------------------------------------------------------|--------------------|
| Department of Behavioral Neurology & Cognitive Neuroscience, Tohoku University Graduate School of Medicine | 2                  |
| Department of Cerebrovascular Medicine, Kohnan Hospital                 | 1                  |
| Department of Cerebrovascular Medicine, Steel Memorial Yawata Hospital  | 1                  |
| Department of Neurology, Anjo Kosei Hospital                            | 1                  |
| Department of Neurology, Asoka Hospital                                 | 1                  |
| Department of Neurology, Brain Research Institutes, Niigata University   | 11                 |
| Department of Neurology, Central Hospital Hyogo Rehabilitation Center    | 2                  |
| Department of Neurology, Chiba University Hospital                      | 2                  |
| Department of Neurology, Chikamori Hospital                             | 1                  |
| Department of Neurology, Ebara Hospital                                 | 1                  |
| Department of Neurology, Ehime Prefectural Central Hospital             | 1                  |
| Department of Neurology, Fukuoka Mirai Hospital                         | 1                  |
| Department of Neurology, Fukuoka University Hospital                    | 1                  |
| Department of Neurology, General Tokyo Hospital                         | 1                  |
| Department of Neurology, Gunma University Hospital                      | 1                  |
| Department of Neurology, Hiroshima Red Cross Hospital & Atomic-bomb Survivors Hospital | 1 |
| Department of Neurology, Hitachinaka General Hospital                  | 1                  |
| Department of Neurology, Japanese Red Cross Nagoya Daini Hospital       | 1                  |
| Department of Neurology, Japanese Red Cross Osaka Hospital              | 6                  |
| Department of Neurology, Kagawa Prefectural Central Hospital            | 1                  |
| Department of Neurology, Kanazawa Medical University                    | 1                  |
| Department of Neurology, Kanazawa University Hospital                  | 3                  |
| Department of Neurology, Kawasaki Medical School                        | 2                  |
| Department of Neurology, Keio University Hospital                       | 2                  |
| Department of Neurology, Kido Hospital                                  | 1                  |
| Department of Neurology, Kitamurayama Hospital                         | 1                  |
| Department of Neurology, Kobe city Medical Center General Hospital | 1 |
| Department of Neurology, Kyoto Prefectural University of Medicine | 1 |
| Department of Neurology, Mie University Hospital | 1 |
| Department of Neurology, Minami-Okayama Medical Center | 1 |
| Department of Neurology, Murakami General Hospital | 2 |
| Department of Neurology, Nagano Red Cross Hospital | 1 |
| Department of Neurology, Nagaoka Red Cross Hospital | 4 |
| Department of Neurology, Nagoya University Hospital | 1 |
| Department of Neurology, Nakamura Memorial Hospital | 1 |
| Department of Neurology, Nara Medical University Hospital | 1 |
| Department of Neurology, National Cerebral and Cardiovascular Center | 1 |
| Department of Neurology, National Hakone Hospital | 1 |
| Department of Neurology, Niigata City General Hospital | 7 |
| Department of Neurology, Niigata Prefectural Shibata Hospital | 1 |
| Department of Neurology, Nippon Medical School Musashi Kosugi Hospital | 1 |
| Department of Neurology, Okayama Kyokuto Hospital | 2 |
| Department of Neurology, Okayama University Hospital | 2 |
| Department of Neurology, Osaka City General Hospital | 1 |
| Department of Neurology, Osaka City University Hospital | 2 |
| Department of Neurology, Osaka General Medical Center | 1 |
| Department of Neurology, Osaka University Hospital | 3 |
| Department of Neurology, Osaka city Kosai-in fuzoku hospital | 1 |
| Department of Neurology, Public Central Hospital of Matto Ishikawa | 1 |
| Department of Neurology, Saiki Hospital | 1 |
| Department of Neurology, Saitama Medical University International Medical Center | 1 |
| Department of Neurology, Shiseikai Daini Hospital | 2 |
| Department of Neurology, Showa General Hospital | 1 |
| Department of Neurology, Soseikai General Hospital | 1 |
| Department of Neurology, Southern Tohoku General Hospital | 1 |
| Department of Neurology, Takasaki General Medical Center | 1 |
| Department of Neurology, Tane Second Hospital | 1 |
| Hospital                                                                 | Count |
|---|---|
| Department of Neurology, The Jikei University Hospital                  | 1     |
| Department of Neurology, Tokyo Metropolitan Bokutoh Hospital            | 1     |
| Department of Neurology, Tokyo National Hospital                        | 1     |
| Department of Neurology, Tokyo Women's Medical University Hospital      | 1     |
| Department of Neurology, Toranomon Hospital                             | 2     |
| Department of Neurology, Toyama Rosai Hospital                          | 1     |
| Department of Neurology, Tsubame Rosai Hospital                         | 1     |
| Department of Neurology, University of Tsukuba Hospital                 | 1     |
| Department of Neurosciences, Tane General Hospital                      | 1     |
| Division of Gastroenterology, Hepato-biliary-pancreatology and Neurology, Akita University School of Medicine | 1     |
| Kitakanto Neurologic Disorders Research Center, Shinozuka Hospital       | 2     |
| Stroke Center, Kyorin University Hospital                                | 1     |

**Supplementary Table 1. Hospitals participated in this study.**
Supplementary Table 2. Basic information of CSVD patients with age of onset of stroke, GD and/or CI/dementia ≤ and > 55 years old.

| Items                                         | Age of onset ≤ 55 years old (Group1) | Age of onset > 55 years old (Group2) |
|-----------------------------------------------|-------------------------------------|-------------------------------------|
|                                              | n = 75                              | n = 31                              |
| Family history                               |                                     |                                     |
| First relatives (%)                           | 34 (45.3), 0                        | 25 (80.6), 0                        |
| Second relatives (%)                          | 17 (22.7), 0                        | 16 (51.6), 0                        |
| First and/or second relatives (%)             | 41 (54.7), 0                        | 31 (100), 0                         |
| Consanguity marriage of parents (%)           | 6 (8), 0                            | 4 (12.9), 0                         |
| Male (%)                                      | 53 (70.7), 0                        | 16 (51.6), 0                        |
| Risk factors                                 |                                     |                                     |
| HT (%)                                        | 36 (48), 0                          | 21 (67.7), 0                        |
| DM (%)                                        | 6 (8), 0                             | 5 (16.1), 0                         |
| DL (%)                                        | 28 (37.3), 0                        | 10 (32.3), 0                        |
| Alcohol (%)                                   | 6 (8.2), 2                          | 6 (20), 1                           |
| Smoking (%)                                   | 31 (43.7), 4                        | 10 (33.3), 1                        |
| Neurological symptoms/signs                  |                                     |                                     |
| CI (MoCA-J ≤ 26) (%)                          | 64 (92.8), 6                         | 22 (81.5), 4                        |
| Dementia (%)                                  | 49 (65.3), 0                         | 19 (61.3), 0                        |
| CI and/or dementia (%)                        | 69 (92), 0                           | 27 (87.1), 0                        |
| GD (%)                                        | 47 (62.7), 0                         | 22 (71), 0                          |
| Stroke (%)                                    | 45 (60), 0                           | 14 (45.2), 0                        |
| Migraine (%)                                  | 17 (23.3), 2                         | 7 (22.6), 0                         |
| Pseudobulbar palsy (%)                        | 26 (35.6), 2                         | 9 (29), 0                           |
| Extraneurological symptoms/signs | | |
|---------------------------------|---|---|
| Alopecia (%), NA | 18 (26.1), 6 | 3 (10.3), 2 |
| Lumbago and/or spondylosis deformans (%), NA | 33 (45.2), 2 | 13 (41.9), 0 |
| Hemorrhage in fundus (%), NA | 4 (8.7), 29 | 1 (6.3), 15 |
| Brain imaging findings | | |
| Fazekas grade of WMHs | | |
| Grade 3 (PVH) (%), NA | 69 (92), 0 | 31 (100), 0 |
| Grade III (DWMH) (%), NA | 75 (100), 0 | 30 (96.8), 0 |
| LI | | |
| Positive LI (%), NA | 63 (84), 0 | 25 (80.6), 0 |
| at semiovale (%), NA | 52 (69.3), 0 | 18 (58.1), 0 |
| at BG (%), NA | 33 (44), 0 | 14 (45.2), 0 |
| at thalamus (%), NA | 41 (54.7), 0 | 13 (41.9), 0 |
| at brainstem (%), NA | 26 (34.7), 0 | 6 (19.4), 0 |
| at cerebellum (%), NA | 8 (10.7), 0 | 2 (6.5), 0 |
| Multiple LIs (%), NA | 59 (78.7), 0 | 21 (67.7), 0 |
| dPVS (%), NA | 72 (97.3), 1 | 31 (100), 0 |
| Severe ECL (%), NA | 39 (52), 0 | 18 (58.1), 0 |
| Severe ATL (%), NA | 21 (28), 0 | 4 (12.9), 0 |
| MBs | | |
| Positive MBs (%), NA | 48 (73.8), 10 | 20 (74.1), 4 |
| Multiple MBs (%), NA | 42 (64.6), 10 | 13 (48.1), 4 |
| Strict lobar (%), NA | 2 (3.1), 10 | 2 (7.4), 4 |
| Non lobar (%), NA | 9 (13.8), 10 | 4 (14.8), 4 |
| Age of genetic test (years old) | | |
| mean ± sd | 52.2 ± 8.6 | 65.8 ± 6.0 |
| median (minimum-maximum) | 53 (32-79) | 64 (56-79) |
| Age of onset of stroke, GD and/or CI/dementia (years old) | | |
| mean ± sd | 46.0 ± 7.5 | 61.4 ± 4.0 |
### Supplementary Table 2. Basic information of CSVD patients with age of onset of stroke, GD and/or CI/dementia ≤ and > 55 years old.

|                | Median (minimum-maximum) |
|----------------|--------------------------|
|                | 48 (23-55)               |
|                | 60 (56-70)               |

CSVD indicates cerebral small vessel disease; HT, hypertension; DM, diabetes mellitus; DL, dyslipidemia; MoCA-J, Montreal cognitive assessment battery in Japanese edition; CI, cognitive impairment; GD, gait disturbance; WMH, white matter hyperintensity; PVH, periventricular hyperintensity; DSWMH, deep and subcortical white matter hyperintensity; LI, lacunar infarction; dPVS, dilated perivascular spaces; ECL, external capsular lesion; ATL, anterior temporal lesion; MBs, microbleeds; sd, standard deviations; NA, number of patients who was not available.
## Supplementary Table 3. Summary of identified mutations.

| cDNA, p.AA | SNP ID | Exon n. | Group1 n. | Group2 n. | MAF of gnomAD | In Silico PolyPhen2 | SIFT | Provean | CADD PHRED | ACMG | PVS1 | PS1-4 | PM1-6 | PP1-5 | Classification |
|------------|--------|---------|------------|-----------|---------------|-------------------|------|---------|------------|------|------|-------|-------|-------|-----------------|
| NOTCH3     |        |         |            |           |               |                   |      |         |            |      |      |       |       |       |                 |
| c.163T>C, p.C55R | NA     | 2       | 1          | 0         | NA            | probably damaging | DELETERIOUS | Deleterious | 27          | -    | -    | 1, 2   | 1, 2, 3, 4 | Likely Pathogenic |
| c.224G>C, p.R75P | NA     | 3       | 1          | 1         | NA            | probably damaging | TOLERATED | Deleterious | 20.9        | -    | -    | 1, 2   | 1, 2, 3, 4 | Likely Pathogenic |
| c.397C>T, p.R133C | rs137852642 | 4     | 1          | 0         | 0.00004515    | probably damaging | TOLERATED | Deleterious | 28.8        | -    | -    | 1, 2   | 1, 2, 3, 4 | Likely Pathogenic |
| c.544G>C, p.R182C | rs28933697 | 4     | 1          | 0         | 0.0003185     | probably damaging | DELETERIOUS | Deleterious | 29.5        | -    | -    | 1, 2   | 1, 2, 3, 4 | Likely Pathogenic |
| c.548G>C, p.C183S | NA     | 4       | 1          | 1         | NA            | probably damaging | DELETERIOUS | Deleterious | 26.2        | -    | -    | 1, 2   | 1, 2, 3, 4 | Likely Pathogenic |
| c.635G>A, p.C212Y | NA     | 4       | 1          | 0         | NA            | probably damaging | DELETERIOUS | Deleterious | 24.4        | -    | -    | 1, 2   | 1, 2, 3, 4 | Likely Pathogenic |
| c.953G>A, p.C318Y | NA     | 6       | 1          | 1         | NA            | probably damaging | DELETERIOUS | Deleterious | 26.8        | -    | -    | 1, 2   | 1, 2, 3, 4 | Likely Pathogenic |
| SNP          | Reference Allele | Effect Allele | Allele Count | Predicted Effect | Ranks | Pathogenicity |
|--------------|------------------|---------------|--------------|------------------|-------|---------------|
| c.994G>T, p.R332C | NA               | 6             | 1            | NA               |       | TOLERATED     |
| c.1063T>G, p.C355G | NA               | 7             | 1            | NA               |       | DELETTERIOUS  |
| c.1249T>A, p.C417S | NA               | 8             | 1            | NA               |       | DELETTERIOUS  |
| c.1279C>T, p.R427C | rs201118034      | 11            | 1            | 0.0002947        |       | TOLERATED     |
| c.1357T>G, p.A446F | NA               | 8             | 1            | NA               |       | DELETTERIOUS  |
| c.1501G>T, p.G501C | NA               | 10            | 1            | NA               |       | DELETTERIOUS  |
| c.1630C>T, p.R544C | rs201118034      | 11            | 1            | 0.0002947        |       | TOLERATED     |
| c.1672C>T, p.R558C | rs75068032       | 11            | 1            | 0.00003187       |       | TOLERATED     |
| c.1789T>A, p.C597S | NA               | 11            | 0            | NA               |       | DELETTERIOUS  |
| c.1819C>T, p.R607C | rs777751363      | 11            | 1            | 0.00003998       |       | TOLERATED     |
| SNP          | Reference ID | Sample Size | Minor Allele | Minor Allele Frequency | Prediction | Evidence | Classification |
|--------------|--------------|-------------|--------------|------------------------|------------|----------|----------------|
| c.2149C>T, p.R717C | rs144163298  | 14          | 0            | 0.00003637             | DELETERIOUS | -        | 1, 2, 3, 4 Likely Pathogenic |
| c.2861A>G, p.Y954C | NA          | 18          | 1            | 0                      | DELETERIOUS | -        | 1, 2, 3, 4 Likely Pathogenic |
| c.3010T>G, p.C1004G | NA          | 19          | 1            | 0                      | TOLERATED   | -        | 1, 2, 3, 4 Likely Pathogenic |
| c.3062A>G, p.Y1021C | rs1167405466 | 19          | 2            | 0.00003187             | TOLERATED   | -        | 1, 2, 3, 4 Likely Pathogenic |
| c.3091C>T, p.R1031C | NA          | 19          | 1            | 0                      | TOLERATED   | -        | 1, 2, 3, 4 Likely Pathogenic |
| c.3226C>T, p.R1076C | rs1438626607 | 20          | 1            | 0.00005194             | DELETIOUS   | -        | 1, 2, 3, 4 Likely Pathogenic |
| c.3427C>T, p.R1143C | rs60373464  | 21          | 1            | 0.00001769             | DELETIOUS   | -        | 1, 2, 3, 4 Likely Pathogenic |
| c.4111C>T, p.R1371C | NA          | 24          | 1            | 0                      | TOLERATED   | -        | 1, 2, 3, 4 Likely Pathogenic |
| HTRA1        | c.496C>T, p.R166C | NA        | 2            | 1                      | TOLERATED   | -        | 1, 2, 3, 4 Pathogenic |

**Classifications:**
- **DELETERIOUS:** Evidence of a potentially deleterious effect.
- **TOLERATED:** Evidence of a tolerated effect.
- **Pathogenic:** Evidence of a pathogenic effect.
| Gene       | Chromosome | rsID   | PhenoType | p | Prediction | Reference | Frequency | Consequence | Frequency | Function | Pathogenicity |
|------------|------------|--------|-----------|---|------------|-----------|-----------|-------------|-----------|----------|--------------|
| Uemura et al 17 |
| ARCC6     | c.497G>T, p.R166L | NA     | 2         | 1 | 0          | NA        | NA        | probably damaging | DELETERIOUS | 32       | Pathogenic   |
|           | c.758T>G, p.L253R | NA     | 3         | 1 | 0          | NA        | NA        | probably damaging | DELETERIOUS | 24       | Pathogenic   |
|           | c.835G>A, p.V279M | rs745305935 | 4         | 1 | 0          | NA        | NA        | probably damaging | DELETERIOUS | 28       | Pathogenic   |
|           | c.854C>T, p.P285L | rs177745564 | 4         | 1 | 0          | NA        | NA        | probably damaging | DELETERIOUS | 29       | Pathogenic   |
|           | c.890G>A, p.V297M | NA     | 4         | 1 | 0          | NA        | NA        | probably damaging | DELETERIOUS | 31       | Pathogenic   |
|           | c.904C>T, p.R302X | rs113993970 | 4         | 2 | 1          | NA        | NA        | NA          | NA        | 40       | Pathogenic   |
|           | c.905G>A, p.R302Q | NA     | 4         | 2 | 0          | NA        | NA        | probably damaging | DELETERIOUS | 32       | Pathogenic   |
|           | ABCC6      | c.41G>A, p.W14X | NA     | 2 | 1          | NA        | NA        | NA          | NA        | 36       | Pathogenic   |
|           | c.1132C>T, p.Q378X | rs72650699 | 9         | 1 | 0          | NA        | NA        | NA          | NA        | 37       | Pathogenic   |
|           | c.2542delA, p.V848fs | rs7687306 | 19        | 4 | 0          | NA        | NA        | NA          | NA        | 1        | Pathogenic   |
|           | p.3936_3937insG | NA     | 28        | 1 | 0          | NA        | NA        | NA          | NA        | 1        | Pathogenic   |
|           | p.L1313fs | NA     | 28        | 1 | 0          | NA        | NA        | NA          | NA        | 1        | Pathogenic   |
Supplementary Table 3. Summary of identified mutations.

| Deletion of exons | NA | 7-10, 16-17, 21-25 | 0 | 1 | NA | NA | NA | NA | 1 | 2, 4 | - | Pathogenic |
|-------------------|----|---------------------|---|---|----|----|----|----|---|-----|---|-------------|
| COL4A1            | NA |                     | 1 | 0 | NA | NA | NA | NA | 19.89 | - | 3 | 2 | - | Undetermined significance |
| c.*33T>A          | NA |                     |   |   | NA | NA | NA | NA |       |   |   |   |   | Likely Pathogenic |
| COL4A2            | NA |                     | 19 | 1 | 0 | NA | NA | NA | NA | - | - | 1, 4 | - | Undetermined significance |
| c.3527_3529delGAG | NA |                     |   |   | NA | NA | NA | NA |       |   |   |   |   | Likely Pathogenic |
| TREX1             | NA |                     | 2 | 1 | 0 | NA | NA | NA | NA | 1 | - | 1, 2, 4 | - | Likely Pathogenic |
| c.858dupG         | p.L287LAfs*38 | NA | 6 | 1 |   | NA | NA | NA | NA | 1 | - | 1, 2, 4 | - | Likely Pathogenic |
1 AA indicates amino acids; MAF, minor allele frequency; gnomAD, genome aggregation database; CADD, combined annotation dependent depletion; ACMG, American College of Medical Genetics and Genomics; NA, not available.
### Supplementary Table 4. Summary of non-CADASIL mgCSVD

| Non-CADASIL mgCSVD | Heterozygous HTRA1 | CARASIL | PXE | Heterozygous ABCC6 | COL4A1 | COL4A2 | RVCL |
|--------------------|--------------------|---------|-----|--------------------|--------|--------|------|
|                    | n = 9              | n = 2   | n = 3 | n = 3              | n = 1  | n = 1  | n = 1 |

**Family history**

|                      | First relatives (%) | NA     | Second relatives (%) | NA     | First and/or second relatives (%) | NA     | Consanguity marriage of parents (%) | NA     | Male (%) | NA     |
|----------------------|---------------------|--------|----------------------|--------|-----------------------------------|--------|-------------------------------------|--------|----------|--------|
|                      | 7 (77.8), 0         | 0 (0), | 2 (66.7), 0          | 1 (33.3), 0 | 0 (0), 0                         | 1 (100), 0 | 1 (100), 0                         | 0 (0), 0 | 6 (66.7), 0 | 1 (50), 0 |
|                      | 1 (11.1), 0         | 0 (0), | 2 (66.7), 0          | 0 (0), 0 | 0 (0), 0                         | 0 (0), 0 | 1 (100), 0                         | 0 (0), 0 | 1 (100), 0 | 0 (0), 0 |
|                      | 7 (77.8), 0         | 0 (0), | 2 (66.7), 0          | 1 (33.3), 0 | 0 (0), 0                         | 0 (0), 0 | 1 (100), 0                         | 0 (0), 0 | 0 (0), 0 | 0 (0), 0 |

**Risk factors**

|                      | HT (%) | NA     | DM (%) | NA     | DL (%) | NA     | Alcohol (%) | NA     | Smoking (%) | NA     |
|----------------------|--------|--------|--------|--------|--------|--------|-------------|--------|-------------|--------|
|                      | 4 (44.4), 0 | 0 (0), | 0 (0), | 1 (33.3), 0 | 0 (0), | 0 (0), | 0 (0), | 0 (0), | 2 (22.2), 0 | 1 (50), |
|                      | 0 (0), | 0 (0), | 0 (0), | 0 (0), 1 | 0 (0), | 0 (0), | 0 (0), 0 | 0 (0), 0 | 0 (0), 0 | 0 (0), 0 |
| Neurological symptoms/signs | CI (MoCA-J ≤ 26) (%) | NA | 7 (77.8), 0 | 2 (100), 0 | 3 (100), 0 | 2 (100), 0 | 0 (0), 1 | 1 (100), 0 | 1 (100), 0 |
|----------------------------|----------------------|----|-------------|-------------|-------------|-------------|----------|-------------|-------------|
| Dementia (%) | 6 (66.7), 0 | 2 (100), 0 | 2 (66.7), 0 | 3 (100), 0 | 1 (100), 0 | 0 (0), 0 | 1 (100), 0 |
| CI and/or dementia (%) | 7 (77.8), 0 | 2 (100), 0 | 3 (100), 0 | 1 (100), 0 | 1 (100), 0 | 1 (100), 0 |
| GD (%) | 7 (77.8), 0 | 2 (100), 0 | 1 (33.3), 0 | 2 (66.7), 0 | 1 (100), 0 | 1 (100), 0 |
| Stroke (%) | 7 (77.8), 0 | 0 (0), 0 | 2 (66.7), 0 | 1 (33.3), 0 | 1 (100), 0 | 0 (0), 0 | 1 (100), 0 |
| Migraine (%) | 3 (37.5), 1 | 0 (0), 0 | 2 (66.7), 0 | 2 (66.7), 0 | 0 (0), 0 | 0 (0), 0 |
| Pseudobulbar palsy (%) | 4 (44.4), 0 | 1 (50), 0 | 2 (66.7), 0 | 1 (50), 1 | 1 (100), 0 | 0 (0), 0 | 0 (0), 0 |
| Extraneurological symptoms/signs | Alopecia (%) | 3 (37.5), 1 | 1 (50), 0 | 2 (100), 1 | 1 (33.3), 0 | 0 (0), 0 | 0 (0), 0 | 1 (100), 0 |
| Lumbago and/or spondylosis deformans (%) | 7 (77.8), 0 | 2 (100), 0 | 3 (100), 0 | 1 (33.3), 0 | 0 (0), 0 | 0 (0), 0 | 0 (0), 0 |
| Hemorrhage in fundus (%) | 0 (0), 6 | 0 (0), 0 | 1 (50), 1 | 0 (0), 2 | 0 (0), 1 | 0 (0), 1 | 0 (0), 0 |
| Brain imaging findings | Fazekas grade of WMHs | Grade 3 (PVH) (%) | 8 (88.9), 0 | 2 (100), 0 | 2 (66.7), 0 | 3 (100), 0 | 1 (100), 0 | 1 (100), 0 |
| | Grade III (DWMH) (%) | 9 (100), 0 | 2 (100), 0 | 3 (100), 0 | 3 (100), 0 | 1 (100), 0 | 1 (100), 0 |
| | Positive LI (%) | 9 (100), 0 | 2 (100), 0 | 2 (66.7), 0 | 2 (66.7), 0 | 1 (100), 0 | 0 (0), 0 | 1 (100), 0 |
| | at semiovale (%) | 8 (88.9), 0 | 2 (100), 0 | 1 (33.3), 0 | 1 (33.3), 0 | 0 (0), 0 | 0 (0), 0 | 1 (100), 0 |
| | Fazekas grade of WMHs | Grade 3 (PVH) (%) | 8 (88.9), 0 | 2 (100), 0 | 2 (66.7), 0 | 3 (100), 0 | 1 (100), 0 | 1 (100), 0 |
| | Grade III (DWMH) (%) | 9 (100), 0 | 2 (100), 0 | 3 (100), 0 | 3 (100), 0 | 1 (100), 0 | 1 (100), 0 |
| | Positive LI (%) | 9 (100), 0 | 2 (100), 0 | 2 (66.7), 0 | 2 (66.7), 0 | 1 (100), 0 | 0 (0), 0 | 1 (100), 0 |
| | at semiovale (%) | 8 (88.9), 0 | 2 (100), 0 | 1 (33.3), 0 | 1 (33.3), 0 | 0 (0), 0 | 0 (0), 0 | 1 (100), 0 |
|                          | 3 (33.3), 0 | 0 (0), 0 | 1 (33.3), 0 | 1 (33.3), 0 | 1 (100), 0 | 0 (0), 0 | 0 (0), 0 |
|-------------------------|-------------|---------|-------------|-------------|-------------|---------|---------|
| at BG (%) , NA          |             |         |             |             |             |         |         |
| at thalamus (%) , NA    | 3 (33.3), 0 | 1 (50), 0 | 1 (33.3), 0 | 1 (33.3), 0 | 1 (100), 0 | 0 (0), 0 | 0 (0), 0 |
| at brainstem (%) , NA   | 1 (11.1), 0 | 2 (100), 0 | 2 (66.7), 0 | 1 (33.3), 0 | 0 (0), 0 | 0 (0), 0 | 0 (0), 0 |
| at cerebellum (%) , NA  | 0 (0), 0 | 0 (0), 0 | 1 (33.3), 0 | 0 (0), 0 | 0 (0), 0 | 0 (0), 0 | 0 (0), 0 |
| Multiple LIs (%) , NA   | 8 (88.9), 0 | 2 (100), 0 | 2 (66.7), 0 | 2 (66.7), 0 | 1 (100), 0 | 0 (0), 0 | 1 (100), 0 |
| dPVS (%) , NA           | 9 (100), 0 | 2 (100), 0 | 3 (100), 0 | 3 (100), 0 | 1 (100), 0 | 0 (0), 0 | 1 (100), 0 |
| Severe ECL (%) , NA     | 7 (77.8), 0 | 2 (100), 0 | 1 (33.3), 0 | 0 (0), 0 | 1 (100), 0 | 0 (0), 0 | 0 (0), 0 |
| Severe ATL (%) , NA     | 0 (0), 0 | 1 (50), 0 | 0 (0), 0 | 0 (0), 0 | 1 (100), 0 | 0 (0), 0 | 0 (0), 0 |

**MBs**

| Positive MBs (%) , NA  | 5 (71.4), 2 | 2 (100), 0 | 2 (100), 1 | 1 (50), 1 | 1 (100), 0 | 0 (0), 0 | 1 (100), 0 |
| Multiple MBs (%) , NA  | 4 (57.1), 2 | 2 (100), 0 | 2 (100), 1 | 1 (50), 1 | 1 (100), 0 | 0 (0), 0 | 1 (100), 0 |
| Strict lobar (%) , NA  | 1 (14.3), 2 | 0 (0), 0 | 0 (0), 1 | 1 (50), 1 | 0 (0), 0 | 0 (0), 0 | 0 (0), 0 |
| Non lobar (%) , NA     | 0 (0), 2 | 0 (0), 0 | 0 (0), 1 | 1 (50), 1 | 0 (0), 0 | 0 (0), 0 | 1 (100), 0 |

**Age of genetic test (years old)**

| mean ± sd | 57.6 ± 11.1 | 40.0 ± 9.9 | 47.7 ± 13.5 | 52.0 ± 12.1 | 51 | 63 | 41 |
| median (minimum-maximum) | 58 (32-71) | 40 (33-47) | 48 (34-61) | 45 (45-66) | - | - | - |

**Age of onset of stroke, GD and/or CI/dementia (years old)**

| mean ± sd | 50.4 ± 9.4 | 36.5 ± 9.2 | 42.7 ± 7.8 | 45 ± 19.7 | 30 | 45 | 33 |
| median (minimum-maximum) | 51 (32-68) | 36.5 (30-43) | 45 (34-49) | 42 (27-66) | - | - | - |
### Supplementary table 4. Summary of non-CADASIL mgCSVD

1. mgCSVD indicates monogenic cerebral small vessel disease; HT, hypertension; DM, diabetes mellitus; DL, dyslipidemia; MoCA-J, Montreal cognitive assessment battery in Japanese edition; CI, cognitive impairment; GD, gait disturbance; WMH, white matter hyperintensity; PVH, periventricular hyperintensity; DSWMH, deep and subcortical white matter hyperintensity; LI, lacunar infarction; dPVS, dilated perivascular spaces; ECL, external capsular lesion; ATL, anterior temporal lesion; MBs, microbleeds; sd, standard deviations; NA, number of patients who was not available; Heterozygous HTRA1, heterozygous high-temperature requirement A serine peptidase 1 (HTRA1)-related CSVD, CARASIL, cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy; PXE, pseudoxanthoma elasticum; Heterozygous ABCC6, heterozygous mutation in ATP Binding Cassette Subfamily C Member 6 (ABCC6); COL4A1, COL4A1-related CSVD; COL4A2, COL4A2-related CSVD; RVCL, retinal vasculopathy with cerebral leukoencephalopathy.
### Supplementary Table 5. Comparison between mgCSVD including CADASIL and non-CADASIL mgCSVD and undetermined in Group 1

|                  | mgCSV D | CADASIL | Non-CADASIL mgCSV | Undetermined | p-value, 2 groups | p-value, 3 groups |
|------------------|---------|---------|-------------------|--------------|------------------|------------------|
| n                | n = 41  | n = 23  | n = 18            | n = 34       |                  |                  |

**Family History**

|                  | mgCSV D | CADASIL | Non-CADASIL mgCSV | Undetermined | p-value, 2 groups | p-value, 3 groups |
|------------------|---------|---------|-------------------|--------------|------------------|------------------|
|                  |         |         |                   |              |                  |                  |
| First relatives (%) | NA      | 25 (61.0), 0 | 15 (65.2), 0 | 10 (55.6), 0 | 9 (26.5), 0 | 0.0028 | 0.0095 |
| Second relatives (%) | NA      | 10 (24.4), 0 | 6 (26.1), 0 | 4 (22.2), 0 | 7 (20.6), 0 | 0.6954 | 0.8872 |
| First and/or second relatives (%) | NA      | 27 (65.9), 0 | 17 (73.9), 0 | 10 (55.6), 0 | 14 (41.2), 0 | 0.0326 | 0.0513 |
| Consanguity marriage of parents (%) | NA      | 3 (7.3), 0 | 2 (8.7), 0 | 1 (5.6), 0 | 3 (8.8), 0 | 0.8108 | 0.9082 |

**Risk factors**

|                  | mgCSV D | CADASIL | Non-CADASIL mgCSV | Undetermined | p-value, 2 groups | p-value, 3 groups |
|------------------|---------|---------|-------------------|--------------|------------------|------------------|
|                  |         |         |                   |              |                  |                  |
| HT (%) | NA      | 14 (34.1), 0 | 7 (30.4), 0 | 7 (38.9), 0 | 22 (64.7), 0 | 0.0084 | 0.0267 |
| DM (%) | NA      | 4 (9.8), 0 | 2 (8.7), 0 | 2 (11.1), 0 | 2 (5.9), 0 | 0.5382 | 0.7949 |
| DL (%) | NA      | 15 (36.6), 0 | 11 (47.8), 0 | 4 (22.2), 0 | 13 (38.2), 0 | 0.8831 | 0.2404 |
| Alcohol (%) | NA      | 2 (5.0), 1 | 2 (8.7), 0 | 0 (0), 1 | 4 (12.1), 1 | 0.2702 | 0.3336 |
| Smoking (%) | NA      | 16 (41.0), 2 | 10 (45.5), 1 | 6 (35.3), 1 | 15 (46.9), 2 | 0.621 | 0.7236 |

**Neurological symptoms/signs**

|                  | mgCSV D | CADASIL | Non-CADASIL mgCSV | Undetermined | p-value, 2 groups | p-value, 3 groups |
|------------------|---------|---------|-------------------|--------------|------------------|------------------|
|                  |         |         |                   |              |                  |                  |
| CI (MoCA-J ≤ 26) (%) | NA      | 35 (89.7), 2 | 20 (87.0), 0 | 15 (93.8), 2 | 29 (96.7), 4 | 0.2715 | 0.3951 |
| Dementia (%) | NA      | 28 (68.3), 0 | 14 (60.9), 0 | 14 (77.8), 0 | 21 (61.8), 0 | 0.5543 | 0.4439 |
| CI and/or dementia (%) | NA      | 37 (90.2), 0 | 20 (87), 0 | 17 (94.4), 0 | 32 (94.1), 0 | 0.5382 | 0.5632 |
| GD (%) | NA      | 23 (56.1), 0 | 9 (39.1), 0 | 14 (77.8), 0 | 24 (70.6), 0 | 0.1965 | 0.0173 |
| Stroke (%) | NA      | 25 (61.0), 0 | 15 (65.2), 0 | 10 (55.6), 0 | 20 (58.8), 0 | 0.8498 | 0.8071 |
| Migraine (%) | NA      | 9 (22.5), 1 | 3 (13), 0 | 6 (35.3), 1 | 8 (24.2), 1 | 0.8608 | 0.2541 |
| Pseudobulbar palsy (%) | NA      | 13 (33.3), 2 | 4 (18.2), 1 | 9 (52.9), 1 | 13 (38.2), 0 | 0.6626 | 0.0727 |

**Extraneurological symptoms/signs**

|                  | mgCSV D | CADASIL | Non-CADASIL mgCSV | Undetermined | p-value, 2 groups | p-value, 3 groups |
|------------------|---------|---------|-------------------|--------------|------------------|------------------|
|                  |         |         |                   |              |                  |                  |
| Alopecia (%) | NA      | 9 (23.7), 3 | 2 (9.1), 1 | 7 (43.8), 2 | 9 (29), 3 | 0.6148 | 0.0492 |
| Lumbago and/or spondylosis deformans (%) | NA      | 17 (43.6), 2 | 5 (23.8), 2 | 12 (66.7), 0 | 16 (47.1), 0 | 0.7664 | 0.0263 |
| Hemorrhage in fundus (%) | NA      | 2 (8.3), 17 | 1 (6.3), 7 | 1 (12.5), 10 | 2 (9.1), 12 | 0.7898 | 0.9168 |
## Brain imaging findings

|                | Fazekas grade of WMHs |                |                |                |
|----------------|-----------------------|----------------|----------------|----------------|
|                | Grade 3 (PVH) (%)     | Grade 3 (PVH)  | Grade 3 (PVH)  | Grade 3 (PVH)  |
|                | NA                    | NA             | NA             | NA             |
|                |                       | 37 (90.2) 0    | 21 (91.3) 0    | 16 (88.9) 0    | 32 (94.1) 0    |
|                |                       | 38 (92.7) 0    | 23 (100) 0     | 15 (83.3) 0    | 25 (73.5) 0    |
| Positive LI (%)| NA                    | NA             | NA             | NA             |
|                |                       | 36 (87.8) 0    | 21 (100) 0     | 15 (83.3) 0    | 23 (67.6) 0    |
| Multiple LI (%)| NA                    | NA             | NA             | NA             |
|                |                       | 40 (97.6) 0    | 23 (100) 0     | 17 (94.4) 0    | 32 (97.0) 1    |
| dPVS (%)       | NA                    | NA             | NA             | NA             |
|                |                       | 25 (61) 0      | 15 (65.2) 0    | 10 (55.6) 0    | 14 (41.2) 0    |
| Severe ECL (%) | NA                    | NA             | NA             | NA             |
|                |                       | 10 (24.4) 0    | 9 (39.1) 0     | 1 (5.6) 0      | 11 (32.4) 0    |
| Multiple MBs (%)| NA                    | NA             | NA             | NA             |
|                |                       | 29 (80.6) 5    | 17 (81.0) 2    | 12 (80.0) 3    | 19 (65.5) 5    |
| MBs            |                        |                |                |                |
|                | Positive MBs (%)      |                |                |                |
| Strict lobar (%)| NA                    | NA             | NA             | NA             |
|                |                       | 2 (5.6) 5      | 0 (0) 2        | 2 (13.3) 3     | 0 (0) 5        |
| Non lobar (%)  | NA                    | NA             | NA             | NA             |
|                |                       | 8 (22.2) 5     | 6 (28.6) 2     | 2 (13.3) 3     | 1 (3.4) 5      |
| Mean ± sd     |                        |                |                |                |
| Age of genetic test (years old) | 53.8 ± 10.1 | 56.1 ± 9.0 | 50.8 ± 10.9 | 50.4 ± 6.0 | 0.0457 | 0.0335 |
| Median (minimum-maximum) | 54 (32-79) | 57 (40-79) | 52.5 (32-69) | 51 (38-64) |

### Supplementary Table 5. Comparison between mgCSVD including CADASIL and non-CADASIL mgCSVD and undetermined in Group1

- Monogenic indicates the group of patients with monogenic cerebral small vessel disease; mgCSVD, monogenic cerebral small vessel disease; CADASIL, cerebral
autosomal dominant arteriopathy with subcortical infarct and leukoencephalopathy;
Undetermined, the group of patients with undetermined cause; HT, hypertension; DM, diabetes mellitus; DL, dyslipidemia; MoCA-J, Montreal cognitive assessment battery in Japanese edition; CI, cognitive impairment; GD, gait disturbance; WMH, white matter hyperintensity; PVH, periventricular hyperintensity; DSWMH, deep and subcortical white matter hyperintensity; LI, lacunar infarction; dPVS, dilated perivascular spaces; ECL, external capsular lesion; ATL, anterior temporal lesion; MBs, microbleeds; sd, standard deviations; NA, number of patients who was not available.
Supplementary Table 6. Logistic regression analysis to investigate association of mgCSVD in Group1 and clinical/imaging findings by using stepwise methods

| Items                          | Odds ratio | 95% Confidence interval | p-value |
|-------------------------------|------------|-------------------------|---------|
| **Family history**            |            |                         |         |
| first relatives               | 1.3325     | 1.0914-1.6268           | 0.0055  |
| **Risk factors**              |            |                         |         |
| HT                            | 0.7408     | 0.6031-0.9098           | 0.0048  |
| DM                            | 1.5370     | 1.0496-2.2508           | 0.0278  |
| **Neurological symptoms/signs** |           |                         |         |
| Gait disturbance              | 0.7470     | 0.5953-0.9372           | 0.0125  |
| **Neuroimaging findings**     |            |                         |         |
| Multiple LIs                  | 1.4184     | 1.1069-1.8177           | 0.0064  |
| severe ECLs                   | 1.1866     | 0.9574-1.4708           | 0.1163  |

mgCSVD indicates monogenic cerebral small vessel disease; HT, hypertension; DM, diabetes mellitus; LI, lacunar infarction; ECL, external capsular lesion.
Supplementary Table 7. Classification of CSVD patients in Group1 by using results of decision tree

| Items of classification | First relatives | Negative | Positive |
|-------------------------|----------------|----------|----------|
|                         | HT             | Positive | Negative | Positive |
| Age of onset of stroke, GD and/or CI/dementia | - | > 43 years old | ≤ 43 years old | - |
| Diagnosis               |                |          |          |          |
| CADASIL (%)             | 3 (15.0)       | 3 (33.3) | 2 (16.7) | 15 (44.1) |
| Heterozygous HTRA1 (%)  | 1 (5.0)        | 0        | 1 (8.3)  | 6 (17.6)  |
| CARASIL (%)             | 0              | 0        | 2 (16.7) | 0         |
| PXE (%)                 | 0              | 0        | 1 (8.3)  | 2 (5.9)   |
| Heterozygous ABCC6 (%)  | 0              | 0        | 2 (16.7) | 0         |
| COL4A1 (%)              | 0              | 0        | 1 (8.3)  | 0         |
| COL4A2 (%)              | 0              | 0        | 0        | 1 (2.9)   |
| RVCL (%)                | 0              | 0        | 0        | 1 (2.9)   |
| Undetermined (%)        | 16 (80.0)      | 6 (66.7) | 3 (25.0) | 9 (26.5)  |

Supplementary Table 7. Classification of CSVD patients in Group1 by using results of decision tree

CSVD indicated cerebral small vessel disease; HT, hypertension; GD, gait disturbance;
CI, cognitive impairment; CADASIL, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; Heterozygous HTRA1, heterozygous high-temperature requirement A serine peptidase 1 (HTRA1)-related CSVD,
CARASIL, cerebral autosomal recessive arteriopathy with subcortical infarcts and
leukoencephalopathy; PXE, pseudoxanthoma elasticum; Heterozygous ABCC6,

heterozygous mutation in ATP Binding Cassette Subfamily C Member 6 (ABCC6);

COL4A1, COL4A1-related CSVD; COL4A2, COL4A2-related CSVD; RVCL, retinal vasculopathy with cerebral leukoencephalopathy.
## Supplementary Table 8. Comparison between mgCSVD and undetermined among patients with age of onset > 55 years old (Group2)

|                        | Monogenic | Undetermined | mgCSVD vs Undetermined |
|------------------------|-----------|--------------|------------------------|
| **n**                  | n=9       | n=22         |                        |
| **Family History**     |           |              |                        |
| First relatives (%)    | 8 (88.9), 0 | 17 (77.3), 0 | 0.4574                |
| Second relatives (%)   | 5 (55.6), 0 | 11 (50), 0   | 0.7787                |
| First and/or second relatives (%) | 9 (100), 0 | 22 (100), 0 | 0                  |
| **Consanguity marriage of parents (%)** | 0 (0), 0 | 4 (18.2), 0 | 0.1705                |
| **Male (%)**           | 6 (66.7), 0 | 10 (45.5), 0 | 0.2834                |
| **Risk factors**       |           |              |                        |
| HT (%)                 | 4 (44.4), 0 | 17 (77.3), 0 | 0.0759                |
| DM (%)                 | 1 (11.1), 0 | 4 (18.2), 0   | 0.6271                |
| DL (%)                 | 5 (55.6), 0 | 5 (22.7), 0   | 0.0759                |
| Alcohol (%)            | 1 (12.5), 1 | 5 (22.7), 0   | 0.5537                |
| Smoking (%)            | 2 (25.0), 1 | 8 (36.4), 0   | 0.5993                |
| **Neurological symptoms/signs** |          |              |                        |
| CI (MoCA-J ≤ 26) (%)   | 6 (75.0), 1 | 16 (84.2), 3 | 0.5737                |
| Dementia (%)           | 5 (55.6), 0 | 14 (63.6), 0  | 0.675                 |
| CI and/or dementia (%) | 7 (77.8), 0 | 20 (90.9), 0  | 0.3222                |
| GD (%)                 | 5 (55.6), 0 | 17 (77.3), 0  | 0.2266                |
| Stroke (%)             | 6 (66.7), 0 | 8 (36.4), 0   | 0.1238                |
| Migraine (%)           | 2 (22.2), 0 | 5 (22.7), 0   | 0.9756                |
| Pseudobulbar palsy (%) | 3 (33.3), 0 | 6 (27.3), 0   | 0.7358                |
| **Extraneurological symptoms/signs** |          |              |                        |
| Alopecia (%)           | 2 (22.2), 0 | 1 (5.0), 2    | 0.1589                |
| Condition                        | Group 1 | Group 2 | p-value |
|---------------------------------|---------|---------|---------|
| Lumbar and/or spondylolisthesis deformans (%) | 4 (44.4), 0 | 9 (46.9), 0 | 0.8563 |
| Hemorrhage in fundus (%)        | 1 (33.3), 0 | 0 (0), 0 | 0.0929 |
| Brain imaging findings          |         |         |         |
| Fazekas grade of WMHs           |         |         |         |
| Grade 3 (PVH) (%)               | 9 (100), 0 | 22 (100), 0 | - |
| Grade III (DWMH) (%)            | 9 (100), 0 | 21 (95.5), 0 | 0.5156 |
| LI                              |         |         |         |
| Positive LI (%)                 | 8 (88.9), 0 | 17 (77.3), 0 | 0.4574 |
| at semiovale (%)                | 8 (88.9), 0 | 10 (45.5), 0 | 0.0261 |
| at BG (%)                       | 4 (44.4), 0 | 10 (45.5), 0 | 0.9991 |
| at thalamus (%)                 | 3 (33.3), 0 | 10 (45.5), 0 | 0.5347 |
| at brainstem (%)                | 2 (22.2), 0 | 4 (18.2), 0 | 0.7961 |
| at cingulate (%)                | 2 (22.2), 0 | 0 (0), 0 | 0.0223 |
| Multiple LIs (%)                | 7 (77.8), 0 | 14 (63.6), 0 | 0.4445 |
| dPVS (%)                        | 9 (100), 0 | 22 (100), 0 | - |
| Severe ECL (%)                  | 6 (66.7), 0 | 12 (54.5), 0 | 0.5347 |
| Severe ATL (%)                  | 1 (11.1), 0 | 3 (13.6), 0 | 0.8449 |
| MIBs                            |         |         |         |
| Positive MIBs (%)               | 6 (75.0), 1 | 14 (73.7), 3 | 0.9432 |
| Multiple MIBs (%)               | 5 (62.5), 1 | 8 (42.1), 3 | 0.3328 |
| Strict lobar (%)                | 0 (0), 1 | 2 (10.5), 3 | 0.3403 |
| Non lobar (%)                   | 1 (12.5), 1 | 3 (15.8), 3 | 0.8261 |
| Age of genetic test (years old) |         |         |         |
| mean ± sd                       | 66.1 ± 5.4 | 65.7 ± 6.3 | 0.7102 |
| median (minimum-maximum)        | 66 (57-73) | 64 (56-79) |         |
| Age of onset of stroke, GD and/or CI/dementia (years old) |         |         |         |
| mean ± sd                       | 61.1 ± 4.6 | 61.5 ± 3.9 | 1 |
| median (minimum-maximum)        | 58 (57-68) | 60.5 (56-79) |         |
Supplementary Table 8. Comparison between mgCSVD and undetermined among patients with age of onset > 55 years old (Group2)

CADASIL indicates cerebral autosomal dominant arteriopathy with subcortical infarct and leukencephalopathy; HT, hypertension; DM, diabetes mellitus; DL, dyslipidemia; MoCA-J, Montreal cognitive assessment battery in Japanese edition; CI, cognitive impairment; GD, gait disturbance; WMH, white matter hyperintensity; PVH, periventricular hyperintensity; DSWMH, deep and subcortical white matter hyperintensity; LI, lacunar infarction; dPVS, dilated perivascular spaces; ECL, external capsular lesion; ATL, anterior temporal lesion; MBs, microbleeds; sd, standard deviations; NA, number of patients who was not available.
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SUPPLEMENTARY FIGURES

Supplementary figure 1

Total variants detected by WES: 110056

Exclude variants with MAF > 0.01

Remaining variants: 16573

Exclude the intronic variants

Remaining variants: 8103

Exclude the synonymous variants

Remaining variants: 6810

Investigate mutations of causative genes
FOXC1, PITX2, COL4A1, COL4A2, CTSA, GLA,
CECR1, ABCC6, NF1, CBS, IKBK, TREX1,
COLGALT1 and c-terminus of LAMB1

Four mutations were identified

| Gene    | Mutations               | SNP ID    | ClinVar         |
|---------|-------------------------|-----------|-----------------|
| FOXC1   | c.1359_1361dupCGG, p.G454dup | rs398123612 | Benign/Likely Benign |
| COL4A2  | c.4441G>T, p.R1472C       | rs779703009 | Benign          |
| ABCC6   | c.2542delG, p.M848fs     | rs67978306  | Pathogenic      |
| ABCC6   | c.410>G>A, p.T14X         | NA        | Not reported    |

Supplementary Figure 1. Workflow of identifying causative mutations detected by WES.
First, we excluded detected variants with a MAF > 0.01. Variants with intronic and synonymous variants were excluded from the remaining variants. We investigated mutations in the causative genes among the remaining variants. Finally, we evaluated the pathogenicity of each mutation using the ClinVar website or previous reports. This figure shows the results of WES for the patient with PXE. We identified four mutations among the causative genes of CSVD. Two of these were benign or likely benign in ClinVar. p.M848fs mutation in ABCC6 is the most frequent causative mutations of PXE in Japan.4
Supplementary Figure 2. Protease activity of each mutant HTRA1 protein

(A) Protease activities of missense HTRA1s identified in this study. Activities were calculated from the slope of the linear portion of normalized fluorescence vs. time plots. The boxplots show the values from two independent experiments of three samples of each protein. WT and S328A indicate the positive and negative controls, respectively. Protease activities were statistically compared between the WT and the other three HTRA1s with an ANOVA followed by the Bonferroni correction. ***p-value < 0.0001 for protease activities of each HTRA1 relative to WT.
(B) SDS-PAGE of WT and missense mutant HTRA1 proteins used in the protease assay. Black arrows indicate the full-length band of HTRA1 tagged with myc-His6.
Supplementary Figure 3. ABCC6 exon 25 CNVs

The copy number of ABCC6 exon 25 is shown as a bar graph. The vertical axis indicates the copy number of exon 25 in ABCC6. Green and red bars indicate the copy number of exon 25 of ABCC6 in a patient with decreased copy numbers of 7–10, 16–17, and 21–25 of ABCC6 and controls. Mean values from three samples for each ddPCR are shown. I-bars indicate the standard error (SE).