Original Investigation

A Novel Mutation in ELOVL4 Leading to Spinocerebellar Ataxia (SCA) With the Hot Cross Bun Sign but Lacking Erythrokeratodermia A Broadened Spectrum of SCA34

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**IMPORTANCE** Although mutations in 26 causative genes have been identified in the spinocerebellar ataxias (SCAs), the causative genes in a substantial number of families with SCA remain unidentified.

**OBJECTIVE** To identify the causative gene of SCA in 2 Japanese families with distinct neurological symptoms and radiological presentations.

**DESIGN, SETTING, AND PARTICIPANTS** Clinical genetic study at a referral center of 11 members from 2 Japanese families, which started in 1997.

**MAIN OUTCOMES AND MEASURES** Results of neurological examinations and radiological evaluations. The causative mutation was identified using genome-wide linkage analysis and next-generation sequencing.

**RESULTS** Affected members (9 of 11 members [81.8%]) showed slowly progressive cerebellar ataxia (all 9 members [100%]), ocular movement disturbance (all 9 members [100%]), and pyramidal tract signs (8 of 9 members [88.9%]) with an age at onset between the second and sixth decades of life. Besides cerebellar and pontine atrophy, magnetic resonance imaging of the brain revealed the hot cross bun sign (4 of 6 members [66.7%]), pontine midline linear hyperintensity (2 of 6 members [33.3%]), or high intensity in the middle cerebellar peduncle (1 of 6 members [16.7%]), which are all reminiscent of multiple system atrophy in tested patients. Using linkage analysis combined with exome and whole-genome sequencing, we identified a novel heterozygous mutation in the ELOVL fatty acid elongase 4 (ELOVL4) gene (c.736T>G, p.W246G) in both families. Haplotype analysis indicated that it was unlikely that these 2 Japanese families shared a common ancestor. Although a missense mutation in ELOVL4 (c.504G>C, p.L168F) was recently reported to be associated with SCA with erythrokeratodermia variabilis (SCA34) in a French-Canadian family, signs of erythrokeratodermia variabilis were absent in our families.

**CONCLUSIONS AND RELEVANCE** Combined with the results of the family with SCA34 reported previously, this report confirms that mutations in ELOVL4 can cause dominantly inherited neurodegeneration severely affecting the cerebellum and brainstem. We should be aware that the presence of multiple system atrophy-like features on magnetic resonance imaging scans, together with cerebellar and brainstem atrophy, suggests SCA34, even when erythrokeratodermia variabilis is absent. The present study further broadened the spectrum of the clinical presentations of SCA34 associated with mutations in ELOVL4, which is involved in the biosynthesis of very long-chain fatty acids.

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Spinocerebellar ataxias (SCAs) are autosomal dominant neurodegenerative disorders that show progressive cerebellar ataxia, often associated with various phenotypes of neurological dysfunction, such as ocULAR movement disturbances, pyramidal tract or extrapyramidal signs, and peripheral neuropathy. Twenty-six causative genes of SCAs have been described to date, some of which have been identified by exome or whole-genome sequencing in combination with linkage analysis. However, causative mutations in a significant number of families with SCA still remain to be identified.

Herein we report on 2 Japanese families with SCA whose members present with a slowly progressing gait ataxia, ocular movement disturbances (such as horizontal gaze nystagmus and supranuclear gaze palsy), dysarthria, and pyramidal tract signs. Magnetic resonance imaging (MRI) of affected family members showed not only cerebellar atrophy but also marked pontine atrophy with the hot cross bun sign or pontine midline linear hyperintensity, resembling multiple system atrophy. Using genome-wide linkage analysis and exome and whole-genome sequencing, we identified a novel mutation in the ELOVL fatty acid elongase 4 (ELOVL4) gene (c.736T>G, p.W246G) that segregated with the disease in these 2 families.

Methods

Participants
We first studied family A (Figure 1), with 7 of 9 members who have slowly progressing ataxia, pyramidal tract signs, and cerebellar and pontine atrophy detected on MRI scans of their brains. We conducted neurological and dermatological examinations for all 9 family members of family A (II-1, II-2, II-3, II-4, III-1, III-2, III-3, III-4, and III-5), extensive ophthalmologic examinations for only 2 affected family members (II-1 and III-1), and electrophysiological examinations for only 1 affected family member (II-1). A neurological examination was also conducted for 2 additional participants from another Japanese family (II-1 and III-1 in family B) with similarly similar clinical and radiological features. These 2 families are unrelated and originated from distant regions of Japan.

Our study was approved by the local ethics committee of Tokyo Medical and Dental University, the University of Tokyo, and Yokohama City University. After obtaining written informed consent from the 11 members of the 2 families, blood samples were obtained, and genomic DNA extracted, using standard protocols. The initial screening by genetic testing excluded SCA types 1, 2, 3, 6, and 31 and denatatorubral-pallidolysian atrophy in these 2 families. Plasma levels of very long-chain fatty acids (VLCFAs; represented by the ratios of C24:0 to C22:0, C25:0 to C22:0, and C26:0 to C22:0 [where C24:0 denotes a saturated fatty acid with C24 carbon chain length]) were measured in 2 affected family members in family A (II-1 and III-1) by use of gas chromatography (SRL Inc).

Linkage Analysis
Genome-wide single-nucleotide polymorphism (SNP) genotyping, using the Genome-Wide Human SNP 6.0 Array (Affymetrix), was performed on the genomic DNA of members II-1, II-2, II-3, II-4, III-1, III-2, and III-3 in family A. Experimental procedures were performed according to the manufacturer’s instructions. Acquired data (.cgh files) were further processed by use of the high-throughput linkage analysis system SNP HitLink, and parametric linkage analysis was performed by assuming autosomal dominant inheritance using Merlin8 (eAppendix in the Supplement).

Evaluation of Copy Number Variations
The Genome-Wide Human SNP 6.0 Array data were used to detect copy number variations with PennCNV (an integrated hidden Markov model designed for detecting high-resolution copy number variations in whole-genome SNP genotyping data) according to the manual’s default settings. The copy number variations detected were then filtered, with the candidate regions determined by the linkage analysis.

Exome and Whole-Genome Sequencing, Data Processing, and Validation
Genomic DNA from 3 affected members in family A (II-1, III-1, and III-2) were further subjected to exome sequencing using exome capture kits (SureSelect Human All Exon kit; Agilent Technologies) and next-generation sequencers (HiSeq2000; Illumina). Whole-genome sequencing was performed on the genomic DNA of 1 affected member in family A (III-1) using HiSeq2000. The acquired reads were mapped by use of the Burrows-Wheeler Alignment tool10 to human genome GRCh37, and the calling of single-nucleotide variations (SNVs) was performed using SAMtools.11 The SNVs were filtered to exclude known variants in several databases: dbSNP (build 135), 1000 Genomes, National Heart, Lung, and Blood Institute Exome Sequencing Project, and HapMap. The SNVs that were either in segmental duplicated regions...
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Results

Clinical Evaluation

A summary of the clinical characteristics of the patients in the 2 Japanese families (as well as of patients in French-Canadian2,24 and European2 families) are shown in our Table, and detailed neurological and radiological findings of each examined patient are described in eTable 1 in the Supplement. All the 9 affected members in the 2 families (7 in family A and 2 in family B) showed slowly progressive gait ataxia as the cardinal manifestation. The mean age at onset was 33.9 years (range, 13-56 years). All the affected members subsequently developed dysarthria.

Disease progression was very slow, and it was not until the age of 60 years or older that affected members required a cane or walker. On neurological examination, truncal and limb ataxia and atactic dysarthria were observed in all 9 affected family members. Horizontal gaze nystagmus was observed in 7 affected members (77.8%), and supranuclear ophthalmoplegia, more obvious in the vertical than horizontal direction, was observed in 3 affected members (33.3%) (II-1, II-3, and II-4 in family A). Pyramidal tract signs, such as elevated deep tendon reflexes in the limbs, or positive Babinski signs were observed in 8 affected members (88.9%). Autonomic symptoms, such as bladder disturbance (44.4%) and constipation (22.2%), were also observed, but none of the affected members had obvious orthostatic hypotension. Notably, we could not find any present evidence or history of skin lesions characteristic of erythrokeratodermia variabilis (EKV) in any of the 9 affected members. The MRI scans of the brains of the 6 affected members who could be investigated (II-1, II-4, III-1, III-4, and III-5 in family A and III-1 in family B) all showed cerebellar and marked pontine atrophy (Figure 2A-H). It is noteworthy that axial T2-weighted images showed either cruciform hyperintensity (which is referred to as the hot cross bun sign) in 4 of these 6 affected members (66.7%) (II-1, III-1, and III-5 in family A and III-1 in family B) or pontine midline linear hyperintensity in the other 2 affected members (33.3%) (II-4 and III-4 in family A) (Figure 2I-M), both of which often appear in patients with multiple system atrophy.25-27

Furthermore, high intensity in the middle cerebellar peduncle, another radiological feature of multiple system atrophy, was observed in 1 of the 6 affected family members (16.7%) (II-4 in family A) on a fluid-attenuated inversion recovery MRI scan (Figure 2N). Extensive ophthalmologic evaluations, which included a visual acuity test, a color sensation test, and fundoscopy, revealed no abnormalities in 2 affected members (II-1 and III-1 in family A). The results of the Goldmann perimeter test, which was only performed for member III-1 in family A, were normal. A nerve conduction study performed for 1 affected member (II-1 in family A) showed normal conduction velocity and amplitude of compound motor action potentials in the median and tibial nerves and of sensory nerve action potentials in the median and sural nerves. The motor evoked potentials and somatosensory evoked potentials were normal for the upper and lower extremities.

Linkage Analysis, Exome Sequencing, and Validation

A genome-wide linkage analysis of 7 members (II-1, II-2, II-3, II-4, III-1, III-2, and III-3 in family A) revealed multiple chromosomal regions of possible genetic linkage with log of odds scores higher than 1.45, with total lengths of 33.4, 34.7, 58.7, and 18.0 megabases, respectively. We next performed exome sequencing of 3 affected members (II-1, III-1, and III-2 in family A) and whole-genome sequencing of 1 member (III-1 in family A) (eTable 2 in the Supplement), to detect candidate mutations within the above-mentioned candidate regions determined by the linkage analysis.

Data from whole-genome sequencing were used to detect variants in the coding sequences and rare variants outside the coding sequences for haplotyping. After selecting variants within the candidate regions, we excluded variants already recorded as SNPs or those within regions of segmental duplication. The selection of variants present in at least 2 affected members resulted in 4 novel nonsynonymous heterozygous SNVs in GCFC2 (NM_001203134.1; c.1298G>A; p.S433N), ELOVL4 (NM_022726.3; c.736T>G; p.W246S), ZBTB24 (NM_014797.2; c.1216C>T; p.P406S), and ENPP3 (NM_005021.3; c.1787C>T; p.T596I) remaining as candidates.

Analysis of copy number variations using SNP array data obtained from family A did not reveal any novel copy number variations that cosegregate with the disease. We further had the opportunity to analyze 2 more affected members of
family A (III-4 and III-5), and we tested them for the cosegregation of each of the 4 candidate SNVs. This revealed that the novel variant identified in exon 6 of \textit{ELOVL4} (c.736T>G, p.W246G) was the only SNV cosegregating with the disease in family A (Figure 1 and Figure 3A). We next investigated whether the same or allelic variant of \textit{ELOVL4} was present in the 2 affected members of family B (II-1 and III-1) because these members showed clinical and MRI findings highly similar to those of the affected members of family A. Sanger sequencing confirmed the same heterozygous mutation in \textit{ELOVL4} (c.736T>G, p.W246G). We screened 513 healthy controls in the extended Japanese in-house exome database and confirmed that none of them harbored this mutation.

To investigate whether the mutation identified in the 2 families (c.736T>G, p.W246G) shares a common ancestral origin, we analyzed the haplotypes around the \textit{ELOVL4} mutation locus in the 2 families. We used rare (mean allele frequency <0.10) SNVs identified by whole-genome sequencing in member III-1 of family A, and we reconstructed the haplotypes in the 2 families. Haplotyping around the \textit{ELOVL4} locus suggested that the 2 families are unlikely to share a common ancestor (eFigure in the Supplement) because the haplotype of a region of at least 211 kilobases in length around the \textit{ELOVL4} locus was not shared by the 2 families.

The bioinformatics functional prediction of the p.W246G mutation indicated that the amino acid residue W246 is highly conserved and that the mutation is damaging (Polyphen-2: 0.963 [damaging], SIFT: 0.000 [damaging], MutationTaster: 1.000 [damaging], and likelihood ratio test: 1.000 [damaging]; Figure 3B). We measured VLCFA contents in the blood samples obtained from 2 affected members (II-1 and III-1 in family A) and found that the ratios of C24:0 to C22:0, C25:0 to C22:0, and C26:0 to C22:0 were normal.

### Table. Comparison of Clinical Characteristics Among Patients With Mutations in \textit{ELOVL4} and \textit{ELOVL5}

| Characteristic | Source | Our Study | Giroux et al. 
2017; Cardieux-Dion et al. 
2014 | Di Gregorio et al. 
2014 |
<table>
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<tr>
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<tbody>
<tr>
<td>Patients</td>
<td>Japanese with SCA</td>
<td>French-Canadian family with Ataxia and EKV (original family with SCA34)</td>
<td>European families with SCA3B</td>
<td></td>
</tr>
<tr>
<td>Mutation</td>
<td>\textit{ELOVL4} (c.736T&gt;G, p.W246G); heterozygous</td>
<td>\textit{ELOVL4} (c.504G&gt;C, p.L168F); heterozygous</td>
<td>\textit{ELOVL5} (p.G230V; cosegregated in 3 families; p.L74V; cosegregation to be confirmed)</td>
<td></td>
</tr>
<tr>
<td>Mean or approximate age at onset of gait disturbance, y</td>
<td>33.9</td>
<td>51</td>
<td>~40</td>
<td></td>
</tr>
<tr>
<td>Progression</td>
<td>Very slow (required cane or walker in 60s or later)</td>
<td>Very slow (required wheelchair in 70s)</td>
<td>Very slow (required cane in 50s)</td>
<td></td>
</tr>
<tr>
<td>Ataxia</td>
<td>Gait and limb ataxia and dysarthria (100.0%)</td>
<td>Gait ataxia (12 of 19 members [63.2%]); limb ataxia and dysarthria also observed</td>
<td>Gait ataxia and dysarthria (100.0%)</td>
<td></td>
</tr>
<tr>
<td>Oculomotor signs</td>
<td>Horizontal gaze nystagmus (7 of 9 members [77.8%]); supranuclear gaze palsy (3 of 9 members [33.3%]); mildly impaired smooth pursuit (5 of 9 members [55.6%])</td>
<td>Nystagmus (36.8%); slow saccade and slow pursuit described</td>
<td>Gaze-evoked nystagmus (100%); slow saccades (5 of 9 member [55.6%]); diplopia also described</td>
<td></td>
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<tr>
<td>Pyramidal tract signs</td>
<td>Elevated deep tendon reflexes in limbs or positive Babinski signs (8 of 9 members [88.9%])</td>
<td>Consistently decreased deep tendon reflexes, even in very young patients</td>
<td>Not reported</td>
<td></td>
</tr>
<tr>
<td>Autonomic disturbances</td>
<td>Bladder disturbances (4 of 9 members [44.4%]); constipation (2 of 9 members [22.2%])</td>
<td>Unremarkable</td>
<td>Not reported</td>
<td></td>
</tr>
<tr>
<td>Skin lesion</td>
<td>None</td>
<td>Current or previous skin lesions of EKV (14 of 19 members [73.7%])</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Radiological characteristics</td>
<td>Pontine and cerebellar atrophy (8 of 8 members) on MRI or CT scan; hot cross bun sign (4 of 6 members [66.7%]) or pontine midline linear hyperintensity (2 of 6 members [33.3%]); hyperintensities of middle cerebellar peduncles on FLAIR scan (1 of 6 members [16.7%]).</td>
<td>Pontine, cerebellar, and cerebral atrophy (4 of 9 members [44.4%]); pontine and cerebellar atrophy (1 of 9 member [11.1%]); mild cerebellar atrophy (1 of 9 members [11.1%]); no abnormality on MRI scan (3 of 9 members [33.3%]).</td>
<td>Cerbellar atrophy without brainstem involvement (7 of 7 members)</td>
<td></td>
</tr>
<tr>
<td>Abnormalities</td>
<td>Ophthalmologic</td>
<td>None</td>
<td>None</td>
<td>Not reported</td>
</tr>
<tr>
<td>Electrophysiological</td>
<td>None</td>
<td>Mild peripheral axonal neuropathy (4 of 8 members [50.0%])</td>
<td>Peripheral neuropathy (5 of 9 members [55.6%])</td>
<td></td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>None</td>
<td>Slightly high linoleic acid levels</td>
<td>Reduced serum arachidonic acid and docosahexaenoic acid levels</td>
<td></td>
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</tbody>
</table>

**Abnormalities:** CT, computed tomographic; EKV, erythrokeratodermia variabilis; FLAIR, fluid-attenuated inversion recovery; MRI, magnetic resonance imaging; SCA, spinocerebellar ataxia.
Discussion

The present genetic study identified a novel \textit{ELOVL4} mutation (p.W246G) as the sole missense mutation cosegregating with the disease in 2 independent Japanese families with SCA. This mutation, which involved a highly conserved amino acid residue, was predicted to be functionally deleterious using bioinformatics tools. The allelic mutation p.L168F in \textit{ELOVL4} has recently been described in a French-Canadian SCA pedigree with EKV (SCA34; OMIM133190) (Figure 3C).24 Our findings further confirmed that heterozygous missense mutations in \textit{ELOVL4} cause SCA.

The previously reported French-Canadian family and our Japanese families are clinically similar in the cardinal clinical feature of slowly progressive cerebellar ataxia. However, several important differences are noted (Table). First, none of our mutation carriers had skin lesions of EKV, which were found with a high frequency (14 of the 19 mutation carriers [73.7%]) in the French-Canadian family. Second, neurological signs, such as pyramidal tract signs, ocular disturbances (gaze palsy and nystagmus), and autonomic symptoms (bladder disturbance and constipation), were more pronounced in our Japanese families compared with the French-Canadian family (Table). Thus, SCA34 caused by the p.W246G mutation did not result in skin lesions but showed broader neurological phenotypes than that caused by the p.L168F mutation. Intriguingly, missense mutations in \textit{ELOVL5}, another member of the elongase family, have recently been reported to cause SCA38 (OMIM 615957).2 In SCA38, a slowly progressive course of gait ataxia is also a common clinical feature, whereas pyramidal tract signs, autonomic symptoms, and EKV skin lesions are absent (Table).

Of clinical importance, the MRI scans of the brains of the members in our Japanese families showed not only cerebellar atrophy but also marked pontine atrophy with the hot cross bun sign (blue arrowheads) detected on T2-weighted MRI scans, and sagittal views of member II-1 (C and G) in family A reveal marked pontine (white arrowheads) and cerebellar atrophy (blue arrowheads) detected on fluid-attenuated inversion recovery MRI scans. Axial T2-weighted MRI scans reveal the hot cross bun sign (blue arrowheads) in members II-1 (I and M) and III-1 (K) in family A and in member III-1 (L) in family B, for whom an MRI scan (M) was taken 15 years before the present study. An axial T2-weighted MRI scan of the brain of member II-4 in family A reveals pontine midline linear hyperintensity (J). Middle cerebellar peduncles (blue arrowheads) in member II-4 in family A reveal hyperintensity on a fluid-attenuated inversion recovery MRI scan (N).
line linear hyperintensity (33.3%), these MRI findings could be a marker for patients with SCA34 with the p.W246G mutation. In contrast, these MRI findings were not noted in the French-Canadian family with SCA34 with the p.L168F mutation or in SCA38 families (Table). Considering the pathological basis of the hot cross bun sign in multiple system atrophy, the pres-ence of these 2 MRI findings might indicate that pontocerebel-lar fibers and reticular formation are affected much intensely in SCA34 caused by the p.W246G mutation compared with SCA34 caused by the p.L168F mutation and SCA38.

**ELOVL4** encodes a 314 amino acid protein (namely, elongation of very long chain fatty acids protein 4 [ELOVL4]) that is expressed in the retina, brain, thymus, testis, and skin. ELOVL4 is mainly localized to the endoplasmic reticulum membrane and in humans there are 7 elongases (ELOVL1-7) that are involved in the elongation of C26 fatty acids to C28 or longer, which are subsequently utilized for the biosynthesis of substances such as phosphatidylcholine, sphingomyelin, and ceramides. Small amounts of phosphatidylcholine and sphingomyelin containing very long-chain polyunsaturated fatty acids exist in the retina, brain, and testis, although their physiological roles in the brain are unclear. In the skin, ceramides containing VLCFAs of C28 or longer are important components of the epidermidis, and the loss of ELOVL4 by homozygous nonsense mutations in humans (ichthyosis, spastic quadriplegia, and mental retardation [OMIM 614457]) and in *Elov4* knockout mice leads to severe neonatal skin symptoms. These results suggest that a loss-of-function mechanism might partially explain the occurrence of EKV skin lesions, which are observed soon after birth in *ELOVL4* and SCA34 caused by the p.W246G mutation compared with SCA34 caused by the p.L168F mutation and SCA38. **Research Original Investigation**

**Figure 3. Genetic Data on 2 Japanese Families With the Novel Mutation (p.W246G) in ELOVL4**

A, The mutation c.736T>G, p.W246G in ELOVL4 was detected in the affected member, as indicated by the arrowhead. B, The affected amino acid residue is highly conserved from zebrafish to humans, as indicated by the red rectangle. C, The brown boxes indicate transmembrane domains as predicted in previous reports and the Uniprot Knowledgebase; the green box indicates the dioxy iron-binding motif (HXXH); the yellow box indicates the dilysole motif for the retention of transmembrane proteins in the endoplasmic reticulum. The mutations (p.W246G and p.L168F) lead to spinocerebellar ataxia (black arrowheads). The p.W246G mutation was identified in our study (the red rectangle). Two recessive mutations (p.R216X and p.I230MfsX22 [blue arrowheads]) were reported to cause ichthyosis, spastic quadriplegia, and mental retardation. Three mutations (N264LfsX9, N264fsX10, and Y270X [orange arrowheads]) cause autosomal dominant Stargardt-like macular dystrophy.

When considering the differences between the p.W246G and p.L168F mutations in *ELOVL4* and the p.G230V mutation in *ELOVL5* causing SCA38 (Table) from a molecular standpoint, we find that the sites of their mutated amino acids relative to the transmembrane helices are of particular importance because elongases are multipass transmembrane...
proteins. The ELOVL4 protein was previously predicted by Zhang et al.,\(^{28}\) using the classic method of Kyte and Doolittle,\(^{41}\) to have 5 transmembrane helices. However, considering the lack of a crystallographic study, there is uncertainty about its topology, and furthermore, Sur4p/Elo3p, the yeast homologue of ELOVL4, was recently predicted to be a 7-pass transmembrane protein.\(^{42}\) Therefore, we sought to predict the topology of ELOVL4 using recently developed and more accurate bioinformatics tools (see Methods).\(^{19-23}\) All the tools unexpectedly predicted ELOVL4 and ELOVL5 to be 7-pass transmembrane helix proteins (Figure 4A and B; see details in eTables 3 and 4 in the Supplement) and suggested that both W246 in ELOVL4 and G230 in ELOVL5 are on the border between the small loop in the endoplasmic reticulum lumen and helix 7, whereas L168 in ELOVL4 is within helix 4.

Furthermore, alignment of ELOVL4 and ELOVL5 sequences showed that W246 in ELOVL4 and G230 in ELOVL5 are only 1 amino acid away from each other (Figure 4C). Considering that mutations in both of these elongases lead to SCA without skin or retinal lesions, the similar location of these 2 mutated amino acids suggest a common molecular mechanism selectively resulting in SCA. In addition, the brain might be more vulnerable to the effects of these mutations than the skin or retina, possibly owing to the disturbed production of VLCFAs or the aberrant protein trafficking. On the other hand, because L168 is only 6 amino acids after the dioxy-binding consensus motif, ELOVL4 and the recently identified mutation in ELOVL5 are predicted to be localized in the endoplasmic reticulum lumen and helix 7, whereas L168 in ELOVL4 is within helix 4.

Conclusions

We described the clinical features of 2 Japanese families with SCA with multiple system atrophy-like features detected on MRI scans and discovered the novel causative mutation p.W246G in ELOVL4. Along with the study\(^7\) of the original French-Canadian family with SCA34, our study confirms that mutations in ELOVL4 can cause SCA34 and broadens its clinical spectrum. These mutations in ELOVL4 and the recently identified mutations in ELOVL5 comprise a spectrum of mutations in elongases that lead to SCAs.
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