More recently, a drosophila model of AxD, in which a mutant GFAP gene is expressed in the glia, has been created. In this model, protein aggregation and oxidative stress were induced. This drosophila model is a simple genetic model that may greatly aid in our understanding of the genetics of AxD, as well as in drug screening.[39]

CONCLUSION

Over 60 years has passed since Alexander reported the first case of AxD. Although the characteristics of its pathology, patterns on MRI, and most recently gene mutations are widely known, there may still be undiagnosed cases because AxD is a very rare disease with a variety of clinical phenotypes. The new guidelines we have proposed to classify AxD into three types on the basis of neurological and MRI findings are expected to assist physicians in making an accurate diagnosis. The researches using animal or cell models suggest that the pathology of AxD involves not only mere functional abnormalities in intermediate filaments but also functional abnormalities in astrocytes as well as in neurons. To date, the mechanisms mediating AxD remain unclear. However, the elucidation of these mechanisms using cellular, murine and most recently, drosophila models, continues apace.

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Glial fibrillary acidic protein mutations in adult-onset Alexander disease: clinical features observed in 12 Japanese patients

Yoshida T, Sasayama H, Mizuta I, Okamoto Y, Yoshida M, Riku Y, Hayashi Y, Yonezu T, Takata Y, Ohnari K, Okuda S, Aiba I, Nakagawa M. Glial fibrillary acidic protein mutations in adult-onset Alexander disease: clinical features observed in 12 Japanese patients. Acta Neurol Scand: 2011: 124: 104–108.

Objective – To clarify the clinical manifestations of adult-onset Alexander disease (AOAD) in Japanese patients with glial fibrillary acidic protein (GFAP) gene mutations. *Methods and materials* – Twelve patients of AOAD with *GFAP* mutations detected in our centre were examined for neurological and magnetic resonance imaging (MRI) findings. *Results* – Major symptoms were pyramidal and bulbar signs. In addition, three patients presented abnormal behaviour and/or memory disturbance. Two of the three patients also had Parkinsonism and had been diagnosed with fronto-temporal dementia or progressive supranuclear palsy until *GFAP* mutations were detected. Abnormalities of the medulla oblongata and cervical spinal cord were observed on MRI in all patients. *Conclusions* – Patients presenting with pyramidal and/or bulbar signs with abnormalities of the medulla oblongata and cervical spinal cord on MRI should be considered for *GFAP* analysis as this is the typical presentation of AOAD. Abnormal behaviour and cognitive disorders including deterioration of memory were rare symptoms but could be an obstacle to diagnosing Alexander disease.

T. Yoshida[1], H. Sasayama[1], I. Mizuta[1], Y. Okamoto[2], M. Yoshida[3], Y. Riku[4], Y. Hayashi[5], T. Yonezu[6], Y. Takata[7], K. Ohnari[8], S. Okuda[9], I. Aiba[10], M. Nakagawa[1]

[1]Department of Neurology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto; [2]Department of Neurology and Geriatrics, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima; [3]Department of Neuropathology, Institute for Medical Science of Aging, Aichi Medical University, Aichi; [4]Department of Neurology, Japanese Red Cross Nagoya Daiichi Hospital, Aichi; [5]Department of Neurology and Geriatrics, Division of Neuroscience, Research field of Neurosciences, Gifu University Graduate School of Medicine, Gifu; [6]Department of Neurology, Graduate School of Medicine, Chiba University, Chiba; [7]Department of Neurology, National Hospital Organization Okinawa Hospital, Okinawa; [8]Department of Neurology, University of Occupational and Environmental Health School of Medicine, Fukuoka, Japan; [9]Department of Neurology, National Hospital Organization Nagoya Medical Centre, Nagoya, Japan; [10]Department of Neurology, National Hospital Organization Higashi Nagoya Hospital, Nagoya, Japan

Key words: Alexander disease; glial fibrillary acidic protein; gene analysis; MRI

T. Yoshida, Department of Neurology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kawaramachi Hirokoji, Kajii-chou 465, Kamigyo-ku, Kyoto 602-0841, Japan
Tel.: +81 75 251 5793
Fax: +81 75 211 8645
e-mail: toyoshid@koto.kpu-m.ac.jp

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Introduction

Alexander disease is a rare neuro-degenerative disorder characterized by white matter degeneration and formation of cytoplasmic inclusions called Rosenthal fibres, which have been observed in astrocytes in previous pathological studies (1). Glial fibrillary acidic protein (GFAP) gene mutations have been recently reported in patients with various clinical forms of Alexander disease (2–6). Adult-onset Alexander disease (AOAD) was first recognized after identification of *GFAP* mutations, and it demonstrates different clinical symptoms and magnetic resonance imaging (MRI) findings from infantile forms (6). The correlation between the genotype and various phenotypes of AOAD, however, remains unresolved in Japan, because there were no reports based on a series of cases.

Since 2001, *GFAP* mutations have been analysed in patients suspected of Alexander disease in our centres throughout Japan. The aim of this study was to investigate the neurological manifestations of AOAD in Japanese patients with *GFAP* mutations and to characterize the clinical features observed in these patients.

Materials and methods

In this study, AOAD was defined as the onset of Alexander disease in individuals older than 12 years of age (6). Twenty-eight patients suspected of AOAD were referred from hospitals all over Japan to Kagoshima University (2001–2002) and Kyoto Prefectural University of Medicine (2002) for analysis of possible *GFAP* mutations. The patients were suspected of AOAD because of leuko-encephalopathy without brainstem abnormalities (nine patients); motor disturbance and/or bulbar signs with atrophy of the medulla oblongata and upper cervical spinal cord present on MRI (16 patients); pathological findings demonstrating Rosenthal fibres accumulation, particularly in the astrocyte end-feet in the subpial and perivascular zones (one patient) and motor disturbance and/or bulbar signs without atrophy of the brainstem (two patients).

After obtaining written informed consent from all patients, genomic DNA was extracted from their peripheral blood. Sequence analysis of the genomic DNA was performed to detect the presence of any *GFAP* mutations. Briefly, the coding region and adjacent splice sites were amplified by direct sequence analysis using an ABI PRISM 3100 auto-sequencer (PE Applied Biosystems, Foster City, CA, USA) and Big Dye terminators according to the manufacturer's instructions. The mutations identified were described according to recent nomenclature recommendations. The presence of mutations was confirmed either by other strands or restriction enzyme digestion. Each nucleotide variant detected was tested in 100 unrelated healthy individuals, a comparison that made it possible to distinguish between disease-causing mutations and neutral common variants.

Information on genetic analysis as well as that on the neurological and MRI findings of patients with Alexander disease was obtained from neurologists and neuro-radiologists of each hospital.

Results

Twelve of the 28 referred patients showed heterozygous missense mutations in *GFAP*, of which 11 patients were referred because of atrophy of the medulla oblongata and upper cervical spinal cord and one patient was referred because of pathological findings consistent with Alexander disease. *GFAP* mutations were not detected in any of the nine patients who presented with leuko-dystrophy without atrophy of the medulla oblongata and upper cervical spinal cord.

The clinical features of AOAD patients with neurological manifestations are summarized in Table 1. Patient age at the time of gene analysis ranged from 24 to 73 years and that at AOAD, onset ranged from 18 to 64 years. Patients 1–5 were from the same family, wherein patients 1 and 4 were siblings; patients 2 and 3 were the daughter and son of patient 1, respectively, and patient 5 was a son of patient 4. Each family member presented with different clinical forms of Alexander disease of which three patients presented with adult form (patients 1, 2 and 4), two patients presented with only pyramidal signs and atrophy of the medulla oblongata and spinal cord on MRI (patients 3 and 5) and one patient, who was the other son of patient 4, presented with the juvenile form. The details of clinical symptoms in patient 1–3 have been described (7). Patient 6 has been described as the first case of AOAD with *R416W* mutation (8). Patient 7, who carried *M74T* mutation and has also been described, who clinically presented with bulbar and pyramidal signs, showed abnormal high intensities in the ventral medulla oblongata and marked atrophy of the medulla oblongata and spinal cord (9). Patient 11 also carried the *M74T* mutation and showed bulbar and pyramidal signs with atrophy of the medulla oblongata and spinal cord. However, patients 7 and 11 did not have a blood relationship. *R258C* mutation, which was detected in patient 8, is a novel mutation. Patient 8 seemed to have a family history of AOAD. The son of patient 8 had gait disturbance and dysphagia for several years, but gene analysis could not be performed for him because he had committed suicide at the age of 45. A brother of the patient exhibited abnormal behaviour at the age of 58 and was later diagnosed with progressive supranuclear palsy (PSP). He passed away at the age of 68 because of brain infarction. The patient 8 presented with abnormal behaviour at the age of 59. Visual and auditory hallucinations occurred at the age of 62. One year later, gait disturbance occurred. At the age of 64, she presented with vertical supra-nuclear palsy, balance difficulty, falling and retrocollis, and she was, therefore, diagnosed with PSP. She also showed euphoria, confabulation and positive snout reflex. The score of Hasegawa dementia scale revised, the maximum

Table 1 Neurological and MRI findings in 12 adult-onset Alexander disease

Case	1	2	3	4	5	6	7	8	9	10	11	12
Age of onset	53	27	–	44	–	24	51	59	64	36	51	18
Age of gene analysis	58	38	32	58	33	30	53	73	67	37	55	24
Sex	F	F	M	F	M	M	M	F	M	M	M	F
Neurological findings												
DTR UL/LL	↑/↑	↑/↑	↑/↑	↑/↑	↑/↑	↑/↑	↑/↑	N~↓/↑	↑/↑	N/N	↑/↑	↑/↑
Muscle weakness	+	+	–	–	–	+	–	–	–	–	+	+
Babinski	+/+	+/+	+/+	+/+	+/+	+/+	–/–	–/–	+/+	–/–	+/+	+/+
Sensory disturbance	+	–	–	+	–	+	–	+	+	+	+	–
Dysarthria	+	+	–	–	–	+	+	–	+	–	–	–
Dysphagia	–	–	–	+	–	–	+	–	+	–	–	+
Ataxia	+	+	–	–	–	+	–	–	+	+	–	+
Nystagmus	–	–	–	–	–	+	–	+	–	+	+	–
Palatal myoclonus	+	+	–	–	–	+	–	–	–	–	–	–
Autonomic dysfunction	+	–	–	+	–	+	–	–	+	+	–	–
Dementia	–	–	–	–	–	–	–	+	+	+	–	–
Convulsion	–	–	–	–	–	–	–	–	–	–	–	–
Muscle rigidity	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	+	–	n.d.	–
Other symptoms		Scoliosis						Blindness		Strabismus		Scoliosis
MRI findings												
Atrophy of medulla oblongata	+	+	+	+	+	+	+	+	+	+	+	+
Atrophy of spinal cord	+	+	+	+	+	+	+	+	+	+	+	+
Atrophy of cerebellum	–	–	–	–	–	–	–	–	–	+	+	+
White matter lesion	+	–	+	+	+	+	–	+	+	+	–	–
Periventricular rim	+	–	+	+	+	–	–	–	+	–	–	–
Abnormalities of thalamus or basal nuclei	–	–	–	–	–	–	–	–	–	+	–	+
Contrast enhancement	–	–	–	–	–	–	–	n.e	–	–	n e	–
GFAP mutation	V87G	V87G	V87G	V87G	V87G	R416W	M74T	R258C	R70W	R79H	M74T	L357P
References	(7)	(7)	(7)	This study	This study	(8)	(9)	This study	This study	This study	This study	This study

↑, increased; ↓, decreased; n.d., not described; n.e., not examined.

of which is 30 points and <20 points is considered consistent with dementia, was 13 points. MRI showed atrophy of the medulla oblongata, spinal cord and cerebellum and showed hyperintensities and atrophy of cerebral white matter with frontal predominance. Single photon emission computed tomography (SPECT) showed a marked decrease in cerebral blood flow in the frontal and temporal lobes. She passed away at the age of 74, and autopsy findings showed numerous Rosenthal fibres, a hallmark of Alexander disease, in the subpial region, frontal white matter, substantia nigra, the dentate nucleus, etc. Therefore, the pathological findings were a clue to *GFAP* analysis. *GFAP* mutations, seen in patient 9, 10 and 12, were already known to exist in Alexander disease [(p.R70W (10, 11), p.R79H (2, 12–15), L357P (16)]. Patient 9 with an *R70W* mutation showed cognitive disorders like fronto-temporal dementia and lead-pipe rigidity in the neck and upper extremities in addition to bulbar and pyramidal signs. His cognitive dysfunction started at the age of 64. MRI showed atrophy of medulla oblongata and spinal cord (Fig. 1). In addition, small foci of age-related signal changes were present in the cerebral white matter. Patient 10 showed ataxia, autonomic dysfunction and dementia without bulbar and pyramidal signs. Atrophy of the medulla oblongata and spinal cord

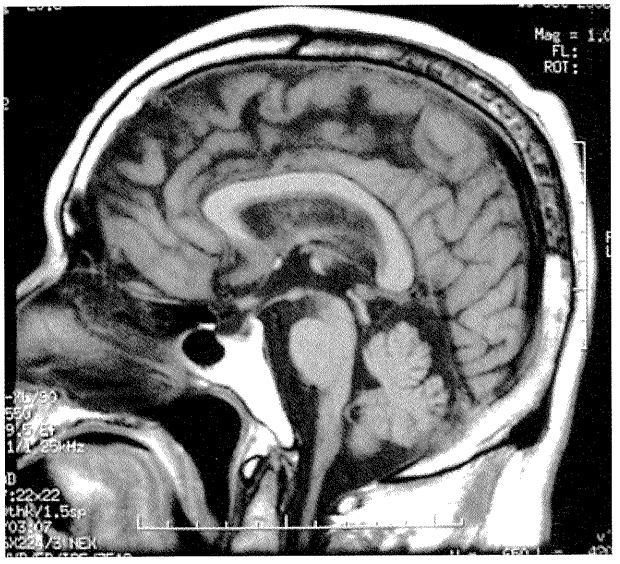

Figure 1. T1-weighted image of patient 9 with R70W mutation shows marked thinning of the medulla and upper cervical spinal cord.

were a clue to perform *GFAP* analysis. Patient 12 showed bulbar and pyramidal signs as well as scoliosis with atrophy of the medulla oblongata and spinal cord on MRI.

Eleven of 12 patients (92%) with heterozygous missense mutations in GFAP presented with hyper-reflexia of the lower limbs. Babinski sign was present in 9 of 12 patients (75%). Muscle weakness, which is often asymmetric in early stages, was present in five patients (42%). Dysarthria (five patients, 42%), truncal and/or limb ataxia (six patients, 50%), nystagmus (four patients, 33%) and autonomic dysfunction, including orthostatic hypotension and bladder dysfunction (five patients, 42%), were all relatively common. Dysphagia was present in four patients (33%). Palatal myoclonus was seen in only three patients (25%). Sensations tended to be impaired in some patients – six patients had decreased vibration sense in the lower limbs (50%) and one patient presented with decreased tactile sensation in the mandibular nerve area. Parkinsonism, including lead-pipe rigidity and bradykinesia, was present in two patients. Two patients had scoliosis. Patient 8 and 9 showed abnormal behaviour, cognitive disorder and deterioration of memory. In both cases, SPECT showed a marked decrease in cerebral blood flow in the frontal and temporal lobes. Sleep disorders, including sleep apnoea and restless legs syndrome, were not observed in any patients. All patients presented with mild to severe atrophy of the medulla oblongata and upper cervical spinal cord on MRI (Fig. 1). Cerebral white matter abnormalities were observed in eight patients (67%). However, distribution of these abnormalities was not frontal dominant but was diffuse and mild. Contrast enhancement was not observed in this study. Signal abnormalities of the basal ganglia and thalami and atrophy or signal abnormalities of the cerebellum were observed in three patients and two patients, respectively.

Discussion

Because *GFAP* mutations were detected as a cause of Alexander disease, various symptoms have been reported in AOAD (3–6), such as those mimicking multiple sclerosis or brain tumour (6).

In the present study, pyramidal signs, including hyper-reflexia and/or the Babinski sign in the lower limbs (observed in 92% of the patients), could be considered characteristic neurological manifestations. Bulbar signs, including dysarthria and dysphagia, cerebellar ataxia and nystagmus were relatively frequent in Japanese patients examined and could therefore be considered cardinal symptoms of AOAD. Palatal myoclonus, which has been regarded as a frequent symptom of AOAD (6), was relatively rare in the cases examined. Symptoms of Parkinsonism, including lead-pipe rigidity and bradykinesia, were also relatively rare, but it is an important symptom in terms of mimicking PSP. Abnormal behaviour and cognitive dysfunction could be an obstacle to consideration of Alexander disease. Sleep disorders, including sleep apnoea and restless legs syndrome, which are often present in non-Japanese patients (17), were not present in any of the cases examined. However, this might not indicate that sleep disorders are a rare symptom in Japan, but that they have not been fully recognized among Japanese neurologists. Therefore, it is necessary for Japanese neurologists to have a better understanding of sleep disorders. In the present study, brainstem lesions, including atrophy of the medulla oblongata, were present in all patients examined. Cerebral white matter abnormalities were shown in 8 patients, and periventricular rim had been present in five patients; however, no case satisfied the criteria proposed for MRI diagnosis of Alexander disease (18). Farina et al. (19) described that atrophy and changes in signal intensity in the medulla oblongata and upper spinal cord are MRI diagnostic features of AOAD. A characteristic finding on MRI in our series was mild to severe atrophy of the medulla oblongata and upper cervical spinal cord, which supports the observation that signal abnormalities or atrophy of the medulla or spinal cord on MRI are a hallmark of late-onset Alexander disease with *GFAP* mutation (4, 19) rather than the MRI criteria proposed in 2001 (18).

Cerebral white matter abnormalities were observed in two-thirds of the cases examined. However, these lesions were atypical because they were symmetrical and mild and did not always show frontal predominance. These findings also support the findings of a previous study which found that cerebral white matter abnormalities observed on MRI were minimal to moderate in patients with AOAD (19). Atrophy or signal abnormalities of the cerebellum were relatively less common in the cases examined in the present study, although ataxia was a frequent clinical symptom.

In this study, three patients who showed typical symptoms of AOAD, including bulbar signs, pyramidal signs in the lower limbs and typical MRI findings indicating atrophy of the medulla oblongata and upper cervical spinal cord, did not have *GFAP* mutations. These patients also did not have any *GFAP* polymorphisms. No *GFAP* muta-

tions were observed in approximately 10% of patients with Alexander disease, in whom other genetic or as yet unknown environmental factors have been hypothesized to influence the phenotype (3, 6). These cases, therefore, require prudent diagnosis including brain biopsy.

A limitation of this study is that information about the clinical manifestations of AOAD in patients with *GFAP* mutations was based not on strict clinical criteria but on reporting by each neurologist. Sleep disorders, which are often present in non-Japanese patients, were not noted or reported in the cases examined in the present study. To clarify the clinical features in more detail, a population-based study with more detailed clinical criteria including sleep disorders is needed.

Pyramidal signs, including hyper-reflexia, Babinski sign with or without bulbar signs, autonomic failure or ataxia, could warrant *GFAP* analysis and could be useful to diagnose AOAD in Japanese patients, which has been reported in previous studies as well (6, 19). Besides, in Japan, it should be noted that abnormal behaviour and cognitive disorders including deterioration of memory could be a conspicuous symptom of AOAD.

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Case report

Serial MRI changes in a patient with infantile Alexander disease and prolonged survival

Takashi Shiihara [a,b,*], Toru Yoneda [a], Ikuko Mizuta [c], Tomokatsu Yoshida [c], Masanori Nakagawa [c], Nobuzo Shimizu [a,d]

[a] *Department of Neurology, Gunma Children's Medical Center, Gunma, Japan*
[b] *Department of Pediatrics, Dokkyo Medical University, Saitama, Japan*
[c] *Department of Neurology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan*
[d] *Gunma Rehabilitation Centre for the Physically Handicapped Children, Gunma, Japan*

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Abstract

Alexander disease is a major entity of leukodystrophy; magnetic resonance imaging (MRI) studies of the brain typically show extensive changes in the cerebral white matter with frontal predominance. Heterozygous missense mutations of *GFAP* are thought to be sufficient for the molecular diagnosis, which has widened the Alexander disease entity beyond the classical one. We report the patient, a 16-year-old Japanese boy, with infantile-onset Alexander disease, showing striking MRI findings; extreme white matter loss of cerebrum through cerebellum, severe atrophy of basal ganglia, cerebellum, brain stem, and cervical spinal cord. Molecular analysis showed a heterozygous mutation R239L (c.730G > T) in *GFAP*. A relative long disease course, over 15 years, with the help of mechanical ventilation revealed the striking MRI progression.

Keywords: Infantile Alexander disease; *GFAP*; R239L; White matter; Cerebellum

1. Introduction

Alexander disease (OMIM #203450), a major entity of leukodystrophy, was originally defined pathologically by an accumulation of rod-shaped eosinophilic deposits, recognized as Rosenthal fibers within astrocytes, and demyelination of the white matter [1,2]. Magnetic resonance imaging (MRI) studies of the brain typically show extensive changes in the cerebral white matter with frontal predominance [3]. Since Brenner et al. revealed that heterozygous missense mutations of glial fibrillary acidic protein (GFAP) gene were associated with Alexander disease, showing a heterozygous mutation in *GFAP* is thought to be sufficient for the molecular diagnosis [1,4]. This relative ease for the diagnosis has widened the Alexander disease entity beyond the classical one [1,5]. Here, we report on an Alexander disease patient with a heterozygous mutation R239L (c.730G > T) in *GFAP*, showing a striking MRI progression over 15 years.

2. Case report

The patient (a 16-year-old boy) was the first child of non-consanguineous parents. There was no history of neurological illness in previous generations. He was born at 38 weeks of gestation by normal delivery after an uncomplicated pregnancy. At the time of birth, he weighed 2940 g (−0.24, standard deviation (SD)), his

* Corresponding author at: Department of Neurology, Gunma Children's Medical Center, 779 Shimohakoda Hokkitsu-machi, Shibukawa, Gunma 377-8577, Japan. Tel.: +81 279 52 3551; fax: +81 279 52 2045.
E-mail address: shiihara-ind@umin.net (T. Shiihara).

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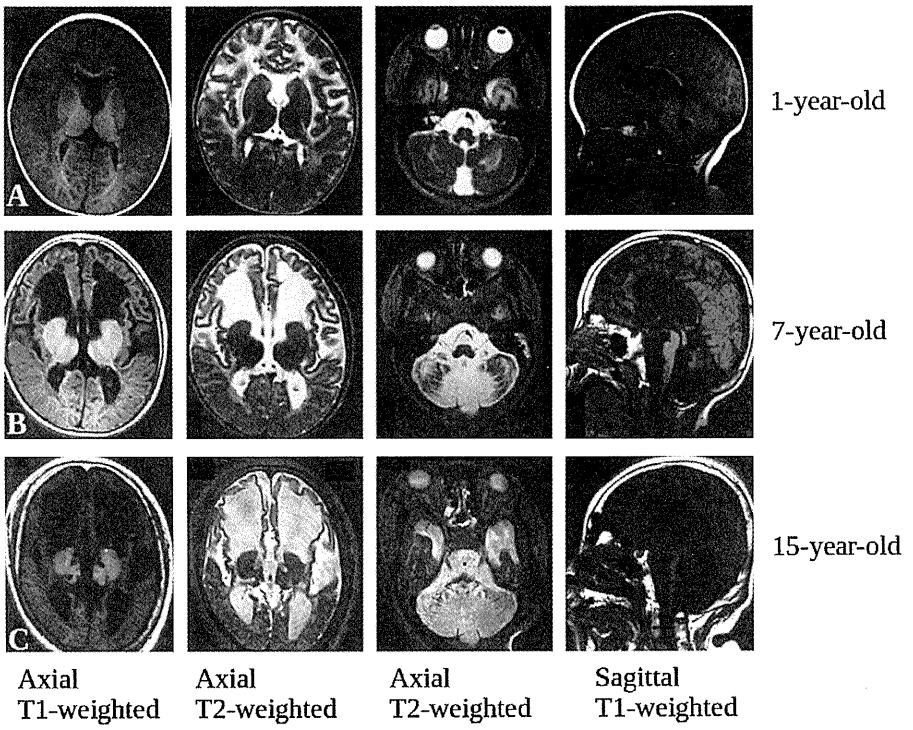

Fig. 1. The rows A–C show MRI studies of our patient (axial T1-weighted image at the level of basal ganglia, axial T2-weighted image at the level of basal ganglia, axial T2-weighted image at the level of cerebellum, and mid-sagittal T1- weighted image), performed at age 1 year-6 months, 7 years-2 months, and 15 years-11 months. Cerebral white matter signal-intensity abnormalities (low T1-weighted and high T2-weighted) with frontal predominance involving the arcuate fibers, the external and the extreme capsule, and basal ganglia symmetric signal-intensity abnormalities involving mainly the caudate and putamen, are already apparent in A. Cerebellar white matter signal-intensity abnormalities are also noted in A. Frontal dominant cerebral white matter cavitation, cerebral and cerebellar white matter volume loss, and brain-stem and cerebellar atrophy are apparent in B. Cerebral and cerebellar white matter have almost vanished in C. Severe brain-stem, cerebellar, and cervical spinal cord atrophy are also noted in C.

length was 51.5 cm (+1.19, SD), and his head circumference (HC) was 32.0 cm (−0.93, SD); serial HC measurements through age 4 years were all within normal limits (i.e., 48.6 cm (+0.27, SD), 48.8 cm (−0.25, SD), and 50.0 cm (−0.29, SD) at age 18 months, 2 years-8 months, and 4 years-5 months, respectively). His developmental milestones were delayed during infancy (i.e., visual tracking at age 2 months, head control at 5 months, sitting unsupported at 8 months, walking with aid at 1 year-7 months and speaking at 1 year-3 months). At 1 year-6 months, after a bout of convulsion, he came to our hospital. Brain computed tomography showed symmetric low density areas in the white matter of the frontal lobe extending into the parietal lobes (figure not shown). Thorough examinations, such as brain magnetic resonance imaging (MRI), electrophysiologic studies, cerebro-spinal-fluid examination, and lysozomal enzyme assays, were done, remained inconclusive. The treatment with anticonvulsants was not successful. Subsequently, he showed psychomotor regression. Around age 6 years, he became bedridden and gradually respiratory insufficiency was evident. At age 8 years, he received a tracheotomy, becoming ventilator dependent.

Since age 1 year-6 months, brain MRIs were done at various intervals until age 7, which showed broad symmetrical white matter involvements, extending from the cerebrum through the cerebellum, with progressive white matter cavitation or loss (Fig. 1A and B). Cerebral frontal lobe dominant white matter abnormalities, symmetrical basal ganglia involvements, periventricular rims, and cerebellar atrophy were also noted.

At age 15 years, he showed macrocephaly; his HC was 59.5 cm (+3.2, SD), repetitive facial myoclonus, poor visual fixation or tracking, auditory startle response, severe muscle atrophy of all extremities, and the loss of deep-tendon reflexes. He had a sleep wake cycle and could show some vague facial expressions, indicating comfort or discomfort. Brain MRI revealed extreme white matter loss of the cerebrum through the cerebellum, severe atrophy of basal ganglia, cerebellum, brain stem, and cervical spinal cord (Fig. 1C). Table 1 summarizes serial MRI changes in this patient (Table 1). After obtaining parental informed consent, gene analysis revealed a heterozygous mutation R239L (c.730G > T) in *GFAP* (Fig. 2).

Table 1
Serial MR1 changes in our patient.

Age	Main findings	
	Signal-intensity abnormalities	Volume abnormalities
1-year-6-month	Cerebral white matter (frontal dominant), basal ganglia (symmetrical), cerebellar white matter and periventricular rims	
2-year-8-month		Cerebral white matter atrophy and glimpse of cerebral white matter cavitation (frontal lobe)
3-year-1-month		Glimpse of basal ganglia atrophy
4-year-1-month		White matter cavitation and atrophy progressed (frontal lobe)
5-year-0-month	Cerebellar white matter abnormally progressed	White matter cavitation and atrophy progressed (frontal lobe) and glimpse of cerebellar and brain-stem atrophy
7-year-2-month		Cerebral white matter cavitation (extending to parietal lobe), cerebral, basal ganglia, cerebellar and brain-stem atrophy progressed
15-year-11-month	White matter signal abnormalities less visible (due to extensive cavitation or atrophy)	Almost vanished cerebral and cerebellar white matter, severe brain-stem, cerebellar, and cervical spinal cord atrophy

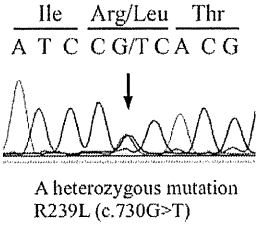

Fig. 2. Direct sequencing analysis of the patient shows a heterozygous mutation R239L due to a G to T transversion (c.730G > T; arrow) of *GFAP*.

3. Discussion

The clinical presentation of Alexander disease can be divided into three groups, infantile, juvenile, and adult [2]. van der Knaap et al. defined the five MRI criteria for infantile or juvenile Alexander disease, such as extensive cerebral white matter changes with frontal predominance, a periventricular rim with high signal on T1-weighted images and low signal on T2-weighted images, abnormalities of basal ganglia and thalami, brain stem abnormalities, and contrast enhancement of particular gray and white matter structures [3]. We did not employ gadolinium enhancement, however; the four MRI criteria out of five, other than the contrast enhancement, were fulfilled on his early MRI. Although, some of infantile Alexander disease patients did not manifest macrocephaly and others manifested it after infancy, the lack of macrocephaly, progressive white matter cavitation and volume loss led us to almost disregard his diagnostic possibility of Alexander disease, and vanishing white matter became a tentative diagnosis [6,7]. R239 is one of hotspots of *GFAP* mutations and an infantile Alexander disease patient with R239L mutation was previously reported [1,4,8]. Thus, the heterozygous R239L mutation in our patient made the Alexander disease diagnosis certain.

Although there are some reports of Alexander disease patients with cerebral white matter cavitation, as far as we know, the extensive cavitation, as though almost all white matter has vanished not only in the cerebrum but also in the cerebellum, seen in our patient, has not been reported [6,9]. Brain-stem and cervical spinal cord involvements, seen in our patient, are common findings in adult-onset Alexander disease [10]. In his early MRI, the supratentorial lesions progressed diffusely preserving the basic characteristics of infantile Alexander disease; a frontal predominance of the white matter abnormalities relatively sparing the frontal cortex and the occipital lobe [2,3]. After infancy, subtentorial lesions gradually developed in the whole rhombencephalon, finally ending in the twiggy brain-stem and the nearly vanishing cerebellum, representing the possible extreme end stage of adult type Alexander disease [10]. So, it would be said that he had serially demonstrated the extreme characteristics of each type Alexander disease in a period of 15 years, with the help of mechanical ventilation (and, of course, his parents' love and dedication). Toxic gain-of-function of GFAP has a pivotal role in Alexander disease pathogenesis [1,2]. The lesional distribution difference in each Alexander disease subtype might result from not only the disease severity but also an age-dependent astrocyte activity in a particular part of brain. Until now the precise data are lacking, therefore, it is a mere speculation, awaiting data from further studies'.

Acknowledgments

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ORIGINAL COMMUNICATION

Late-onset Alexander disease with a V87L mutation in glial fibrillary acidic protein (GFAP) and calcifying lesions in the sub-cortex and cortex

Hidekazu Suzuki · Tomokatsu Yoshida · Mari Kitada · Juri Ichihashi · Hiroshi Sasayama · Yoshiro Nishikawa · Yoshiyuki Mistui · Masanori Nakagawa · Susumu Kusunoki

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Abstract *Glial fibrillary acidic protein (GFAP)* mutation has been reported in Alexander disease. We report a 31-year-old woman suffering from Alexander disease with a V87L mutation in *GFAP*. She showed psychomotor regression and a history of seizures, in addition to pendular nystagmus, dysarthria, spastic gait, and bladder dysfunction. Brain magnetic resonance imaging (MRI) showed atrophy of the medulla oblongata and mild cervical cord atrophy, deep white matter abnormalities, periventricular rim, and signal changes of the medulla oblongata and dentate hilum. Sequence analysis of her *GFAP* gene showed a heterozygous c.273G>C mutation predictive of a p.V87L amino acid substitution. We concluded that she was actually affected with Alexander disease. Twenty months later she fell down and sustained a head contusion. Urgent head computed tomography (CT) showed calcification in the subcortical and cortical regions, which may relate to the psychomotor regression and history of seizures. Calcification in the subcortical and cortical regions on head CT has not been reported in Alexander disease; this may be associated with a V87L mutation in *GFAP*.

Keywords Alexander disease · GFAP · V87L · Calcifications

H. Suzuki (✉) · M. Kitada · J. Ichihashi · Y. Mistui · S. Kusunoki
Department of Neurology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan
e-mail: hide_green@74yahoo.co.jp

H. Suzuki · Y. Nishikawa
Department of Neurology, Otemae Hospital, Osaka, Japan

T. Yoshida · H. Sasayama · M. Nakagawa
Department of Neurology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan

Introduction

Alexander disease is a leukodystrophy that is pathologically characterized by the astrocytic inclusion known as Rosenthal fibers, which accumulate particularly in the end-feet of the astrocytes in the subpial and perivascular zones, consisting of GFAP, heat shock protein 27, and αB-crystallin [1, 2]. Clinically, Alexander disease is classified into three subtypes: infantile, juvenile, and adult forms, based on the age at disease onset. The infantile form is the most common and usually presents before 2 years of age, showing macrocephaly, psychomotor regression, bulbar dysfunction, and seizures, and leads to death before 10 years of age. The juvenile form usually presents between 2 and 12 years of age, and the clinical course progresses more slowly than that of the infantile form. The symptoms of the adult form are similar to those of the juvenile form, with onset occurring later, but it is reported that mental function is normal and seizures are uncommon in the adult form. Recently, *GFAP* mutations have been reported in various forms of Alexander disease [3]. We report a 31-year-old woman suffering Alexander disease with a V87L mutation in *GFAP* who had psychomotor regression and a history of seizures with calcification in the subcortical and cortical regions on head CT.

Case report

A 31-year-old woman presented with slowly progressive gait disturbance of several years. The family history was

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negative for neurological disease. She had had episodes of tonic-clonic seizures and been prescribed anticonvulsants from the ages of 10 months old to 12 years old. Mental function began to regress slowly from around 15 years of age. Gait disturbance appeared at 25 years old. She was referred to our hospital because of her tendency to fall down, which began at approximately 30 years old. Physical examination showed kyphosis. Neurological examination showed psychomotor regression (16/30 in the Mini-mental state examination), pendular nystagmus, dysarthria, dysphagia, cerebellar ataxia, spastic gait, and urine incontinence. However, no palatal tremor was detected. Deep tendon reflexes were systemically increased, and pathological reflexes were bilaterally positive. Brain MRI showed atrophy of the medulla oblongata, and mild atrophy of the cervical cord, deep white matter abnormalities, periventricular rim, and signal changes of the medulla oblongata and dentate hilum (Fig. 1a–f). We considered her to be suffering from Alexander disease. After informed consent had been obtained from her parents, genomic DNA was extracted from the peripheral blood, and DNA sequence analysis of the *GFAP* was performed. Briefly, the coding region and the adjacent splice sites were amplified for direct sequence analysis with an ABI PRISM 310 auto sequencer (PE Applied Biosystems, Foster City, CA) using Big Dye terminators according to the manufacturer's instructions. Sequence analysis of the *GFAP* gene showed a heterozygous c.273G>C mutation, predictive of a p.V87L amino acid substitution (Fig. 2). We concluded that she was actually affected with Alexander disease. Twenty months later she sustained a head contusion due to the progressive gait disturbance. Urgent head CT showed calcifications, which were subtle in the basal ganglia but more relevant in the subcortical and cortical regions (Fig. 1g–i). The other causes of intracerebral calcification, such as hypoparathyroidism, celiac disease, or Fahr's disease, were ruled out on the grounds of clinical findings, i.e., normal serum calcium and parathyroid hormone levels, and the absence of antibodies to gliadin and tissue transglutaminase. Furthermore, 6 months later, when she had aspiration pneumonia, palatal tremor was detected. An endoscopy to evaluate swallowing function showed larynx (including vocal cord) tremor synchronous with palatal, pharynx, and tongue tremor.

Discussion

Bulbar symptoms, pyramidal signs, and cerebellar ataxia are prominent clinical features of Alexander disease in both juvenile and adult forms. The juvenile form shows mental retardation and often seizures [4]. As for brain MRI features in the juvenile form, white matter abnormalities with frontal predominance are observed in approximately 60% [4]. It is reported that mental function is normal and seizures are uncommon in the adult form [3, 4]. As for brain MRI features in the adult form, marked atrophy of the medulla oblongata or upper cervical cord are observed in approximately 90% [4, 5]. Deep white matter abnormalities are observed in approximately only half of the cases in the adult form, and the absence of deep white matter abnormalities are significantly associated with an older onset age. Brainstem signal changes are observed in approximately 40% and may be significantly associated with a younger onset age [5]. Our case could be the juvenile form, considering that she had psychomotor regression and a history of seizures, had deep white matter abnormalities, and brainstem signal changes in the brain MRI. However, although seizures were observed from 10 months old, an attempt to taper anticonvulsants was successful at 12 years old. Thereafter, mental function began to regress slowly from around 15 years of age. Gait disturbance appeared at 25 years old. The specific onset age of her symptoms was not clear. Therefore, the clinical features of this patient may be intermediate between juvenile and adult forms. Recently, a new guideline for diagnosing Alexander disease has been proposed, and three types according to neurological and MRI features instead of onset age are analyzed, which consist of the cerebral form (type 1), bulbospinal form (type 2), and intermediate form (type 3) [6]. Our case was type 3. Sequence analysis of the *GFAP* gene led to diagnosing Alexander disease.

Sequence analysis of the *GFAP* gene in our case showed a heterozygous c.273G>C mutation, predictive of a p.V87L amino acid substitution. Okamoto et al. [7] reported familial cases of suspected adult onset Alexander disease with a V87G mutation in *GFAP*. In that report, three patients with a V87G mutation in *GFAP* were described, two of whom (a 58-year-old woman and a 38-year-old woman) presented with ocular motor abnormality and ataxia, palatal tremor, and pyramidal signs similar to our case with a V87L mutation. Although atrophy of the medulla oblongata and spinal cord, and white matter lesions in brain MRI were detected in both our case and in patients with the V87G mutation, psychomotor regression and seizure were not observed in patients with the V87G mutation.

Palatal tremor is reported to be observed in about 30% of adult form patients and in only 2% of juvenile form patients [4]. In a few cases, palatal tremor, which also involved the tongue, pharynx, platysma, jaw, and diaphragm, was reported [3, 4, 8, 9], while in our case the larynx (including vocal cord) tremor synchronous with palatal, pharynx, and tongue tremor was found by an endoscopy.

The calcifying lesions in the subcortical or cortical regions on head CT have not been reported in Alexander disease. Only three cases with calcifying lesions on head CT have been reported before, in one child with genetically

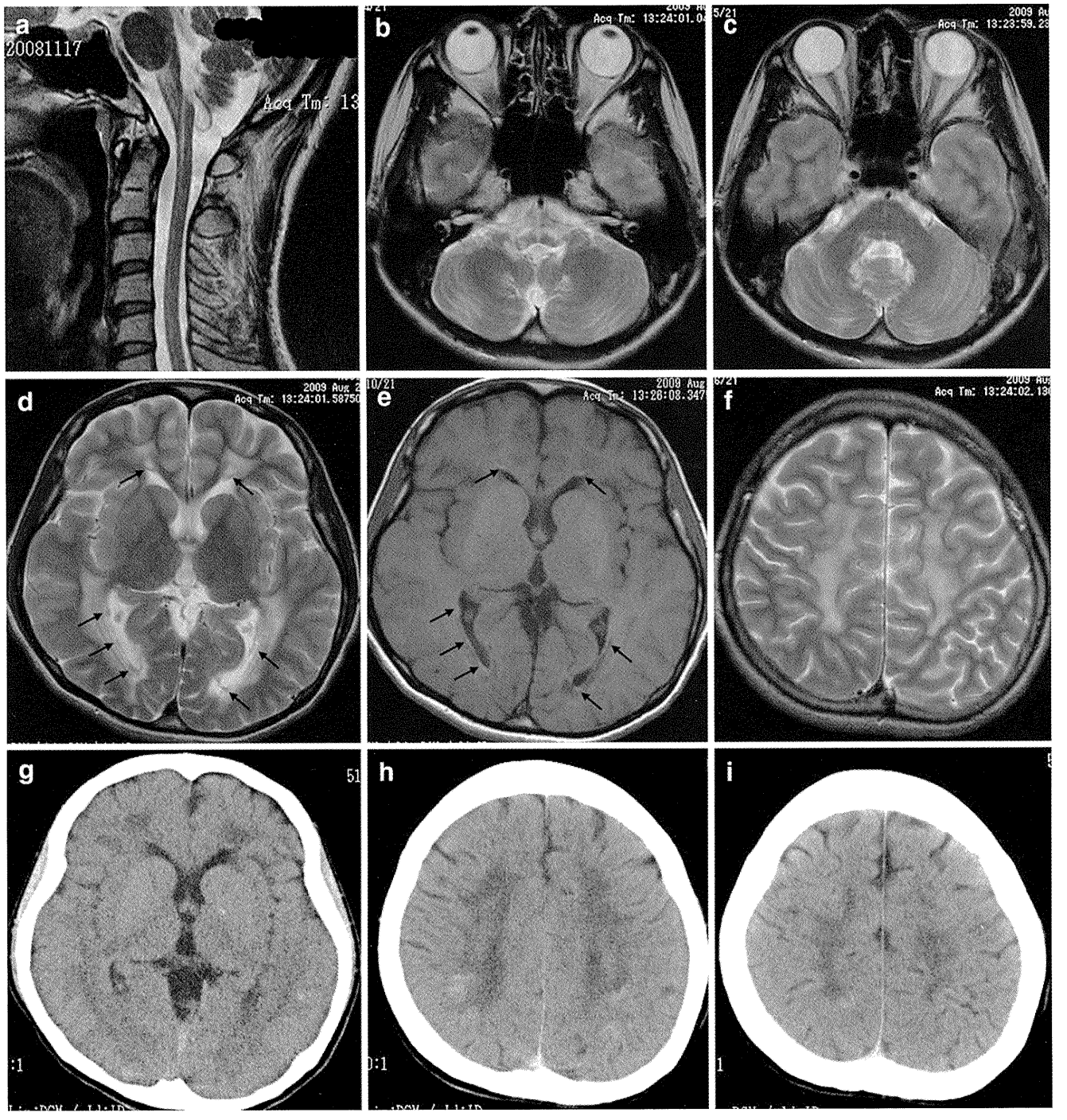

Fig. 1 T2-weighted sagittal section showing marked atrophy of the medulla oblongata and mild cervical spinal cord atrophy (**a**). T2-weighted axial images showing marked atrophy of the medulla oblongata (**b**), with slight cerebellar atrophy and enlargement of the fourth ventricle (**c**). Abnormal high intensities in dentate hilum and medulla oblongata were detected bilaterally (**b**, **c**). Periventricular rim decreased signal on T2-weighted axial images (*arrows*, **d**), increased signal on T1-weighted images (*arrows*, **e**), and marked white matter lesions on T2-weighted images (**d**, **f**). Mild increased signal in putamina was detected bilaterally on T1-weighted images (**e**). Computed tomography showed calcifications, which were subtle in the basal ganglia but more relevant in the subcortical and cortical regions (**g**, **h**, **i**)

confirmed Alexander disease [10] and in two adults reported in the pre-molecular era [11, 12]. In the pediatric case the calcifications were in the periventricular regions [10]. In two adult cases, one was a 28-year-old woman with calcifications in the basal ganglia [11]; the other was a 39-year-old woman with calcifications in the caudate nuclei [12]. In the latter case the calcifications were found in the thalamus and basal ganglia, with fine perivascular

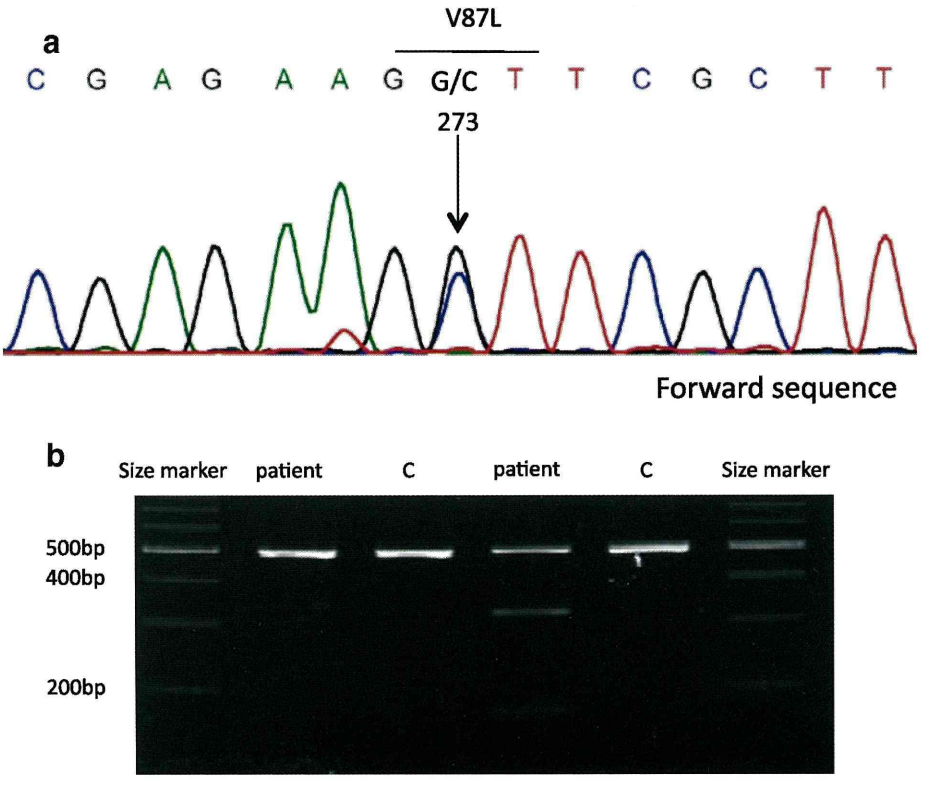

Fig. 2 Direct sequence analysis of the patient showed a heterozygous V87L mutation due to a G to C transversion (c.273G>C, *arrow*) of *GFAP* (**a**). c.273G>C produces a new recognition site for *Hind* III (**b**)

deposits in the cerebral cortex at necropsy [12]. Interestingly, she had mild psychomotor regression and a history of controllable generalized convulsions at the age of 3. Calcification around the cortical regions may relate to the psychomotor regression and history of seizures common to both this patient and our patient. Recently, Sawaishi [13] pointed out the possibility that calcifying lesions in Alexander disease may be overlooked by routine follow-up MRI examination. We actually would not have recognized the calcifying lesions if not for her head contusion episode.

Still, calcification in the brain is a rare finding in Alexander disease and may be associated with a V87L mutation in *GFAP*.

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Conflict of interest None.

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REPORT

Mutations in *POLR3A* and *POLR3B* Encoding RNA Polymerase III Subunits Cause an Autosomal-Recessive Hypomyelinating Leukoencephalopathy

Hirotomo Saitsu,[1,*] Hitoshi Osaka,[2] Masayuki Sasaki,[3] Jun-ichi Takanashi,[4] Keisuke Hamada,[5] Akio Yamashita,[6] Hidehiro Shibayama,[7] Masaaki Shiina,[5] Yukiko Kondo,[1] Kiyomi Nishiyama,[1] Yoshinori Tsurusaki,[1] Noriko Miyake,[1] Hiroshi Doi,[1] Kazuhiro Ogata,[5] Ken Inoue,[8] and Naomichi Matsumoto[1,*]

Congenital hypomyelinating disorders are a heterogeneous group of inherited leukoencephalopathies characterized by abnormal myelin formation. We have recently reported a hypomyelinating syndrome characterized by diffuse cerebral hypomyelination with cerebellar atrophy and hypoplasia of the corpus callosum (HCAHC). We performed whole-exome sequencing of three unrelated individuals with HCAHC and identified compound heterozygous mutations in *POLR3B* in two individuals. The mutations include a nonsense mutation, a splice-site mutation, and two missense mutations at evolutionally conserved amino acids. Using reverse transcription-PCR and sequencing, we demonstrated that the splice-site mutation caused deletion of exon 18 from *POLR3B* mRNA and that the transcript harboring the nonsense mutation underwent nonsense-mediated mRNA decay. We also identified compound heterozygous missense mutations in *POLR3A* in the remaining individual. *POLR3A* and *POLR3B* encode the largest and second largest subunits of RNA Polymerase III (Pol III), RPC1 and RPC2, respectively. RPC1 and RPC2 together form the active center of the polymerase and contribute to the catalytic activity of the polymerase. Pol III is involved in the transcription of small noncoding RNAs, such as 5S ribosomal RNA and all transfer RNAs (tRNA). We hypothesize that perturbation of Pol III target transcription, especially of tRNAs, could be a common pathological mechanism underlying *POLR3A* and *POLR3B* mutations.

Congenital hypomyelinating disorders form a heterogeneous group of central nervous system leukoencephalopathies that is characterized by abnormal myelin formation. Although these conditions are readily recognized by brain magnetic resonance imaging (MRI), many cases are not diagnosed correctly.[1] Several syndromes affecting myelination, such as hypomyelination with hypodontia and hypogonadotropic hypogonadism (4H) syndrome (MIM 612440) and hypomyelination with atrophy of the basal ganglia and cerebellum (H-ABC) (MIM 612438), have been described.[2–5] We have recently reported a hypomyelinating syndrome characterized by diffuse cerebral hypomyelination with cerebellar atrophy and hypoplasia of the corpus callosum (HCAHC).[6] Individuals with HCAHC do not show hypodontia or atrophy of the basal ganglia, which are observed in 4H syndrome and H-ABC; however, diffuse hypomyelination, atrophy, or hypoplasia of the cerebellum and corpus callosum are overlapping features of these three syndromes, suggesting that there might be a common underlying pathological mechanism.

Here, we report on four individuals with HCAHC from three unrelated families (Figure 1A; Table 1). Clinical information and peripheral blood or saliva samples were obtained from the family members after obtaining written informed consent. Experimental protocols were approved by the Institutional Review Board of Yokohama City University. To identify pathogenic mutations, we performed whole-exome sequencing of three probands from three unrelated families (individuals 1, 3, and 4). DNAs were captured with the SureSelect Human All Exon 50Mb Kit (Agilent Technologies, Santa Clara, CA) and sequenced with one lane per sample on an Illumina GAIIx (Illumina, San Diego, CA) with 108 bp paired-end reads. Image analysis and base calling were performed by sequence control software real-time analysis and CASAVA software v1.7 (Illumina). A total of 90,014,368 (individual 1), 86,942,264 (individual 3), and 92,168,758 (individual 4) paired-end reads were obtained and aligned to the human reference genome sequence (GRCh37/hg19) with MAQ[7] and NextGENe software v2.00 with sequence condensation by consolidation (SoftGenetics, State College, PA). This approach resulted in more than 88% of target exomes being covered by ten reads or more (see Table S1, available online). Single nucleotide variants (SNVs) were called with MAQ and NextGENe. Small insertions and deletions were

[1]Department of Human Genetics, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan; [2]Division of Neurology, Clinical Research Institute, Kanagawa Children's Medical Center, 2-138-4 Mutsukawa, Minami-ku, Yokohama 232-8555, Japan; [3]Department of Child Neurology, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi-cho Kodaira, Tokyo 187-8551, Japan; [4]Department of Pediatrics, Kameda Medical Center, 929 Higashi-cho, Kamogawa-shi, Chiba 296-8602, Japan; [5]Department of Biochemistry, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan; [6]Department of Molecular Biology, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan; [7]Department of Neurology, Kameda Medical Center, 929 Higashi-cho, Kamogawa-shi, Chiba 296-8602, Japan; [8]Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi-cho Kodaira, Tokyo 187-8551, Japan
*Correspondence: hsaitsu@yokohama-cu.ac.jp (H.S.), naomat@yokohama-cu.ac.jp (N.M.)
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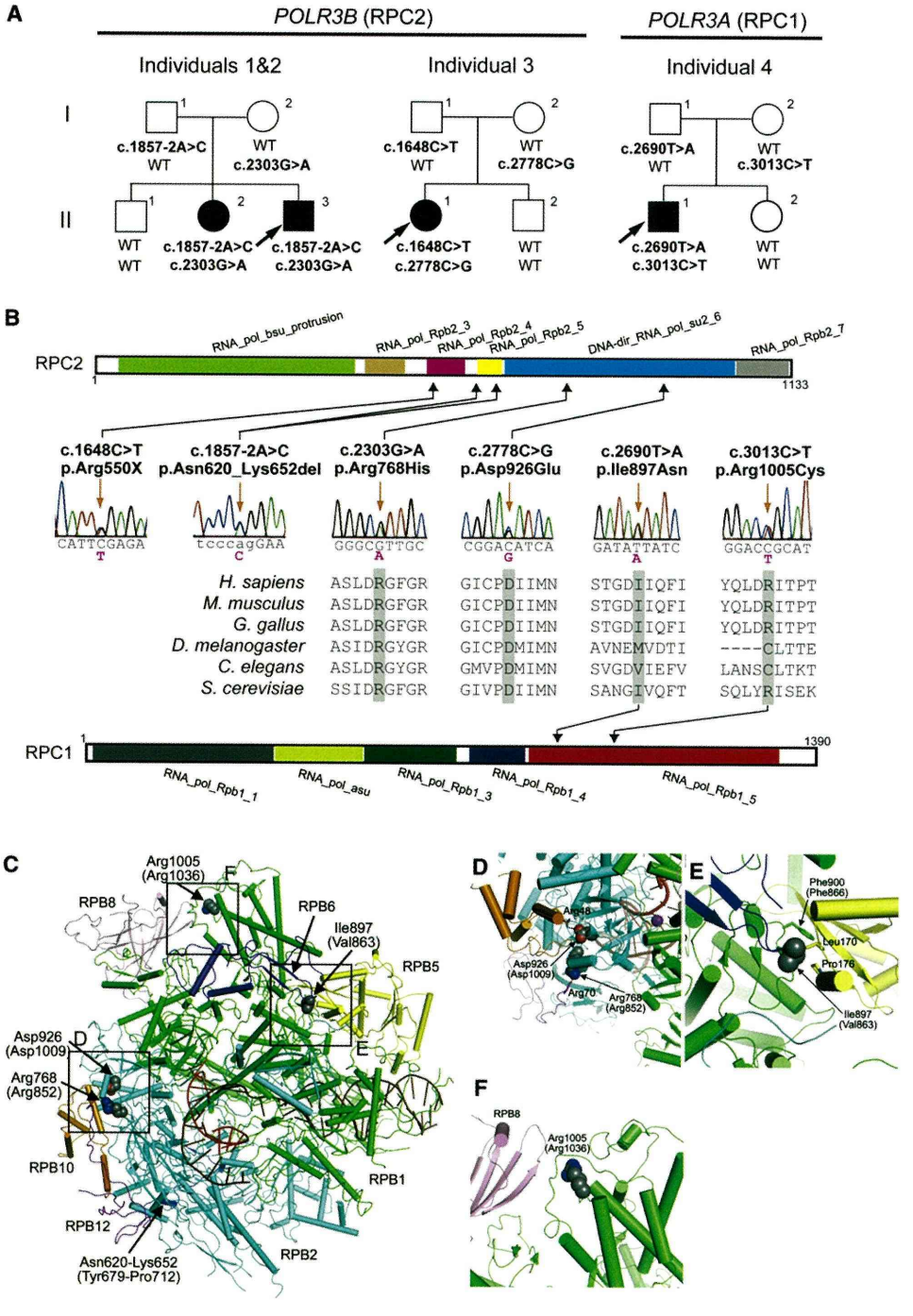

Figure 1. Mutations in *POLR3B* and *POLR3A*

(A) Pedigrees of four kindreds with HCAHC are shown. We identified four mutations in *POLR3B* encoding RPC2 in three individuals from two unrelated families and two mutations in *POLR3A* encoding RPC1 in one family. The segregation of each mutation is shown.

(B) Schematic representation of RPC2 (upper) and RPC1 (lower) proteins with Pfam domains (from Ensembl). Locations of each amino-acid-altering mutation are depicted with electropherograms. All of the missense mutations occurred at evolutionally conserved amino acids. Homologous sequences were aligned with the CLUSTALW website.

(C–F) 3D representations of RPC1 and RPC2 mutations. Mutated amino acids in RPC1 and RPC2 are shown along with their equivalent positions in the homologous RPB1 and RPB2 subunits of RNA Polymerase II (amino acid and its position in parenthesis). The structure and positions of mutations are illustrated by PyMOL with the crystal structure (PDB accession number 3GTP). RPB3, RPB9, and RPB11 subunits, which are specific to RNA Polymerase II, have been omitted from the figure. RPB1 is shown in green, RPB2 in sky blue, RPB5 in yellow, RPB6 in dark blue, RPB8 in pink, RPB10 in orange, RPB12 in purple, DNA in brown, and RNA in red. Amino acids that interact with mutated amino acids are also shown.

Table 1. Clinical Features of the Individuals

Clinical Features	Individual 1	Individual 2	Individual 3	Individual 4
Genes	*POLR3B*	*POLR3B*	*POLR3B*	*POLR3A*
Mutations, DNA	c.1857-2A>C, c.2303G>A	c.1857-2A>C, c.2303G>A	c.1648C>T, c.2778C>G	c.2690T>A, c.3013C>T
Mutations, protein	p.Asn620_Lys652del, p.Arg768His	p.Asn620_Lys652del, p.Arg768His	p.Arg550X, p.Asp926Glu	p.Ile897Asn, p.Arg1005Cys
Gender	M	F	F	M
Current age (years)	27	30	16	17
Intellectual disability	mild	mild	moderate	mild
Cognitive regression	–	–	–	–
Seizures	–	–	–	–
Initial motor development	normal	normal	normal	normal
Age of onset (years)	3	3	2	4
Motor deterioration	–	–	–	+
Wheelchair use	–	–	–	+
Optic atrophy	–	–	–	–
Myopia	+	+	–	+
Nystagmus	+	+	–	–
Abnormal pursuit	+	+	+	–
Vertical gaze limitation	+	+	+	–
Dysphagia	–	–	+	–
Hypersalivation	–	–	–	–
Cerebellar signs	+	+	+	+
Tremor	–	+	+	+
Babinski refex	–	–	–	–
Spasticity	–	–	mild	–
Peripheral nerve involvement	–	–	–	–
Nerve biopsy	NA	NA	NA	NA
Hypodontia	–	–	–	–
Hypogonadism	+	+	–	–

NA is an abbreviation for not available.

detected with NextGENe. Called SNVs were annotated with SeattleSeq Annotation.

We adopted a prioritization scheme to identify the pathogenic mutation in each individual, similar to the approach taken by recent studies (Table S2).[8–10] First, we excluded the variants registered in the dbSNP131 or 1000 Genome Project from all the detected variants. Then, SNVs commonly detected by MAQ and NextGENe analyses were selected as highly confident variants; 364 to 374 SNVs of nonsynonymous (NS) or canonical splice-site (SP) changes, along with 113 to 124 small insertions or deletions (indels), were identified per individual. We also excluded variants found in our 55 in-house exomes, which are derived from 12 healthy individuals and 43 individuals with unrelated diseases, reducing the number of candidate variants to ~250 per individual. Assuming that HCAHC is an autosomal-recessive disorder based on two affected individuals in one pedigree (individuals 1 and 2), we focused on rare heterozygous variants that are not registered in the dbSNP or in our in-house 55 exomes.

We surveyed all genes in each individual for two or more NS, SP, or indel variants. We found three to eight candidate genes per individual (Table S2). Among them, only *POLR3B* encoding RPC2, the second largest subunit of RNA Polymerase III (Pol III), was common in two individuals (individuals 1 and 3). The inheritance of the variants in *POLR3B* (transcript variant 1, NM_018082.5) was examined by Sanger sequencing. In individual 1, we confirmed that a canonical splice-site mutation (c.1857-2A>C [p.Asn620_Lys652del]), 2 bp upstream of exon 18, was

inherited from his father, and that a missense mutation (c.2303G>A [p.Arg768His]) in exon 21 were inherited from his mother (Figure 1A). The two mutations were also present in an affected elder sister (individual 2) but not present in a healthy elder brother. In individual 3, we confirmed that a nonsense mutation (c.1648C>T [p.Arg550X]) in exon 16 was inherited from her father and that a missense mutation (c.2778C>G [p.Asp926Glu]) in exon 24 was inherited from her mother (Figure 1A). The two mutations were not present in a healthy younger brother. To examine the mutational effects of c.1857-2A>C and c.1648C>T, reverse transcription PCR and sequencing with total RNA extracted from lymphoblastoid cells derived from the individuals was performed as previously described.[11] We demonstrated that the c.1857-2A>C mutation caused deletion of exon 18 from the *POLR3B* mRNA (Figures 2A–2C), resulting in an in-frame 33 amino acid deletion (p.Asn620_Lys652del) from RPC2 (Figure 1B). In addition, the mutated transcript harboring the nonsense mutation (c.1648C>T) was found to be expressed at a much lower level compared with the wild-type transcript (Figure 2D). The expression level of the mutated transcript was increased after treatment with 30 μM cycloheximide (CHX),[11] which inhibits nonsense-mediated mRNA decay (NMD), indicating that the mutant transcript underwent NMD (Figure 2D). The two missense mutations (p.Arg768His and p.Asp926Glu) found in the three individuals occurred at evolutionary conserved amino acids (Figure 1B). Among the other candidate genes in individuals 1 and 3, *MSLN* (MIM 601051), encoding mesothelin isoform 1 preproprotein that is cleaved into megakaryocyte potentiating factor and mesothelin, is a potential candidate in the family of individual 1 as its homozygous variant segregated with the phenotype; however, it is expressed in epithelial mesotheliomas, and the mutation affects less conserved amino acid (Table S3). The other candidate genes' variants did not cosegregate with the phenotype. Thus, mutations in *POLR3B* are most likely to cause HCAHC in two families.

In individual 4, in whom no *POLR3B* mutations were found, there were six candidate genes for an autosomal-recessive model. Among them, *POLR3A* (MIM 614258, GenBank accession number NM_007055.3), harboring two missense mutations, appeared to be a primary candidate because it encodes the largest subunit of Pol III (RPC1) (Figure 1A and Table S2). By Sanger sequencing, we confirmed that a missense mutation (c.2690T>A [p.Ile897Asn]) in exon 20 was inherited from his father and that another missense mutation (c.3013C>T [p.Arg1005Cys]) in exon 23 was inherited from his mother (Figure 1A). The two mutations were not present in a healthy younger sister. The two missense mutations (p.Ile897Asn and p.Arg1005Cys) occurred at relatively conserved amino acids (Figure 1B). In total, we found four mutations in *POLR3B* and two mutations in *POLR3A*. Evaluation of the missense mutations by PolyPhen-2 program showed that three mutations (p.Arg768His,

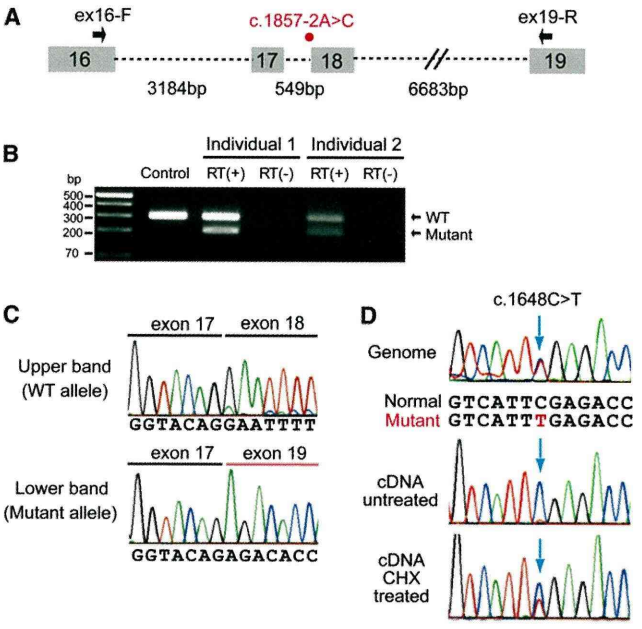

Figure 2. Effects of Splice-Site and Nonsense Mutations in *POLR3B*

(A) Schematic representation of the genomic structure of *POLR3B* from exon 16 to 19. Exons, introns, and primers are shown by boxes, dashed lines, and arrows, respectively. The mutation in intron 17 is depicted as a red dot.

(B) RT-PCR analysis of individuals 1 and 2 with c.1857-2A>C and a normal control. Two PCR products were detected from the individual's cDNA: the upper band is the wild-type (WT) transcript, and the lower band is the mutant. Only a single wild-type amplicon was detected in the control.

(C) Sequence of WT and mutant amplicons clearly showed exon 18 skipping in the mutant allele.

(D) Analysis of the c.1648C>T mutation. Sequence of PCR products amplified with genomic (upper), cDNA from untreated cells (middle), and cDNA from CHX treated cells (lower) as a template. Although untreated cells show extremely low levels of c.1648C>T mutant allele expression, cells treated to inhibit NMD show significantly increased levels of mutant allele expression.

p.Asp926Glu, and p.Ile897Asn) were probably damaging and that p.Arg1005Cys is tolerable. The c.2303G>A mutation (*POLR3B*) was found in one allele out of 540 Japanese control chromosomes. The remaining five mutations were not detected in 540 Japanese control chromosomes, indicating that the mutations are very rare in the Japanese population. Among the other candidate genes in individuals 4, *IGSF10*, a member of immunoglobulin superfamily, is a potential candidate because its variants segregated with the phenotype (Table S3); however, considering a close relationship between *POLR3A* and *POLR3B*, and the fact that *POLR3A* mutations have been recently reported in hypomyelinating leukodystrophy (see below),[12] *POLR3A* abnormality is the most plausible culprit for HCAHC in individual 4.

The structure of Pol III[13,14] and Pol II[15,16] is highly homologous, especially in the largest subunits. Thus, we extrapolated the mutations of RPC1 or RPC2 onto the structure of yeast Pol II (Protein Data Bank [PDB] accession number 3GTP)[17] (Figure 1C). RPB1 and RPB2 subunits of