Original research

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 TREX1 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,1 causes dementia or gait disturbance (GD).2 Although ageing and hypertension (HT) are CSVD risk factors,3,4 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.5,6 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

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFr domains</td>
<td>10</td>
<td>12</td>
<td>24</td>
<td>34</td>
</tr>
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<td>Sex</td>
<td>Male</td>
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<td>Male</td>
</tr>
<tr>
<td>Family history</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
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<td>First relatives</td>
<td>Parents</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Children</td>
<td>None</td>
<td>NA</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Second relatives</td>
<td>Grandparents</td>
<td>NA</td>
<td>NA</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Sisters/brothers</td>
<td>None</td>
<td>NA</td>
<td>Positive</td>
</tr>
<tr>
<td>Neurological symptoms/signs</td>
<td>Stroke (years old)</td>
<td>47</td>
<td>None</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>CI/Dementia (years old)</td>
<td>47</td>
<td>48</td>
<td>79</td>
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<tr>
<td></td>
<td>GD (years old)</td>
<td>None</td>
<td>None</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Migraine</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>MRI findings</td>
<td>WMHs (Fazekas grade)</td>
<td>3 and III</td>
<td>3 and III</td>
<td>3 and III</td>
</tr>
<tr>
<td></td>
<td>LI</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>ECL†‡</td>
<td>Moderate</td>
<td>Severe</td>
<td>Severe</td>
</tr>
<tr>
<td></td>
<td>ATL</td>
<td>Early confluent</td>
<td>Confluent</td>
<td>None</td>
</tr>
<tr>
<td>Pathological findings</td>
<td>GOM</td>
<td>NA</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

*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. 
‡Mild: ≤1/4 of the EC; Moderate: >1/4 and ≤1/2 of the EC; Severe: >1/2 of the EC. 

Table 2 Summary of diagnosis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Group 1 (age of onset of neurological symptoms/signs ≤55 years old)</th>
<th>Group 2 (age of onset of neurological symptoms/signs &gt;55 years old with family history)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (%)</td>
<td>Positive (n=41)</td>
<td>Negative (n=34)</td>
</tr>
<tr>
<td>CADASIL</td>
<td>23 (30.7)</td>
<td>17 (11.6)</td>
</tr>
<tr>
<td>Heterozygous HTRA1</td>
<td>8 (10.7)</td>
<td>6 (9.6)</td>
</tr>
<tr>
<td>CARASIL</td>
<td>2 (2.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PKE</td>
<td>3 (4.0)</td>
<td>2 (4.6)</td>
</tr>
<tr>
<td>Heterozygous ABC6</td>
<td>2 (2.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>COL4A1</td>
<td>1 (1.3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>COL4A2</td>
<td>1 (1.3)</td>
<td>1 (2.4)</td>
</tr>
<tr>
<td>RVCL</td>
<td>1 (1.3)</td>
<td>1 (2.4)</td>
</tr>
<tr>
<td>Undetermined</td>
<td>34 (44.3)</td>
<td>14 (29.3)</td>
</tr>
</tbody>
</table>

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 exomeuclease 1 (TREXI) was also performed. The primer set for these three genes has been previously reported elsewhere. 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, COL4A1, COL4A2, CTSA, GLA, CECR1, ABC6C, NF1, CBS, IKBKG, TREX1, COL4A1, COL4A2, ABCC6, LAMB1, COL4A1-COL4A2, ABCC6, and FOXC1. 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 ABC6C has been previously reported.

In addition, we also investigated COL4A1, COL4A2, ABCC6 and HTRA1 copy number variants (CNVs) in undiagnosed patients and patients with heterogeneous mutations in ABC6C.

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.

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In addition, we also investigated COL4A1, COL4A2, ABCC6 and HTRA1 copy number variants (CNVs) in undiagnosed patients and patients with heterogeneous mutations in ABC6C.

Cerebrovascular disease

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 exomeuclease 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.

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In addition, we also investigated COL4A1, COL4A2, ABCC6 and HTRA1 copy number variants (CNVs) in undiagnosed patients and patients with heterogeneous mutations in ABC6C.

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, dPVS, 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 cognitive impairment (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.
75 CSVD patients with age at onset ≤ 55 y.o.

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 infarct and leukoencephalopathy; CARASIL, cerebral autosomal recessive arteriopathy with subcortical infaracts and leukoencephalopathy; COLA41, COLA4A1-related CSVD; COLA42, COLA42-related CSVD; heterozygous ABC6, 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.

and COLA4A2 using copy number estimation by a Mixture of PoissonS (cn.MOPS) in R software (V.4.1.0). The identified CNVs were verified through a droplet digital PCR (ddPCR) (online supplementals methods and results).

Classifying the identified mutations
Computational analysis of the identified mutations was performed using PolyPhen2, SIFT and Provean on the VaProS website (http://p4d-info.nig.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.

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

<table>
<thead>
<tr>
<th>Family history</th>
<th>Undetermined vs non-CADASIL</th>
<th>Undetermined vs CADASIL</th>
<th>Non-CADASIL vs CADASIL</th>
</tr>
</thead>
<tbody>
<tr>
<td>First relatives</td>
<td>0.1357</td>
<td>0.0179</td>
<td>0.7477</td>
</tr>
<tr>
<td>Risk factors</td>
<td>HT</td>
<td>0.1759</td>
<td>0.0468</td>
</tr>
<tr>
<td>Neurological symptoms/signs</td>
<td>GD</td>
<td>0.746</td>
<td>0.0570</td>
</tr>
<tr>
<td>Extraneurological symptoms/signs</td>
<td>Alopecia</td>
<td>0.3468</td>
<td>0.1943</td>
</tr>
<tr>
<td></td>
<td>Spondylosis deformans or lumbago</td>
<td>0.2452</td>
<td>0.1948</td>
</tr>
<tr>
<td>Neuroimaging findings</td>
<td>LI</td>
<td>0.5075</td>
<td>0.0233</td>
</tr>
<tr>
<td></td>
<td>Severe ATL</td>
<td>0.0780</td>
<td>0.7778</td>
</tr>
<tr>
<td></td>
<td>Strict lobar distribution of MBs</td>
<td>0.3182</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Non lobar distribution of MBs</td>
<td>0.4241</td>
<td>0.0452</td>
</tr>
</tbody>
</table>

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.

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. Multiple MBs were defined as more than four, and the distribution of MBs was classified as strictly lobar or non-lobar. Severe ECLs were defined as the length of hyperintensity in more than half of the external capsule on T2WI/FLAIR images. A severe anterior temporal lesion (ATL) was defined as confluent hyperintensity in the anterior temporal lobe on T2WI/FLAIR images. LIs, dPVS and MBs were defined according to the Standards for Reporting Vascular Changes on Neuroimaging.
stepwise methods to calculate adjusted ORs with 95% CIs were performed using R (V4.1.2). CSVD patients were classified with the items with no missing values using a decision tree of statistics and a machine learning toolbox on MATLAB R2020b Update 3 (9.9.0.1538559). 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).22 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 ABCC6 throughout Japan (online supplemental table 1). Among them, we recruited 109 patients from 70 neurological centres (online supplemental table 1). All four patients had early onset (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.9 24 Genetic tests for HTRA1 revealed two patients with CARASIL and nine with heterozygous HTRA1 mutations.10 23 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 mutants was significantly decreased (online supplements 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.26 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.MOPS17 to examine COL4A1, COL4A2, ABCC6 and HTRA1 CNVs in 52 undiagnosed patients and three patients with non-CADASIL mgCSVD mutations in ABCC6 or COL4A2. Binary alignment map file of five undiagnosed patients were unavaiable. 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. One patient had a heterozygous mutation (p.M848fs) in ABCC6. 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.23 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.9 24 Genetic tests for HTRA1 revealed two patients with CARASIL and nine with heterozygous HTRA1 mutations.10 23 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 mutants was significantly decreased (online supplements methods and results and figure 2).

Table 4 Summary of genetic mutations identified among patients with leukoencephalopathy

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Severe WMHs with CSVD, n (%)</th>
<th>Progressive neurological syndrome with WMHs, n=100</th>
<th>Younger onset of cognitive decline with WMHs, n=45</th>
<th>CI with WMHs, n=35</th>
<th>Leukoencephalopathy, n=60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total patients with mutation, n (%)</td>
<td>50 (47.2)</td>
<td>21 (21.0)</td>
<td>20 (44.4)</td>
<td>14 (40.0)</td>
<td>12 (20.0)</td>
</tr>
<tr>
<td>Patients with CSVD-related gene mutation, n (%)</td>
<td>50 (47.2)</td>
<td>5 (5.0)</td>
<td>19 (42.2)</td>
<td>7 (20.0)</td>
<td>8 (13.3)</td>
</tr>
<tr>
<td>NOTCH3</td>
<td>30 (28.3)</td>
<td>4 (4.0)</td>
<td>17 (37.8)</td>
<td>2 (5.7)</td>
<td>7 (11.7)</td>
</tr>
<tr>
<td>HTRA1</td>
<td>11 (10.4)</td>
<td>–</td>
<td>2 (4.4)</td>
<td>1 (2.9)</td>
<td>–</td>
</tr>
<tr>
<td>ABC6</td>
<td>6 (5.7)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>COL4A1</td>
<td>1 (0.9)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>COL4A2</td>
<td>1 (0.9)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TREX1</td>
<td>1 (0.9)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CTSA</td>
<td>0 (0)</td>
<td>1 (1.0)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GLA</td>
<td>0 (0)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>ITM28</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1 (2.9)</td>
<td>–</td>
</tr>
</tbody>
</table>

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.
In cases with a family history, including those with onset over 55 years of age, 50.0% had mgCSVD. This included 66.7% with CADASIL, 19.4% with HTRA1 mutations and 8.3% with ABCC6 mutations.

**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.

**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’s age of onset of neurological symptoms/signs is >55 years. Furthermore, genetic tests should terminate if the patient has an age of onset of neurological symptoms/signs >43 years and a negative family history of first relatives. Additional genetic tests for COL4A1, COL4A2 or WES should be performed for the remaining CSVD patients. 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 ABC6, heterozygous mutation in ATP binding cassette subfamily C member 6 (ABCC6); mgCSVD, monogenic cerebral small vessel disease; heterozygous HTRA1, heterozygous high-temperature requirement A serine peptidase 1-related CSVD; PXE, pseudoxanthoma elasticum; RVCL, retinal vasculopathy with cerebral leukoencephalopathy; WES, whole-exome sequencing. (-), negative; (+), positive.

Compared with the undetermined patients, the monogenic group had a significantly higher frequency of a family history of first relatives (61.0% vs 25.5%, 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 ABC6-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.

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. The HTRA1 mutations have also been described as risk factors for sporadic CSVD. These findings suggest that the contribution of HTRA1 to CSVD may be higher than that previously thought, especially in Japan. In addition, ABC6 is the third most commonly mutated gene in patients with severe CVSD. We identified three cases of biliary-tract stones and multiple LIs may be useful in hypothesising an mgCSVD generation 2). First, we recommend NOTCH3, HTRA1 and ABC6 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. In addition, we did not evaluate the pathogenicity of COL4A2 mutation such as using skin biopsy. 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. 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 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 ABC6 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


