596

Figure 3 The brain MRI findings of patient 1 examined at 4 years (a–c), patient 2 examined at 3 years (d–f), patient 3 examined at 12 months (g–i), patient 4 examined at 15 months (j–l), patient 5 examined at 5 months (m–o) and patient 6 examined at 3 years (p–r). T1-weighted sagittal views (a, d, g, j, m, p), T1-weighted axial views (b, e, h, k, n, q) and T2-weighted axial views (c, f, i, l, o, r). ACC are noted in all patients. Patient 5 (m), in particular, exhibits complete agenesis of the corpus callosum. Reduced volume of the frontal lobe is seen in all patients. Prominently delayed myelination is noted in patient 3 and 4 (i and l).

— 243 —

triggered by hypoglycemia. Ultrasonography showed enlarged bilateral cerebral ventricles and intraventricular hemorrhage. Echocardiography showed no abnormalities. Ophthalmologic examination displayed exotropia and atrophy of the right retina and optic papilla. Auditory brain-stem response (ABR) showed normal results. Owing to her feeding difficulty, she required tube feeding. At the age of 5 months, she experienced a status convulsive epilepticus, and recurrent spike and waves were noted on EEG at that time. She was treated with several antiepileptic drugs. The brain MRI examined at 1 year of age showed ACC and reduced volume of the brain (Figures 3g–i). Low

intensity of the white matter was only noted on the posterior limb of internal capsule, indicating delayed myelination (Figure 3i).

At 1 year and 6 months, the patient could not control her head by herself. She did not demonstrate eye contact, and her feeding difficulties persisted. Distinct facial features included sparse hair, microcephaly, a flat occipit, a coarse face, a high nasal bridge, low-set ears, micrognathia, inversion of eyelids, esotropia and atopic skin. Immediately after this examination, acute respiratory infectious disease caused hypoxic brain damages, which was confirmed by brain computed tomography that revealed the presence of multi-cystic lesions (data not shown). After this event, she shows spastic quadriplegia.
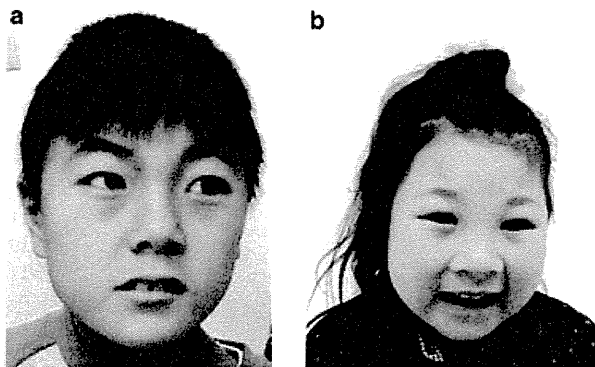
Patient 4

A 3-year and 3-month-old girl was born by induced delivery at 37th week as a first child of healthy, non-consanguineous parents. She showed prenatal growth retardation with her birth weight of 2386 g (<3rd centile), length of 44 cm (<3rd centile) and OFC 31 cm (<3rd centile). Atrial septal defect was revealed by ultrasonography. She displayed psychomotor developmental delay with holding up her head at 5 months, sitting by herself at 14 months and crawling at 20 months. From the age of 14 months, she was prescribed with antiepileptic drugs because of status convulsive that continued for ~1 h. The brain MRI examined at an age of 15 months revealed ACC and hypoplastic brain (Figures 3j–l). Low intensity of the white matter was only noted in the posterior limb of the internal capsule, indicating delayed myelination (Figure 3l). Conventional karyotyping on metaphase spreads prepared from peripheral lymphocytes showed a normal female karyotype.
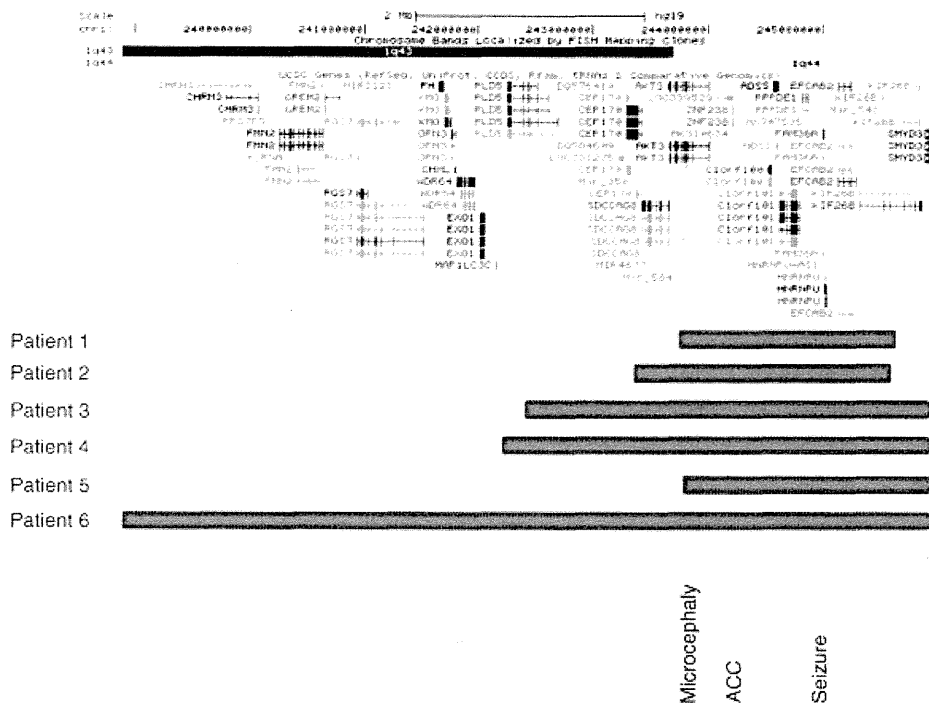

Figure 4 Facial features of patient 1 (a) and patient 2 (b). Arched eyebrows and small mouth are noted in both patients.

Figure 5 Deletions regions of the present patients depicted on a genome map from UCSC Genome Browser. Blue rectangles indicate the deletion region of the interstitial deletions identified on patients 1 and 2. Blue trapezoids indicate the terminal deletions identified on patients 3–6. Directions of the pointed trapezoids indicate continuous deletions to the telomeres. The region responsible for microcephaly, ACC and seizure that were proposed by Ballif et al.[17] are shown by gray rectangles.

Table 1 Summary of the clinical features of six patients in this study

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Frequency reported by van Bon et al.[13]
Karyotype	del (1) (q44q44)	del (1) (q43q44)	del (1) (q43)	del (1) (q43)	del (1) (q44)	del (1) (q43)	
Type of aberrations	Interstitial deletion	Interstitial deletion	Subtelomeric deletion	Unbalanced translocation	Subtelomeric deletion	Subtelomeric deletion	
Deletion region							
Start[a]	243 809 193	243 303 991	242 442 098	242 223 230	243 880 099	238 888 870	
End[a]	245 665 521	245 506 920	249 250 621	249 212 668	249 212 668	249 212 668	
Size (Mb)	1.9	2.2	6.8	7.0	5.3	10.3	
Gender	M	F	F	F	F	F	
Age	9 years	3 years and 1 month	1 year and 6 months	3 years and 3 months	6 years	10 years	
Birth weight (g)	2820	2348	2040	2386	1870	NA	
Controversial points for 1q43 deletions							
Microcephaly	+	+	+	+	+	+	9/11
Abnormalities of the corpus callosum	–	+	–	+	+	+	9/10
Seizures	+	+	–	+	+	+	9/11
Delayed myelination	–	–	+	–	NA	NA	
Severe volume loss of the brain	–	–	+	+	+	+	
General findings							
Developmental delay/mental retardation	+	+	+	+	+	+	11/11
Growth retardation	+	+	NA	+	+	+	7/11
Low birth weight	–	+	+	+	+	NA	5/11
Hypotonia	NA	+	+	NA	+	+	11/11
Feeding problem	+	–	+	–	+	NA	
Facial findings							
Round face	–	+	+	+	NA	NA	7/11
Arched eyebrows	+	+	+	+	NA	NA	
High nasal bridge	+	–	+	–	NA	NA	
Upward-slanting palpebral fissures	+	+	+	+	NA	NA	3/11
Epicanthic folds	+	+	+	+	NA	NA	4/11
Strabismus	+	–	+	–	NA	NA	2/11
Micro/retrognathia	–	–	+	+	NA	NA	0/11
Prominent jaw	+	–	–	–	NA	NA	
Low-set ears	+	+	+	+	NA	NA	
Down-turned corner of mouth	+	+	+	+	NA	NA	
Other features							
Sparse hair	–	–	+	–	NA	NA	
Flat occiput	+	–	+	–	NA	NA	
Tapering fingers	+	–	–	–	NA	NA	
Single palmer creases	+	–	–	–	NA	NA	
Complications of other organs							
Cardiac anomaly	–	–	–	ASD	–	AR	3/11
Kidney/urine pathway anomaly	–	–	–	–	Hydronephrosis	–	2/11

Abbreviations: AR, aortic regurgitation; ASD, atrial septal defect; F, female; M, male; NA, not available.
[a]Genomic positions refer to build 19.

At present, the patient exhibits severe developmental delay as she cannot stand without support and her language usage is limited to babbled words. Her mental and psychomotor developmental index of the Bayley Scales of Infant Development (II) indicate 6 and 8 months, respectively. She demonstrates growth deficiency and microcephaly with her weight of 12.8 kg (25–50th centile), height of 87.7 cm

Subtelomeric deletion of 1q43q44
K Shimojima et al
599

(10th centile) and OFC of 44 cm (<3rd centile). Dysmorphic features include hypertelorism, low-set ears and micrognathia.

Patient 5

A 6-year-old girl was born at 38 weeks of gestation, with birth weight of 1870 g (<3rd centile), length of 42 cm (<3rd centile) and OFC of 28 cm (<3rd centile), indicating intrauterine growth restriction. Since early infancy, she exhibited severe hypotonia, and her psychomotor development was severely delayed. She experienced refractory seizures after 1 year of age. Although hydronephrosis was noted by abdominal ultrasonography, there was no abnormal renal function. The brain MRI examined at an age of 4 months revealed a hypoplastic brain associated with complete agenesis of the corpus callosum (Figures 3m–o). As a result of recurrent aspiration pneumonia, laryngotracheal separation surgery was performed. At present, her height is 99 cm (<3rd centile), weight is 15.3 kg (<3rd centile) and OFC of 42 cm (<3rd centile), indicating severe growth deficiency and microcephaly. She can stand by support, but no meaningful words.

Patient 6

A 10-year-old girl was born at 40 weeks and 3 days of gestation. At the age of 3 years, she was referred to our institution because of intractable epilepsy. At that time, neurological examination revealed generalized hypotonia. EEG showed frequent spikes on the left hemisphere. The brain MRI indicated a hypoplastic brain associated with ACC and delayed myelination (Figures 3p–r). Echocardiography revealed mild aortic regurgitation. At present, her height is 115 cm (<3rd centile), weight is 16.3 kg (<3rd centile) and OFC of 48 cm (<3rd centile), indicating growth delay and microcephaly. She shows severe psychomotor developmental delay with sitting by support and no meaningful word.

DISCUSSION

The characteristics of 1q44 deletions have been recognized as growth deficiency, psychomotor developmental delay, epilepsy, microcephaly, brain malformations including ACC, and distinct facial features.[4,6,7,9,11,18] After chromosomal microarray testing has been available for the identification of submicroscopic chromosomal aberrations, many patients with submicroscopic deletions of 1q44 have been identified.[8,10,12,14,15] Now, precise genotype–phenotype correlation has been evaluated through the accumulation of patients with variable deletion sizes, and a minimal essential region has been proposed for expressing the main characteristics of 1q44 deletion syndrome.[7,8,14–16] Ballif et al.[17] evaluated 22 patients with small interstitial deletions of 1q44 and demonstrated critical regions for microcephaly, ACC and seizures. Consequently, AKT3 was reported to be the gene responsible for microcephaly; zinc finger protein 238 gene (ZNF238) for ACC; and heterogeneous nuclear ribonucleoprotein U gene (HNRNPU) for seizures[17] (Figure 5).

In this study, we identified six patients with the deletions including 1q44. Clinical and genetic findings were summarized in Table 1 and Figure 5, respectively. The presenting patient 1 and 2 showed typical interstitial deletions of 1q44, in which AKT3 and ZNF238 are included, and manifested the typical features of mental retardation, microcephaly, ACC and seizures. This evidence supports the findings reported by Ballif et al. that the essential features of 1q44 deletion syndrome are derived from deletions of the minimal region including AKT3 and ZNF238.[17] In comparison with these two patients, the other four patients displayed terminal deletion of 1q44 that include the minimal region including AKT3 and ZNF238.

Although the chromosomal breakpoint of patient 5 was just on AKT3, phenotypic feature of this patient are extremely severe as compared with those of patient 1 and 2 who had interstitial deletion of 1q44. Although Roos et al.[18] reported that 1.6 Mb terminal region does not have clinical relevance, because it appears to primarily contain many of the olfactory receptors genes, many genes of unknown functions are included in this 2.6 Mb region (chr1:245 000 000–247 600 000). Thus, this additionally deleted region in patient 5 may also have been responsible for his severe neurological manifestations.

Patient 4 had an additional chromosomal aberration, with a partial trisomy of 5p derived from an unbalanced translocation. However, the phenotypic severity of patient 4 was not significantly different from that of the other patients with 1q43 deletions. Thus, the partial trisomy of 5p identified in patient 4 did not demonstrate an important contribution to the patient's manifestations. Patient 3 showed extremely severe developmental delay, which may have been modified by the hypoglycemia and subsequent intraventricular hemorrhage suffered during the early infantile period.

The deletion regions of the remaining three patients (patient 3, 4 and 6) expanded toward the centromere beyond the critical region for 1q44 deletion syndrome, and several more genes are included in the deletion region in these three patients. Severe volume loss of the brain was revealed in the brain MRI of these three patients, similar to patient 5, and delayed myelination was also seen on the brain MRI of patient 3 and 4 (Figures 3i, l). Although patient 5 exhibited high T2 signal intensities in the white matter, this image was obtained when he was 4 months of age, and we cannot ascertain if this finding indicated delayed myelination. Similarly, because the brain MRI of patient 1, 2 and 6 were obtained after 3 years of age, we cannot determine whether these three patients experienced delayed myelinations in their early infancy. However, delayed myelination, as a finding associated with 1q44 deletion, has not yet been reported in the literature.[17] It may therefore be that the characteristic findings of the patients with expanded deletion beyond the 1q43 region may have clinical implications for delayed myelination. Although many UCSC genes are present in these additional regions, some of these genes may have clinical relevance for delayed myelination observed in patient 3 and 4. The centrosomal protein 170 kDa gene (CEP170) is a potential candidate because of its high expression level in the fetal brain.

In this genotype–phenotype correlation study for patients with 1q44 deletions, we revealed that telemetric region beyond the physical position of chr1:245 000 000 may be responsible for severe volume loss of the brain, and the proximal region beyond AKT3 may be responsible for delayed myelination. The neighboring genes surrounding 1q43q44 may have some modifier effects to the severe brain impairments associated with delayed myelination. To identify them, much more information regarding genotype–phenotype correlations will need to be accumulated for patients with terminal deletion of 1q.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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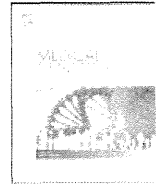
Short clinical report

Pelizaeus-Merzbacher disease caused by a duplication-inverted triplication-duplication in chromosomal segments including the *PLP1* region

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ARTICLE INFO

ABSTRACT

Pelizaeus-Merzbacher disease (PMD; MIM#312080) is a rare X-linked leukodystrophy presenting with motor developmental delay associated with spasticity and nystagmus. PMD is mainly caused by abnormalities in the proteolipid protein 1 gene (*PLP1*), most frequently due to duplications of chromosomal segments including *PLP1*. In this study, a 9-year-old male patient manifesting severe developmental delay and spasticity was analyzed for *PLP1* alteration, and triplication of *PLP1* was identified. Further examination revealed an underlying genomic organization, duplication-inverted triplication-duplication (DUP-TRP/INV-DUP), in which a triplicated segment was nested between 2 junctions. One of the 2 junctions was caused by inverted homologous regions, and the other was caused by non-homologous end-joining. PMD patients with *PLP1* duplications usually show milder-classical forms of the disease compared with patients with *PLP1* missense mutations manifesting severe connatal forms. The present patient showed severe phenotypic features that represent an intermediate form of PMD between classical and connatal forms. This is the first report of a patient with *PLP1* triplication caused by a DUP-TRP/INV-DUP structure. This study adds additional evidence about the consequences of *PLP1* triplication.

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1. Introduction

Pelizaeus-Merzbacher disease (PMD; MIM#312080) is a rare X-linked leukodystrophy presenting with nystagmus, hypotonia that later develops into spasticity, dystonia, ataxia, and developmental delay beginning in the first year of life [4,9]. The proteolipid protein 1 gene (*PLP1*; MIM#300401) is the main responsible gene for PMD and the most common *PLP1* alterations are gene duplications, which are found in 60–70% of PMD patients [4,9]. Nucleotide alterations that affect the amino acid sequences of *PLP1* are found in 20% of PMD patients. The clinical manifestations of patients with *PLP1* alterations are dependent on the genotypes.

Recently, we identified a rare *PLP1* triplication in a patient with PMD. In this study, we revealed the underlying genomic organization with a duplication-inverted triplication-duplication (DUP-TRP/INV-DUP), which is a recently proposed mechanism of chromosomal rearrangement [2]. The clinical implication of *PLP1* gene dosage effects is discussed.

2. Materials, methods, and results

2.1. Clinical report

A 9-year-and-10-month-old boy was born at 38 weeks of gestation with a birth weight of 2400 g. Although his siblings were healthy, his maternal uncle and male cousin showed similar manifestations as his own; his maternal male cousin died at the age of 7 years (Supplemental Fig. S1). After birth, he showed nystagmus, laryngeal stridor, and profound hypotonia, and his subsequent psychomotor development was severely delayed. At the age of 5 months, his brain magnetic resonance imaging (MRI)

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revealed hypomyelination, which became profound at the age of 5 years and 5 months (Fig. 1).

At present, his height is 108 cm (<3rd centile), weight is 13.0 kg (<3rd centile), and head circumference is 47.7 cm (<3rd centile), indicating microcephaly. He shows severe developmental delay. He cannot turn over or sit on his own. Although he shows a lack of stable head control, he does not require head support when sitting in a "chair" position. He can eat minced food orally but with full support. Toilet habit is not acquired. He can vocalize, although very slowly, and can show his intentions by using simple signs or buttons. Mild scoliosis and mildly restricted range of movement in his hip and knee joints are noted. Neurological examination reveals mild muscular hypotonia. Pyramidal tract signs are prominent in his lower extremities; decreased deep tendon reflexes in his upper extremities, diminished abdominal reflex, increased deep tendon reflexes in his lower extremities, and positive Babinski reflex and ankle clonus in both his legs are also noted. Although some cerebellar signs are positive with continuous horizontal nystagmus and trunkal ataxia, the other findings are negative, including intention tremor and dysmetria. No dystonic finding is noted. Because he has never shown epileptic seizures, he has never been evaluated with electroencephalography. Moreover, no nerve conduction velocity tests have been done.

2.2. Cytogenetic analysis

From his clinical features, PMD was suspected as the candidate diagnosis. Then, we analyzed PLP1 gene dosage by multiplex ligation-dependent probe amplification (MLPA) analysis, using Holland SALSA P022 MLPA probe mix (MRC Holland, Amsterdam, the Netherlands) according to the manufacturer's protocol [7]. The peak height of PLP1 in this patient was higher than that of previously evaluated patients with PLP1 duplication, which led us to suspect triplication of PLP1 rather than duplication (data not shown). To confirm this MLPA result, microarray-based comparative genomic hybridization (aCGH) analysis was performed using the Agilent catalog 105K oligonucleotide microarray (Agilent Technologies, Santa Clara, CA) according to the method described elsewhere [6], and the chromosomal aberration region was revealed to consist of duplicated and triplicated regions. To identify the precise aberration regions, a custom-made oligoarray was originally designed using the web-based software Agilent earray (https://earray.chem.agilent.com/earray/), according to the manufacturer's protocol. The array included 11,526 probes between chrX

101,600,412–103,499,946 with an average interval of 147-bp (Agilent Technologies). Finally, genomic copy number gain was identified on Xq22.2 with a size of 984-kb (chrX: 102,321,416–103,305,265) in which a nested aberration of 214-kb including PLP1 (chrX: 103,010,204–103,223,711) was included (Fig. 2A, Table 1).

The identified aberration was further examined by fluorescence in situ hybridization (FISH) as previously described [6]. Metaphase and interphase nuclei were prepared from peripheral blood lymphocyte-stimulated phytohemagglutinin according to the standard method. Human bacterial artificial clones were selected from the UCSC Genome Browser (http://www.genome.ucsc.edu) as described previously [6]. The results of FISH analysis were compatible with that of aCGH and confirmed that the triplicated region including PLP1 was surrounded by duplicated regions (Fig. 2B–D). The patient's mother declined examination for the carrier status of PLP1 triplication.

The underlying genomic organization was investigated using long range PCR-based analyses with Tks Gflex™ DNA Polymerase (Takara, Ohtsu, Japan). The primers used for PCR are listed in Supplemental Table S1. The combinatory use of sense primers A and B generated an appropriately 20-kb PCR product, which fit the predicted architecture of junction 1 (Fig. 3A and B). The combinatory use of antisense primers C and D also generated a 756-bp PCR product (Fig. 3A and B). Direct sequencing analysis identified the junction 2 sequence (Fig. 3C). Because there is no homologous sequence in junction 2, this rearrangement was considered a consequence of non-homologous end-joining (NHEJ) [4].

3. Discussion

We presented a male patient with generalized spasticity, severe developmental delay, and hypomyelination. His condition was diagnosed as PMD on the basis of clinical findings. We detected PLP1 triplication from combined examinations with MLPA, aCGH, and FISH analyses. aCGH analysis confirmed a duplicated region on Xq22.2 in which a nested aberration with triplicated region of PLP1 was identified.

Recently, Carvalho et al. proposed a new mechanism for genomic rearrangement causing the genomic organization DUP-TRP/INV-DUP in 5 patients with MECP2-related disorders [2], in whom various sizes of triplicated segments were found embedded in the neighboring duplicated segments and were inserted in an inverted orientation. The existence of inverted repeats in the
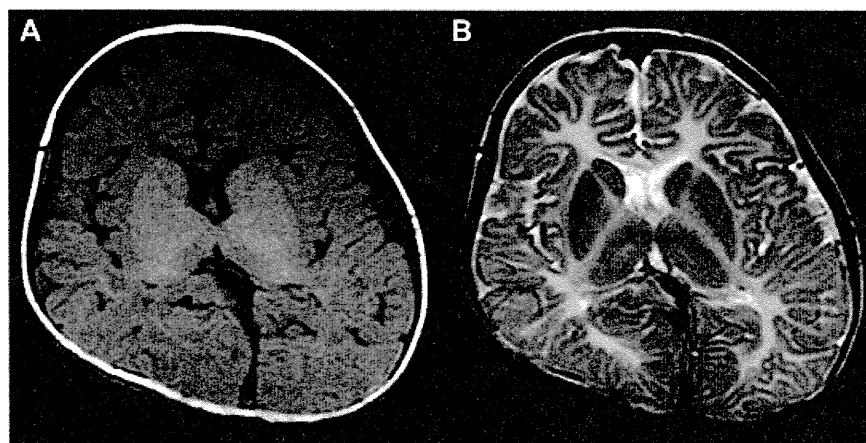
Fig. 1. Brain MRI of the patient performed at 5 years and 5 months of age. The reduction in the contrast of cortex and white matter in T1-weighted image (A) and the diffuse hyper-signal in T2-weighted image (B) show severe hypomyelination. Complete lack of myelination is also shown in the posterior limb of internal capsule.
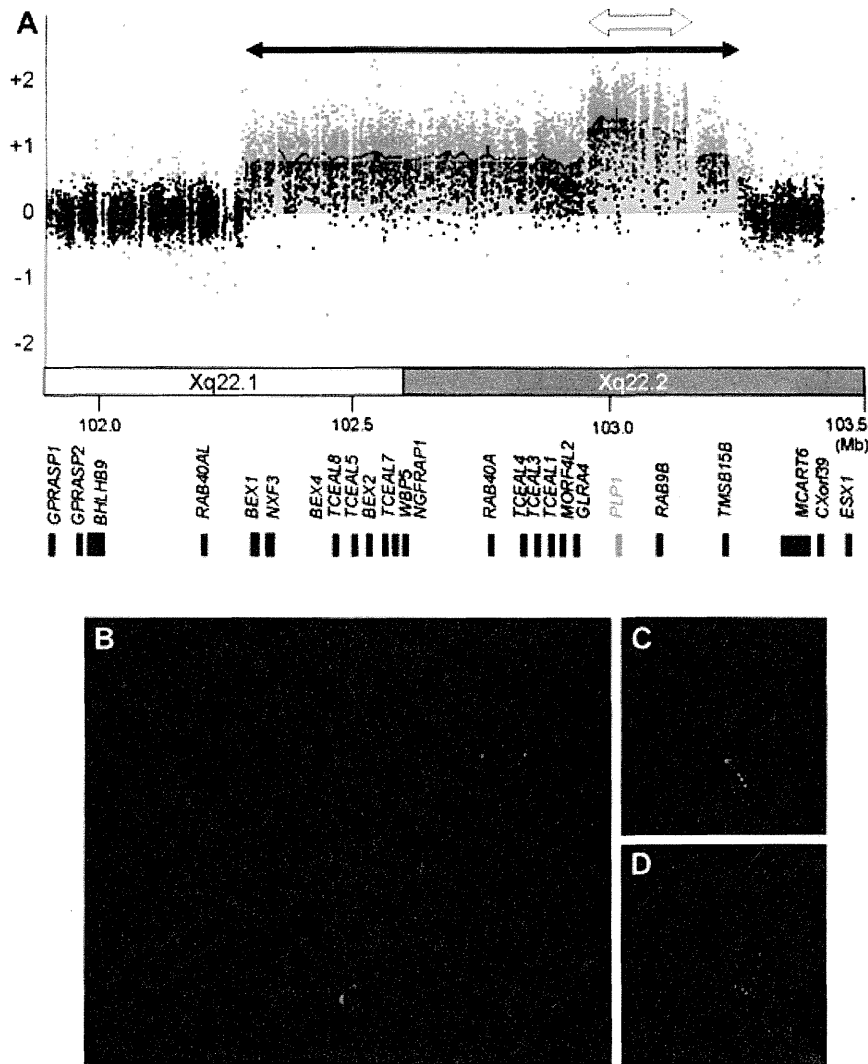
Fig. 2. Results of cytogenetic and molecular analyses. (A) The genome view of the result of custom array represents a chromosomal duplication (black bar with arrow on both ends) and a nested triplication including PLP1 (white bar with arrow on both ends). (B) Two green signals of CTD-2086O16 (chrXq22.2: 102,807,623–102,897,468) were identified in the interphase nuclei, indicating duplication of this region. The red signal of RP11-75D20 (chrXp22.13: 18,314,474–18,506,931) was used as a marker. Both of the signals are on the same X chromosome, indicating that the PLP1 locus is not translocated on the other chromosome. (C, D) Three red signals of RP4-540A13 (chrXq22.2: 103,067,273–10,3152,647) were identified in the interphase nuclei, indicating triplication of this region. The green signal of RP11-106N3 (chrXp22.13: 18,467,339–18,626,601) was used as a marker.

telomeric side mediates these inverted rearrangements [2]. Although the authors also identified a DUP-TRP/INV-DUP structure in the neighboring region of PLP1 in one individual, the triplicated region did not include PLP1 itself. In this study, we identified a DUP-TRP/INV-DUP structure in the present patient with PMD, and PLP1 was included in the triplicated region. Detailed molecular examination revealed that two H2B histone family member X pseudogenes, H2BFXP, were located at each of the telomeric ends of the triplication and duplication (Fig. 3A). According to the UCSC Genome Browser, the two H2BFXP showed 99% identical sequences and were inserted in an inverted orientation (Fig. 3A). The result of this study indicated that the inverted orientation of H2BFXP mediated the first rearrangement (junction 1), and this caused the insertion of an inverted segment including PLP1 and resulted as the triplication of this region. The 2nd rearrangement was caused by NHEJ [4], because there was no homologous sequence in junction 2 (Fig. 3C). From these results, the mechanism of the chromosomal
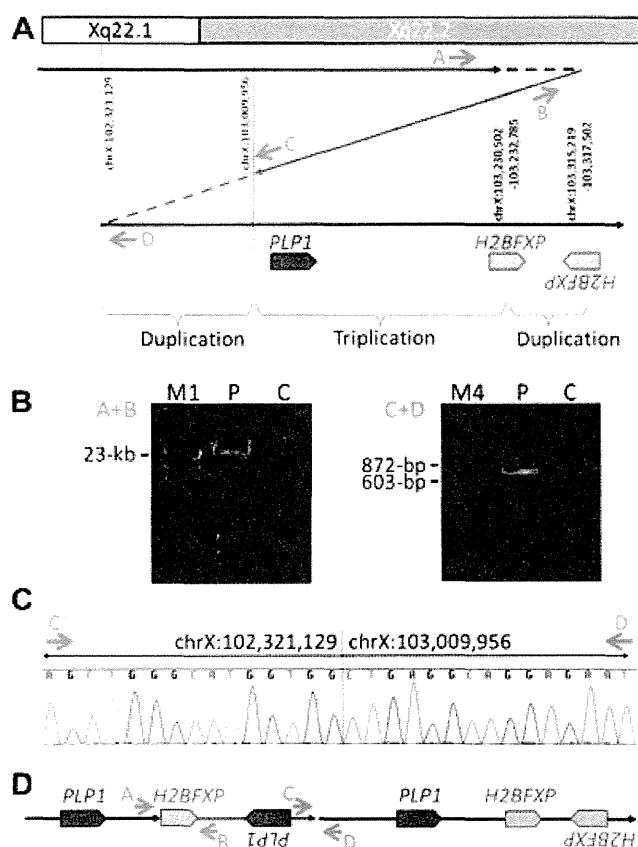
Table 1

The summary of the genomic copy number aberration

Chromosome band		Position	Average log₂ ratio	Locus	
chrX	q22.1	102,320,921	Duplication 0.76		Primer D
		102,321,416			
		102,807,623		CTD-2086O16	
		102,897,468			
		103,009,875			
		103,010,204	Triplication 1.25		Primer C
		103,031,781		PLP1	
		103,047,545			
	q22.2	103,067,273		RP4-540A13	
		103,152,647			
		103,223,711			Primer A
				H2BFXP	
		103,243,004			
		103,305,265			
				H2BFXP	
		103,328,529			Primer B

Genomic position is reffered to build 19

Fig. 3. Genomic organization of the present patient. (A) Predicted chromosomal rearrangements around Xq22.2. Red arrows, locations and directions of primers; trapezoids, locations and directions of genes; blue arrow, inverted genomic region. (B) Results of the electropherogram of the PCR products. The combinatory use of primers A + B generated an approximately 20-kb PCR product of junction 1, and primers C + D generated a 756-bp PCR product. M1, molecular marker of λ/HindIII digest; M4, molecular marker of φX174/HaeIII digest; P, the present patient; C, normal control. (C) The sequencing electropherogram indicates junction 2, generated by PCR using primers C + D. (D) The final conclusion about the genomic organization is shown in the horizontal view.

rearrangement identified in this study can be explained by DUP-TRP/INV-DUP (Fig. 3D) [2].

Traditionally, PMD was classified into 3 categories: connatal form, classical form, and X-linked spastic paraplegia type 2 (SPG2; MIM#312920) [4]. Callioux et al. proposed a new subclassification of PMD as a continuous clinical spectrum [1]. According to these 2 classification systems, PMD patients with simple *PLP1* duplications manifest rather milder phenotypes (classical form/form 1–2), whereas PMD patients with nucleotide alterations often show severe phenotypes (connatal form/form 0). As the present patient has never achieved head control, his clinical condition can be traditionally classified as the connatal type and regarded as form 0 according to Callioux et al.'s classification [1]. However, he can eat

minced food orally and there is no strider at present. Thus, we regarded his condition as an intermediate form of PMD between classical (form 1) and connatal (form 0).

There have been only few reports of PMD patients with more than 2 copies of *PLP1* [3,5,8]. Five male patients with *PLP1* triplication reported by Wolf et al. showed severe clinical features compared with patients with *PLP1* duplications [8]. None of the 5 patients achieved stable head control, and their brain MRI showed an almost complete absence of the normal myelin signal [8]. The present patient also showed lack of stable head control, severe mental retardation, and a complete lack of myelination in the posterior limb of internal capsule was identified by brain MRI. Thus, our study gives evidence that patients with *PLP1* triplications show a more severe phenotype than patients with *PLP1* duplications.

Conflict of interest

The authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmg.2012.02.013.

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BRIEF COMMUNICATIONS

Mutations affecting components of the SWI/SNF complex cause Coffin-Siris syndrome

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By exome sequencing, we found *de novo SMARCB1* mutations in two of five individuals with typical Coffin-Siris syndrome (CSS), a rare autosomal dominant anomaly syndrome. As *SMARCB1* encodes a subunit of the SWItch/Sucrose NonFermenting (SWI/SNF) complex, we screened 15 other genes encoding subunits of this complex in 23 individuals with CSS. Twenty affected individuals (87%) each had a germline mutation in one of six SWI/SNF subunit genes, including *SMARCB1, SMARCA4, SMARCA2, SMARCE1, ARID1A* and *ARID1B*.

Chromatin remodeling factors regulate the gene accessibility and expression by dynamic alteration of chromatin structure. SWI/SNF complexes have important roles in lineage specification, maintenance of stem cell pluripotency and tumorigenesis[1–5]. These complexes are composed of evolutionarily conserved core subunits and variant subunits. Brahma-associated factor (BAF) and Polybromo BAF (PBAF) complexes constitute two major subclasses[1–5]. It has been suggested that the BAF complex is similar to the yeast SWI/SNF complex and that the PBAF complex is more like the chromatin remodelling complex (RSC) in yeast, which is required for cell cycle progression through mitosis[6]. However, several subunits that are common to both BAF and PBAF complexes are predicted to be related to the regulation of lineage- and tissue-specific gene expression[2].

Coffin-Siris syndrome (MIM 135900) is a rare congenital anomaly syndrome characterized by growth deficiency, intellectual disability, microcephaly, coarse facial features and hypoplastic nail of the fifth finger and/or toe (**Fig. 1** and **Supplementary Table 1**)[7]. The majority of affected individuals represent sporadic cases, which is compatible with an autosomal dominant inheritance mechanism. The genetic cause for this syndrome has not been elucidated.

To identify the genetic basis of CSS, we performed whole-exome sequencing of five typical affected individuals (**Supplementary Methods**). Taking into account our model that assumes that an abnormality in a causal gene would be shared in two or more subjects, 51 variants were identified as candidates (**Supplementary Table 2**). All the variants were also examined by Sanger sequencing of PCR products amplified using genomic DNA from the five affected individuals and their parents. Nine variants were found to be false positives, 40 were inherited from either the father or mother, and 2 *de novo* heterozygous mutations of *SMARCB1* were found in 2 affected individuals (c.1130G>A (p.Arg377His) and c.1091_1093del AGA (p.Lys364del)) (**Table 1, Supplementary Fig. 1** and **Supplementary Methods**). Two *de novo* coding-sequence mutations occurring within a specific gene is an extremely unlikely event[8], supporting the idea that *SMARCB1* is a causative gene in CSS. Next, we screened *SMARCB1* in 23 individuals with CSS by high-resolution melting analysis[9] and identified the mutation encoding the p.Lys364del alteration in two additional individuals, including one of Arab descent (subject 22) (**Table 1** and **Supplementary Fig. 1**). As the mutation detection rate was relatively low (4 of 23, only 17.4%), we screened 15 additional genes encoding other SWI/SNF subunits (**Supplementary Table 3**). Unexpectedly, four other subunits, *SMARCA4* (also known as *BRG1*), *SMARCE1, ARID1A* and *ARID1B* were also found to be mutated (**Table 1** and **Supplementary Figs. 2–5**). In subject 10, a c.2144C>T mutation in *ARID1B* (encoding p.Pro715Leu) was found in addition to the c.5632delG mutation in *ARID1B*. RT-PCR products that were amplified from total RNA from this subject's lymphoblastoid cells were cloned into the pCR4-TOPO vector. The two mutations were present on different alleles, according to sequencing of clones containing each allele (data not shown). As the c.5632delG mutation is

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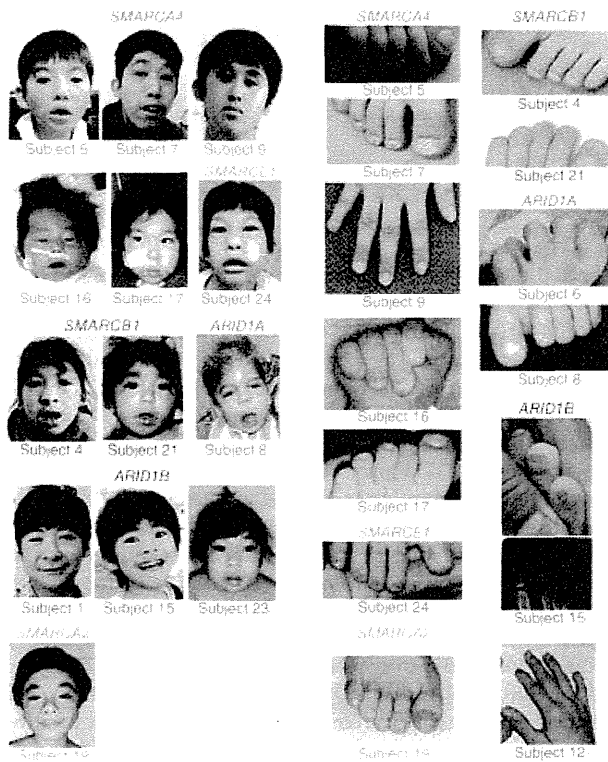
Figure 1 Photographs of individuals with Coffin-Siris syndrome. The faces (left) and hypoplastic-to-absent nail of the fifth finger or toe (right) of affected individuals are shown with the color-coded names of the corresponding mutated genes. The green arrow indicates the absence of the distal phalanx in the fifth toe. No obvious hypoplastic nails were observed in subjects 12 or 19. Consent for all the photographs was obtained from the families of the affected individuals.

in mice[10]. However, in humans, abnormalities in both *SMARCA4* and *SMARCA2* are found in CSS, indicating that the in-frame partial deletion of the gene encoding BRM in subject 19 has a specific mutational effect different from that of simple inactivation in mice. These data support the idea that abnormalities in the BRG1-BAF and BRM-BAF complexes can cause the abnormal neurological development in CSS.

All the mutated genes found in CSS, except for *SMARCE1*, have been reported to be associated with tumorigenesis[1,2]. Among the 23 subjects with CSS, only subject 3 with an *ARID1A* mutation presented with hepatoblastoma. To our knowledge, haploinsufficiency and/or homozygous inactivation of *ARID1A* have been found in several types of cancer but not in hepatoblastoma. Malignancies were not detected in any of the other subjects with CSS examined here. It remains to be seen whether malignancies are robustly associated with CSS.

Given the fact that all the mutations in *ARID1A* and *ARID1B* in CSS were predicted to cause protein truncation, we proposed that haploinsufficiency of these two genes must be able to cause CSS. cDNA analysis of lymphoblastoid cell lines from subjects 1, 6 and 23 indicated that the mutated transcripts were subject to nonsense-mediated mRNA decay (**Supplementary Fig. 8**). In subject 10, the *ARID1B* mutation associated with the creation of a premature stop codon in the last exon did not result in nonsense-mediated mRNA decay as expected (**Supplementary Fig. 8**).

In regard to the other mutated genes, germline heterozygous truncation mutations in *SMARCB1* and *SMARCA4* have been reported

very likely to be deleterious (as it results in a truncated protein), the c.2144C>T mutation is likely to be a rare polymorphism. Of note, subject 12, who presented an atypical facial appearance and indistinct hypoplastic nails, had two interstitial deletions at 6q25.3–q27 involving *ARID1B*, as detected by a SNP array (**Supplementary Fig. 6** and **Supplementary Methods**). Furthermore, subject 14 was found to have an interstitial deletion of *SMARCA2* by a SNP array (**Supplementary Fig. 7** and **Supplementary Methods**). No other copy-number changes involving genes encoding SWI/SNF complex components were found in subjects 2, 14 or 18 by array analysis. The overall mutation detection rate was 87%. In total, 20 of the 23 subjects had a mutation affecting one of the six SWI/SNF subunits.

Mutations in CSS were identified in the BAF-specific subunits *ARID1A* and *ARID1B* but not in PBAF-specific subunits (*BRD7, ARID2* and *PBRM1*) (**Supplementary Table 3**). In addition, mutations were identified in *SMARCA4* (*BRG1*) as well as in *SMARCA2* (*BRM*) (**Supplementary Table 3**). The BRG1 and BRM proteins are mutually exclusive catalytic ATP subunits in mammalian SWI/SNF complexes. Of note, the majority of heterozygous *Smarca4*-null mice survive with susceptibility to neoplasia, with a minority dying after birth because of exencephaly, whereas homozygous *Smarca2*-null mice are viable and fertile[4]. In *Smarca2*-null mice, Brg1 is upregulated, suggesting that Brg1 can functionally replace Brm

Table 1 Mutations in individuals with Coffin-Siris syndrome

Subject ID	Gene	Mutation	Alteration	Type	Control allele frequency[a]
4	*SMARCB1*	c.1091_1093del AGA	p.Lys364del	De novo	0/502
11	*SMARCB1*	c.1130G>A	p.Arg377His	De novo	0/500
21	*SMARCB1*	c.1091_1093del AGA	p.Lys364del	NC	0/502
22	*SMARCB1*	c.1091_1093del AGA	p.Lys364del	NC	0/502
9	*SMARCA4*	c.1636_1638del AAG	p.Lys546del	De novo	0/350
7	*SMARCA4*	c.2576C>T	p.Thr859Met	De novo	0/368
5	*SMARCA4*	c.2653C>T	p.Arg885Cys	De novo	0/368
16	*SMARCA4*	c.2761C>T	p.Leu921Phe	De novo	0/368
25	*SMARCA4*	c.3032T>C	p.Met1011Thr	NC	0/372
17	*SMARCA4*	c.3469C>G	p.Arg1157Gly	De novo	0/368
19	*SMARCA2*	Partial deletion		De novo	–
24	*SMARCE1*	c.218A>G	p.Tyr73Cys	De novo	0/368
3	*ARID1A*	c.31_56del	p.Ser11Alafs*91	NC	0/330
6	*ARID1A*	c.2758C>T	p.Gln920*	NC	0/376
8	*ARID1A*	c.4003C>T	p.Arg1335*	De novo	–
1	*ARID1B*	c.1678_1688del	p.Ile560Glyfs*89	De novo	–
15	*ARID1B*	c.1903C>T	p.Gln635*	De novo	–
23	*ARID1B*	c.3304C>T	p.Arg1102*	De novo	–
10	*ARID1B*	c.2144C>T	p.Pro715Leu	NC	0/368
10	*ARID1B*	c.5632del G	p.Asp1878Metfs*96	NC	0/374
12	*ARID1B*	Microdeletion		NC	

NC, not confirmed because parental samples were unavailable.
[a]The numbers indicate the observed allele frequency (alleles harboring the change/total tested alleles) in Japanese controls. None of the mutations was found in dbSNP132, the 1000 Genomes database or the National Heart, Lung, and Blood Institute (NHLBI) GO exomesequencing project database. –, not tested.

— 253 —

in individuals with rhabdoid tumor predisposition syndromes 1 (RTPS1; MIM 609322) and 2 (RTPS2; MIM 613325)[11,12], and various types of *SMARCB1* mutations (missense, in-frame deletion, nonsense and splice site) have been found in the germline of individuals with familial and sporadic schwannomatosis (MIM 162091)[13,14]. Furthermore, mice with heterozygous knockout of *Smarca4* or *Smarcb1* were prone to tumor development[2]. All the mutations in *SMARCA4* and *SMARCB1* in individuals with CSS were non-truncating (either missense or in-frame deletions), implying that they exert gain-of-function or dominant-negative effects (excluding haploinsufficiency as a cause). It is noteworthy that comparable germline mutations in *SMARCB1* have such different phenotypic consequences in their association with the phenotypes of CSS and schwannomatosis. The *SMARCB1* mutations in CSS and those in schwannomatosis are indeed different according to the Human Gene Mutation Database. With regard to the *SMARCA2* interstitial deletion in CSS, the change maintained the coding sequence reading frame but removed exons 20–27 that encode the HELICc domain. RT-PCR analysis confirmed the deletion of exons 20–27 at the cDNA level (**Supplementary Fig. 7**). These data suggest the importance of the HELICc domain in the SMARCA2 protein.

The various types of mutations in the genes encoding different SWI/SNF components resulted in similar CSS phenotypes. This suggests that the SWI/SNF complexes coordinately regulate chromatin structure and gene expression. This is the first report, to our knowledge, of germline mutations in SWI/SNF complex genes associated with a multiple congenital anomaly syndrome, highlighting new biological aspects of SWI/SNF complexes in humans. Similarly, genes encoding SNF2-related proteins, which are implicated as chromatin remodeling factors outside of SWI/SNF complexes, are mutated in different syndromes, including in α-thalassaemia/mental retardation syndrome X-linked (ATRX; *ATRX* mutations) and in coloboma, heart defect, atresia choanae, retarded growth and development, genital abnormality and ear abnormality (CHARGE) syndrome (*CHD7* haploinsufficiency)[3]. We expect that more mutations affecting chromatin remodeling factors will be found in different human diseases.

URLs. Human Gene Mutation Database, https://portal.biobase-international.com/cgi-bin/portal/login.cgi.

AUTHOR CONTRIBUTIONS
Y.T., S. Miyatake, I.O., H.D., H.S. and N. Miyake performed exome sequencing and Sanger sequencing. Y.T., M.S., K.O., I.O., T.M., H.D., H.S. and N. Miyake performed data management and analysis. N.O., H.O., T. Kosho, Y.I., Y.H.-K., T. Kaname, K.N., H.K., K.W., Y.F., T.H., M.K., Y.H., T.Y., S.Y., S. Mizuno, S.S., T.I., T.N., T.O. and N.N. provided clinical materials after careful evaluation. Y.T., N. Miyake and N. Matsumoto wrote the manuscript. N. Matsumoto designed and oversaw all aspects of the study.

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Homozygous c.14576G>A variant of *RNF213* predicts early-onset and severe form of moyamoya disease

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Supplemental data at
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Supplemental Data

ABSTRACT

Objective: *RNF213* was recently reported as a susceptibility gene for moyamoya disease (MMD). Our aim was to clarify the correlation between the *RNF213* genotype and MMD phenotype.

Methods: The entire coding region of the *RNF213* gene was sequenced in 204 patients with MMD, and corresponding variants were checked in 62 pairs of parents, 13 mothers and 4 fathers of the patients, and 283 normal controls. Clinical information was collected. Genotype-phenotype correlations were statistically analyzed.

Results: The c.14576G>A variant was identified in 95.1% of patients with familial MMD, 79.2% of patients with sporadic MMD, and 1.8% of controls, thus confirming its association with MMD, with an odds ratio of 259 and $p < 0.001$ for either heterozygotes or homozygotes. Homozygous c.14576G>A was observed in 15 patients but not in the controls and unaffected parents. The incidence rate for homozygotes was calculated to be >78%. Homozygotes had a significantly earlier age at onset compared with heterozygotes or wild types (median age at onset 3, 7, and 8 years, respectively). Of homozygotes, 60% were diagnosed with MMD before age 4, and all had infarctions as the first symptom. Infarctions at initial presentation and involvement of posterior cerebral arteries, both known as poor prognostic factors for MMD, were of significantly higher frequency in homozygotes than in heterozygotes and wild types. Variants other than c.14576G>A were not associated with clinical phenotypes.

Conclusions: The homozygous c.14576G>A variant in *RNF213* could be a good DNA biomarker for predicting the severe type of MMD, for which early medical/surgical intervention is recommended, and may provide a better monitoring and prevention strategy. *Neurology* 2012;78:803-810

GLOSSARY

CI = confidence interval; **HRM** = high-resolution melting; **MMD** = moyamoya disease; **OR** = odds ratio; **PCA** = posterior cerebral artery.

Moyamoya disease (MMD) is a cerebrovascular disease, which is now a relatively common cause of pediatric strokes.[1,2] Annual incidence is estimated to be 0.35–0.54 per 100,000 person-years in Japan[3,4] and about one tenth of that in Europe.[5,6] MMD can lead to devastating neurologic deficits and intellectual impairments if left untreated.

Although MMD is a progressive disease, its natural history varies from slow progression to rapid neurologic decline.[7] Preoperative infarctions, early age at onset, intellectual impairment, seizure, and progressive posterior cerebral artery (PCA) stenosis are known prognostic factors.[8–11] Surgical revascularization can improve the cerebrovascular hemodynamics and prevent subsequent attacks in the ischemic type of MMD.[8] Thus, early diagnosis and surgical intervention are very important.

Genetic factors underlying MMD are of clinical relevance. Epidemiologic studies have shown that about 15% of patients had a family history.[12] Anticipation of the disease is

Table 1	Sample demographics of patients with moyamoya disease and controls[a]		
Clinical features	No. of patients (%)		No. of patients without data
MMD	204		
Gender (M/F)	68 (33.5)/135 (66.5)		1
Distribution of age at onset	0–58 y		7
Frequency of childhood onset (<15 y)	143 (72.6)		
Frequency of childhood onset (<4 y)	36 (18.3)		
Clinical manifestation			11
Infarction	87 (45.1)		
TIA	77 (39.9)		
ICH/IVH	17 (8.8)		
Others	12 (6.2)		
With family history	41 (20.1)		0
With intellectual impairment	33 (17.7)		18
With epilepsy	33 (17.6)		16
PCA involvement			52
Unilateral	31 (20.4)		
Bilateral	43 (28.3)		
Total	74 (48.7)		
Bilateral MMD	148 (96.1)		50
Controls	283		
Gender (M/F)	140 (51.5)/132 (48.5)		11

Abbreviations: ICH/IVH = intracranial hemorrhage/intraventricular hemorrhage; MMD = moyamoya disease; PCA = posterior cerebral artery; TIA = transient ischemic attack.
[a] Numbers of patients (%) in each feature are shown, except for the distribution of ages at onset for all patients.

also observed in familial MMD.[13] Recently, the important MMD susceptibility gene, *RNF213*, was identified.[14,15] However, its clinical relevance remains unknown. For this investigation, we conducted a comprehensive genetic study of *RNF213* as well as a clinical phenotype analysis of MMD.

METHODS Study subjects. Blood samples from 204 Japanese patients with MMD were obtained consecutively between January 2008 and February 2011. There were no sample overlaps between ours and those in the previous studies.[14,15] MMD was diagnosed as either definite (bilateral) or probable (unilateral) according to published guidelines.[16] Six patients with probable MMD were female adults and 5 of them had sporadic MMD. The medical charts were completed by the clinicians who were blinded to the genotype of the patients. Sample demographics are shown in table 1. We also obtained either blood or saliva samples from 62 pairs of

parents, as well as 4 fathers and 13 mothers whose partners were unavailable. As many as 94 to 283 samples from healthy Japanese individuals were tested as normal controls for each sequence variant found.

Standard protocol approvals, registrations, and patient consents. Experimental protocols were approved by the Committee for Ethical Issue at Yokohama City University School of Medicine. Written informed consents were obtained from all the patients or their parents.

Mutation screening. Genomic DNA was obtained from peripheral blood leukocytes using QuickGene-610L (Fujifilm, Tokyo, Japan) or from saliva using Oragene DNA (DNA Genotek, Kanata, Canada). DNA was amplified using GenomiPhi version 2 (GE Healthcare, Buckinghamshire, UK). Mutation analysis of exons and exon-intron borders covering the coding region of *RNF213* (GenBank accession number, NM_020914.4), except for exon 61, was performed in all MMD patient samples by high-resolution melting (HRM) analysis on a LightCycler 480 System II (Roche Diagnostics, Basel, Switzerland). Primer sequences, PCR conditions, and HRM settings are available on request. HRM analysis with and without the spike-in method was performed for the detection of homozygous mutations.[17] If samples showed any aberrant melting curve patterns, direct sequencing was performed using an ABI Genetic Analyzer 3100 or 3500xL (Applied Biosystems, Foster City, CA) and analyzed with sequence analysis software version 5.1.1 (Applied Biosystems) and Sequencher 4.10 build 5828 (GeneCodes Corporation, Ann Arbor, MI). For exon 61, which bears the c.14576G>A variant, direct sequencing was performed for all patients with MMD and their parental samples. Additional screenings by HRM analysis were performed for the confirmed mutations in up to 283 normal control Japanese individuals. All variants were confirmed with PCR direct sequencing using either genomic DNA or another DNA, amplified with GenomiPhi separately. No discrepancy was seen in the data between the 2 different conditions of DNA.

Statistical analysis. Patients without information for each clinical feature (listed below) were excluded from the analyses (table 1 and tables e-2 and e-3 on the *Neurology®* Web site at www.neurology.org). All statistical analyses were performed using SPSS Statistics 19 (IBM, New York, NY) software. χ^2 tests were applied to compare each categorical phenotype variable between different genotypes: clinical symptom at onset, with/without family history, intellectual impairment, epilepsy, and the unilateral/bilateral distribution of vasculopathy. Nonnormally distributed continuous variables, such as age at onset and the number of steno-occlusive PCA arteries were compared using the Mann-Whitney *U* test and Kruskal-Wallis test between different genotypes. $p < 0.05$ was considered statistically significant. A Kaplan-Meier curve was used to assess the cumulative incidence with the log-rank test. The Cox regression model was used to test which variables were associated with age at onset. The exact 95% confidence interval (CI) of the incidence rate of MMD was calculated according to the binomial distribution. The comparisons of clinical features between parent-offspring pairs or sibling pairs were performed using the Wilcoxon signed-rank test and McNemar test.

RESULTS Identification of *RNF213* variants. c.14576G>A was identified in 39 of 41 patients with familial MMD (95.1%), in 129 of 163 patients with nonfamilial MMD (79.2%), and in 5 of

— 256 —

Table 2 — Distribution of the c.14576G>A variant among patients with MMD, parents of the patients, and normal control Japanese individuals[a]

		c.14576G>A genotype			
	Total	Wild-type: G/G (%)	Heterozygous: G/A (%)	Homozygous: A/A (%)	GA + A/A (%)
Patients with MMD	204	36 (17.6)	153 (75.0)	15 (7.4)	168 (82.4)
Sporadic	163	34 (20.8)	117 (71.8)	12 (7.4)	129 (79.2)
With no other variant	137	20 (14.6)	105 (76.6)	12 (8.8)	117 (85.4)
With one other variant	25	13 (52)	12 (48)	0	12 (48)
With one other homozygous variants	1	1 (100)	0	0	0
Familial	41	2 (4.9)	36 (87.8)	3 (7.3)	39 (95.1)
With no other variant	36	0	33 (91.7)	3 (8.3)	36 (100)
With one other variant	3	0	3 (100)	0	3 (100)
With 2 other compound heterozygous variants	2	2 (100)	0	0	0
Parents of patients with MMD	141	77 (54.6)	63 (44.7)	1 (0.7)	64 (45.4)
Affected	9	0	8 (88.9)	1 (11.1)	9 (100)
Unaffected	132	77 (58.3)	55 (41.7)	0	55 (41.7)
Normal controls	283	278 (98.2)	5 (1.8)	0	5 (1.8)
OR (patients with MMD vs normal control)			236	ND	259
95% CI			91–615		100–674
p (Fisher exact test)			<0.001	<0.001	<0.001

Abbreviations: CI = confidence interval; MMD = moyamoya disease; ND = not determined; OR = odds ratio.
[a] Numbers of patients in each category (%) are shown.

283 normal control Japanese individuals (1.8%) (table 2 and table e-1). Sixty-two pairs of parents were also tested for the c.14576G>A genotype, with the conclusion that the c.14576G>A variant allele was inherited from either or both parents in all patients tested. Among 168 patients with the c.14576G>A variant, 15 had a homozygous change, whereas none of the controls and unaffected parents did. We conclude that the heterozygous c.14576G>A variant increases the risk for MMD with an odds ratio (OR) of 236 (95% CI 91–615, $p < 0.001$). Because no homozygous mutation was detected in the control samples or unaffected family members, the OR for the homozygote could not be calculated (∞), suggesting its strong effect. The incidence rate of MMD was calculated to be extremely high with a 95% CI of 0.78–1.00 with the homozygous mutation. Eighteen other genetic variants beside c.14576G>A were also identified in RNF213 (figure 1, table e-1). Sixteen of them were novel, which had not been reported in the previous studies.[14,15] Two of the variants were also found in the previous study[14]; however, they were thought to be common single nucleotide polymorphisms because they were also found in the normal controls without the significant difference of frequency. Other genetic variants showed a relatively small OR without any significance (table e-1). Thirty-one patients had these individual variants (table 2). Fifteen of them also had the heterozygous

c.14576G>A, and 4 of 5 patients whose parents' samples were available had these 2 variants existing as compound heterozygotes (for example, one variant from the father and the other from the mother). In the other 16 patients having no c.14576G>A, 1 had a homozygous c.13342G>A variant, and 2 had 2 variants: c.13342G>A and c.14053G>A as a compound heterozygote. Of the novel 16 variants, 11 of them were not found in 188 normal control Japanese individuals and were all private mutations (only once in one family).

Correlation between the c.14576G>A genotype and clinical phenotype. We compared the clinical features of patients with MMD according to the c.14576G>A genotype, the wild type (genotype GG, as group GG), the heterozygote (genotype GA, as group GA), or the mutant homozygote (genotype AA, as group AA). Age at onset was lower in AA than in GA or GG ($p = 0.002$ or $p = 0.007$) (figure 2A and table e-2). Median age at onset was 3 years in AA, 7 years in GA, and 8 years in GG. Among those with childhood onset (age at onset <15 years), in whom the effect of secondary vascular changes in later life could be ignored and therefore a pure genetic effect could be expected, the association between earlier onset age and the homozygous c.14576G>A genotype was clearly replicated (table e-2). Although the clinical manifestation is different

— 257 —

A schematic presentation of the RNF213 protein with 3 conserved domains, the genetic variants we have identified, and the genomic structures of 2 RNF213 isoforms (shown from top to bottom). All missense changes, including R4859K (c.14576G>A as larger characters) are indicated. A.A. = amino acids; AAA = ATPases associated with a variety of cellular activities; RING = really interesting new gene. (Based on National Center for Biotechnology Information Reference sequence, NP_065965.4.)

between the childhood-onset group and the adult-onset group, the rates of the patients with this variant, 83.2% (119 of 143 patients) and 79.6% (43 of 54 patients), respectively, were not significantly different. Among adult patients, there was no significant difference in the rate of having this variant between those with familial history (84.6%, 11 of 13 patients) and those without (78.0%, 32 of 41 patients). The univariate Cox regression analysis showed that only the c.14576G>A genotype was the significant predictive variable for age at onset (table e-4). The cumulative incidence of MMD was higher in AA than GA or GG at almost all age distributions (figure 2B), but this tendency was more apparent in the childhood-onset group. Further investigation with more AA and GG patients is necessary for better statistical accuracy. More AA patients were affected before age 4, compared with GA and GG patients ($p < 0.001$) (figure 3A). All AA patients had infarctions at initial presentation.

Figure 2   Correlation between the c.14576G>A variant and age at onset
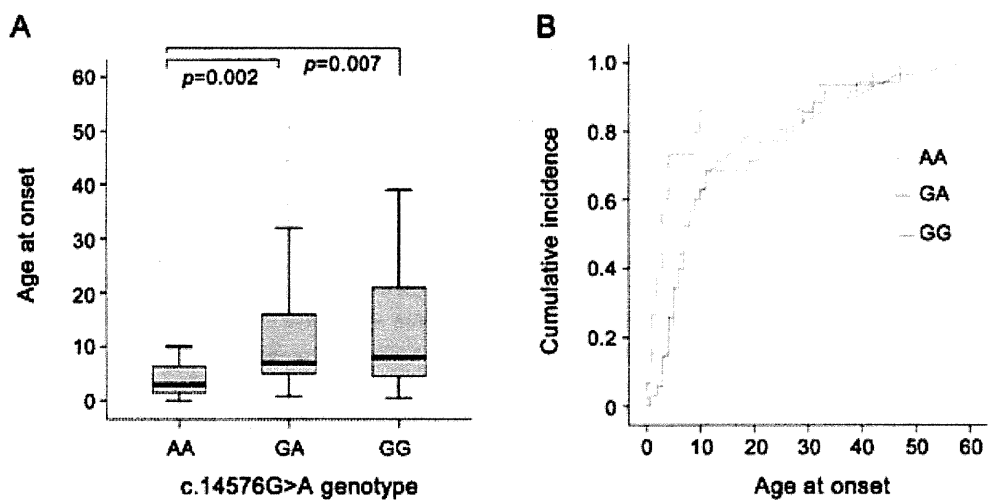
(A) A box plot of age at onset between 3 groups of patients with either the mutant homozygote (AA), heterozygote (GA), or wild type (GG) of the c.14576G>A variant. O indicates mild outliers; △ indicates extreme outliners. (B) Cumulative incidence curve of the 3 groups of patients with either the mutant homozygote (AA), heterozygote (GA), or wild type (GG) of the c.14576G>A variant.

— 258 —
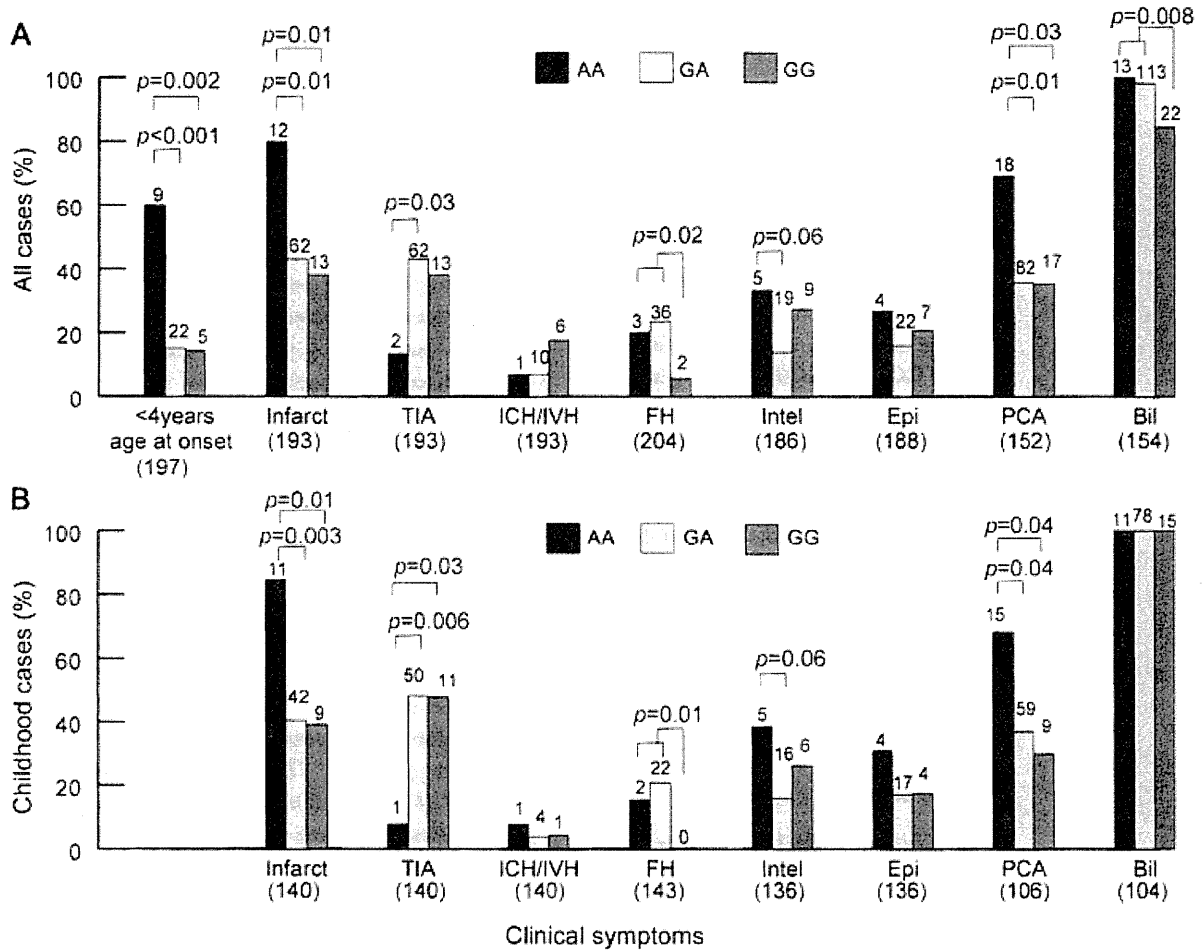
(A) The clinical characteristics of MMD for the 3 groups of patients with either the mutant homozygote (AA), heterozygote (GA), or wild type (GG) of the c.14576G>A variant (204 patients). The numbers of total patients with clinical records regarding either the presence or absence of each characteristic are indicated below the bars, and the numbers of patients in each group are indicated above the respective bars. (B) Clinical characteristics of MMD for the 3 groups of patients with either the mutant homozygote (AA), heterozygote (GA), or wild type (GG) of the c.14576G>A variant among those with age at onset younger than 15 years. The numbers of total patients with clinical records regarding either the presence or absence of each characteristic are indicated below the bars, and the numbers of patients in each group are indicated above the respective bars. Bil = bilateral vasculopathy; Epi = epilepsy; FH = with family history; ICH/IVH = intracranial hemorrhage/intraventricular hemorrhage, Infarct = infarction; Intel = intellectual impairment; PCA = posterior cerebral artery involvement.

The frequencies of other clinical features of MMD in AA, GA, and GG were also compared (figure 3A and table e-2). As the clinical manifestation at diagnosis, infarction was more common in AA than in GA or GG ($p = 0.01$, OR 5.3, 95% CI 1.43–19.56 or $p = 0.01$, OR 6.5, 95%CI 1.53–27.32); TIA was less common in AA than in GA ($p = 0.03$; OR 0.20; 95% CI 0.04–0.94). Bilateral MMD and family history of the disease were more frequent in AA and GA than in GG ($p = 0.008$, OR 11, 95% CI 1.98–66.36 and $p = 0.02$, OR 5.1, 95% CI 1.18–22.36). The number of steno-occlusive PCAs was larger in AA than in GA ($p = 0.01$) (counted as 2 arteries per person). Seventy-four of the 152 patients (48.6%) had PCA lesions, and infarctions and intel-

lectual impairment were more frequent in those with PCA involvement than those without (infarctions 68.9% vs 30.8%, $p < 0.001$; intellectual impairment 26.8% vs 5.3%, $p < 0.001$). Intellectual impairment and epilepsy tended to be a more common complication in AA than in GA, with and without marginal significance. We also compared these clinical features in AA, GA, and GG, excluding 6 patients with unilateral MMD, but the results were not changed (data not shown). Among childhood-onset cases (age at onset <15 years), the associations between the c.14576G>A genotype and these clinical features were generally similar, except for bilateral vasculopathy (all genotypes in childhood-onset cases showed bilateral involvement) (figure 3B).

— 259 —

Correlation between variants other than c.14576G>A and clinical phenotype. We also compared the clinical features of patients with MMD with the other variants, except the c.14576G>A variant, with those without (table e-3). Interestingly, none of the c.14576G>A homozygotes had any other variants. The other patients were categorized into 4 groups, who showed at least one of any individual variants without c.14576G>A (as group GG1), no other variant without c.14576G>A (as group GG0), at least one of any other variants with heterozygous c.14576G>A (as group GA1), and no other variant with heterozygous c.14576G>A (as group GA0). Although there were no differences in age at onset between GG1 and GG0 patients, it was lower in GA0 patients than GA1 patients ($p = 0.03$). Median age at onset was 7 years for GA0 and 12 years for GA1. The frequency of infarctions was lower and that of intracerebral hemorrhage was higher in GA1 than in GA0 ($p = 0.02$, OR 0.19, 95% CI 0.04–0.90 and $p = 0.009$, OR 8.3, 95% CI 2.00–34.19). However, when patients with MMD with another variant, which was predicted to be pathogenic by PolyPhen-2[18] or SIFT[19] algorithms, were compared with those without, consistently no differences in any of these clinical features were observed (data not shown). Further analyses with larger numbers of patients are needed to validate this effect.

Anticipation of MMD. In addition, statistical comparisons of clinical features between 5 parent-offspring pairs having the same *RNF213* genotype (heterozygous c.14576G>A) were performed (table e-5). Age at onset was lower in the second generation than in the first generation ($p = 0.04$). Median age at onset was 5 and 37 years, respectively. This result may support the anticipation of MMD as reported previously.[13] Conversely, age at onset was not different between 6 sibling pairs having the same *RNF213* genotype ($p = 0.67$). Median age at onset was 8 years for the older siblings and 12.5 years for the younger ones. There were no differences in other clinical symptoms among patients from the same pedigree.

DISCUSSION We confirmed a strong association between c.14576G>A in *RNF213* and MMD with the larger number of Japanese patients different from those of the previous studies.[14,15] More importantly, this is the first report showing the significant phenotype-genotype correlation. The OR for the heterozygous c.14576G>A was 236 ($p < 0.001$) and could not be exactly calculated for the homozygote (∞). With the assumption that the effects of both heterozygous and homozygous changes on MMD onset were similar, the homozygous c.14576G>A variant would increase the risk with an OR of 259 (95% CI 100–674, $p < 0.001$). However, the effect of the homozygous variant on MMD onset was expected to be much larger than that of the heterozygote because no homozygote was found in a total of 283 normal controls and 132 unaffected family members in this study and 429 normal controls and 28 unaffected family members in the previous study.[14] We also showed that the risk of being diagnosed with MMD with the homozygous variant was more than 78%. Although this variant does not exactly fit the pure Mendelian inheritance pattern because it is observed to some extent in the normal population, this variant might have a much larger effect on the pathogenesis of MMD than the common variants of complex diseases, considering its extremely high OR. This rare variant could be an example of missing heritability, that is, the majority of heritability of complex traits that are unexplained by common variants with a small effect size.[20,21] Thus, this variant should not be considered as one of common variants contributing to common diseases.

The c.14576G>A variant has not been found among the total number of 55 Caucasian patients in the previous studies on *RNF213*.[14,15] However, 4 other rare variants were identified in 4 of 50 Caucasian patients.[15] The overall variant detection rate for *RNF213* was as high as 90.2% for our Japanese patients, in contrast to 8% for the Caucasian patients in the previous study.[15] Importantly, 82.4% of our patients were accounted for by the c.14576G>A variant. It was reported that c.14576G>A variant was identified in 90% of Japanese patients, 79% of Korean patients, and 23% of Chinese patients.[15] The founder effect widely distributed in some areas of east Asia was likely to be expected, and this variant could explain the difference of prevalence of MMD between Asian and non-Asian populations.

RNF213 is a RING (really interesting new gene) finger protein containing an AAA (ATPases associated with variety of a cellular activities) domain, indicating that it has E3 ubiquitin ligase activity and energy-dependent unfoldase activity.[14,22] Knockdown of *RNF213* in zebrafish leads to the abnormal sprouting and irregular diameter of intracranial vessels, suggesting its possible contribution to vascular formation.[15] More research on its contribution to MMD pathogenesis will be necessary.

Although the number of adult-onset cases was relatively small, the similar rates of the cases with this variant between childhood-onset patients and sporadic adult-onset patients might suggest that the variant apparently contributes to both groups. Either a heterozygous or homozygous c.14576G>A variant increased the risk for adult-onset MMD (OR 217,

95% CI 72–656, $p < 0.001$) compared with that in adult normal controls.

Whether bilateral and unilateral MMD belong to a single entity is a very important question. Of the 6 patients with unilateral MMD, 2 were heterozygotes and the others were wild types, which indicated a lower frequency of heterozygotes than that in the previous study.[14] Because we showed a significant difference in the frequency of bilateral vasculopathy between GG and other genotypes, we speculate that to some extent patients with unilateral MMD share a genetic background, but there could be different genetic backgrounds in these groups. Further investigation is needed to confirm these findings with larger numbers of patients with unilateral MMD.

The recent spread of brain check-up has increased the opportunity to encounter patients with asymptomatic MMD.[23] Whereas our patients in this study all had symptomatic MMD, it is necessary to further examine the *RNF213* variant in the asymptomatic group.

The homozygous c.14576G>A variant carriers showed significantly earlier age at onset, more frequent occurrence of infarctions at initial presentation, and PCA involvement. The association of PCA involvement and infarction or intellectual impairment in our data were compatible with the previous report.[11] These features indicate that c.14576G>A homozygotes have more severe and wider vasculopathy in the brain. The other poor prognostic factors, such as intellectual impairment and epilepsy,[8] were probably more frequent in homozygotes but did not reach statistical significance. We speculated that these conditions might be modified or prevented by early diagnosis and by surgical and medical interventions.

Early surgery for young patients with MMD (<3–4 years of age) has been recommended previously,[24] because they often demonstrate a more severe clinical course.[9,10,24] Approximately 80% of these patients had infarction at initial presentation and had subsequent preoperative infarctions more frequently than patients with older age at onset.[24,25] In our study, 77.1% of the patients diagnosed before age 4 had infarctions at diagnosis, whereas 38% of those diagnosed after age 4 had infarctions ($p < 0.001$), results similar to the previous data.

Conversely, it was demonstrated that young age at onset of symptoms did not always herald a poor later outcome. Instead, neurologic deficits due to infarctions at the time of surgery held the most prognostic value.[7,26] It was recently reported that an irreversible infarction was the greatest risk for an unfavorable outcome by multivariate logistic regression analysis.[8] Specific biomarkers, which might be strongly associated with infarction, would be of invaluable clinical importance to provide the appropriate timing for an operation. In our study, 60% of homozygous c.14576G>A individuals were diagnosed with MMD before age 4, and all of them had infarctions at initial presentation. Thus, the homozygous c.14576G>A variant may be a more specific predictor, which would discriminate those with poor prognosis from those with relatively favorable prognosis among patients with young-onset MMD.

We therefore propose that the homozygous c.14576G>A genotype could be an efficient DNA marker predicting the severe type of MMD with a poor prognosis and a strong biomarker for patients requiring early operation. c.14576G>A genotyping could also be useful to predict the actual risk of severe initial infarctions. Careful follow-up of these high-risk homozygotes could make it possible to undertake intervention before the first infarctions and prevent the irreversible neurologic deficits that can occur in these patients. Thus, the homozygous c.14576G>A variants may provide a better monitoring and prevention strategy. Furthermore, this variant could be very useful in genetic counseling.

— 261 —

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