the C, is the hot spot for mutation. Clinically, most families show purely cerebellar ataxia with the age of onset at their fourth decade,[13] although an association of myoclonic tremor has been reported in some families.[14,15] The neuropathology of SCA14 has been described in one family, in which Purkinje cell-dominant degeneration was seen.[9] The immunochemical analysis suggested reduced expression of both PKCγ and ataxin-1 in SCA14. Interestingly, the PKCγ protein concentration is decreased in the transgenic mice that overexpress ataxin-1 and show progressive ataxia.[16] These observations indicate that SCA14 and SCA1 might have a common pathway in the mechanism of neurodegeneration. A recent study indicated at least some mutation in the exon 4 of PRKCG caused the hyperactivation of PKCγ.[17] However, some but not all mutations cause the aggregation of PKCγ in cultured cells under certain circumstances.[18]

Another disease with single nucleotide substitution mutation is the ADCA with a mutation in the fibroblast growth factor 14 (*FGF14*) gene (SCA27).[19,20] Only one Dutch family has been reported as having ADCA with the FGF14 mutation; childhood-onset postural tremor and a slowly progressive ataxia and dyskinesia that develop in early adulthood are the cardinal features. No pathological study has been described so far for this disease. Mutation in the mouse homolog of the *FGF14* gene also causes ataxia.[21]

AUTOSOMAL DOMINANT CEREBELLAR ATAXIA IN WHICH CAUSATIVE GENES REMAIN UNIDENTIFIED

Despite the genetic discovery of many causative genes, there are still many other diseases for which the causes are unassigned to known gene loci. Although SCA6 and Machado-Joseph disease/SCA3 are the two most common forms in Japan, still 20–40% of ADCA is without identifiable mutations.[22]

We have been searching for mutations of ADCA with pure cerebellar syndrome (ADCA type III). We mapped our ADCA type III families to human chromosome 16q13.1–22.1,[23] to which SCA4 has been mapped already.[24,25] Although SCA4 is clinically characterized by prominent sensory neuropathy and pyramidal tract signs, our families lacked these symptoms.[2,23] This clinical difference made us call our families "16q-linked ADCA type III" rather than SCA4. We embarked on positional cloning to discover the gene.[26] In collaboration with many facilities in diverse regions of Japan, we were allowed to analyze more than 50 families, and analyzed the haplotypes of chromosome 16q22.1-linked markers in these families. Narrowing down the candidate region by haplotype-sharing analysis and further screening of mutations in all genes lying within the region led us to identify a genetic change strongly associated with patients with 16q-ADCA type III. (The change was a single nucleotide, C–T substitution in the 5′-UTR of the gene we called *puratrophin-1*. A detailed description of this gene has been published[27]).

During our attempt to recruit 52 families, we were able to examine a patient with 16q-linked ADCA type III neuropathologically. This study turned out to be the first autopsied case, not only for 16q-linked ADCA type III, but also for SCA4.[28] This patient first felt unsteadiness on walking when she was 70-years-old. At that time, she could make a long trip by taking planes to visit her son who lived abroad. However, her gait ataxia gradually worsened. She noticed hearing impairment during her seventh decade. She also noticed a tremor in her upper limb at the age of 80 years, particularly when she intended to reach something by hand. She was admitted to a nursing home at the age of 90 years because of her ataxia. Neurological examination at this age showed that she had prominent gait ataxia, so that she could not walk without assistance. She also had a severe hearing impairment, a mild positional tremor of her head and action tremor in her extremities, cerebellar speech, and limb ataxia. The tendon reflexes were mildly reduced in her upper and lower extremities. The pathological reflexes were not observed. She suffered from acute cholecystitis when she was 92-years-old. Although she recovered from this disease, she became bedridden after this episode. She died of natural causes at the age of 96 years.

The brain and spinal cord of this patient were obtained at autopsy and fixed in 10% neutral buffered formalin for 2 weeks. After fixation, the brain including the brainstem and the cerebellum weighed 1200 g. The macroscopic finding of the brain showed a moderate cortical atrophy in the anterior part of the cerebellar vermis (Fig. 1). The cerebral hemispheres were well-preserved. The brainstem, including the basis pontis and inferior olives, was of normal size. The thoracic and lumbar regions of the spinal cord also appeared to be normal.

Microscopic examination disclosed cerebellar cortical degeneration with neuronal loss predominant in the Purkinje cell as the most prominent neuropathological feature of this patient. The Purkinje cell loss was severe in the anterior part of the vermis, such as the culmen and the declive. In such regions, the numbers of granule cells were moderately reduced (Fig. 2A). In the nodulus, uvula, and lobules quadrangularis, there was moderate Purkinje cell loss with mild granule cell loss. In other areas, the Purkinje cell loss tended to be mild (Fig. 2B). What seemed very remarkable was that many remaining cell bodies of the Purkinje cells were often shrunken and were surrounded by thick eosinophilic amorphous material (Fig. 2C). This amorphous material appeared pale on KB staining

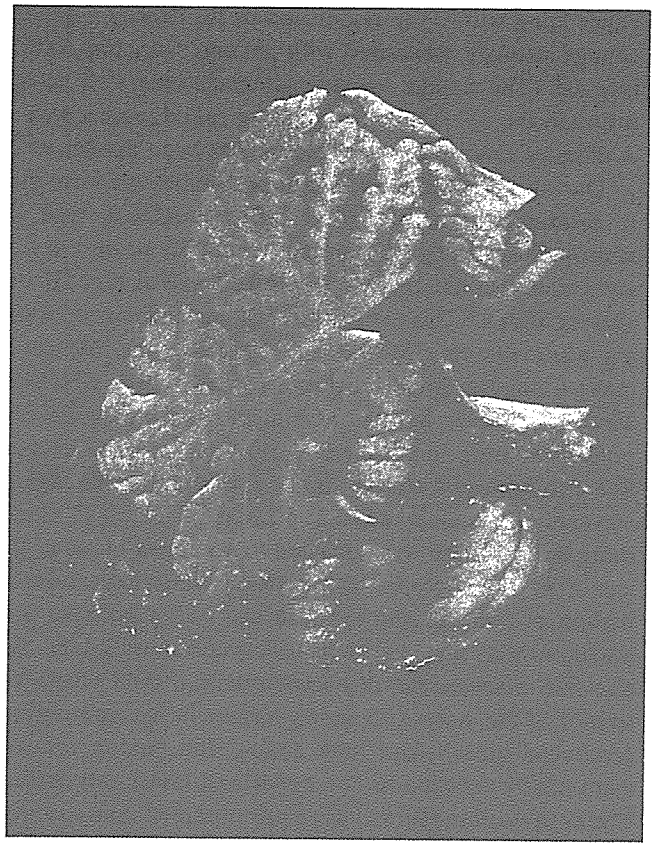

Fig. 1 Macroscopic observation of the cerebellum of a patient with 16q-linked autosomal dominant cerebellar ataxia type III. The anterior part of the cerebellar vermis appears to be atrophic.

(Fig. 2D), but it was not stained by the modified Bielschowsky's method (Fig. 2E). In general, the degree of granule cell loss tended to be milder than that of Purkinje cell loss, indicating that the Purkinje cell is the main target of this disease (Fig. 2B). The cerebellar white matter showed mild to moderate gliosis. No obvious neuronal loss was seen in the dentate and fastigial nuclei, although moderate astrocytic gliosis was seen.

In the brainstem, no obvious neuronal loss was seen, including the inferior olivary nucleus. The auditory system, including the cochlear nuclei, trapezoid body, lateral lemniscus, superior olivary nucleus, inferior colliculus in the brainstem, and the auditory cortex in the transverse temporal gyrus, were not affected. In the spinal cord, no obvious neuronal loss was seen. There was mild myelin pallor in the fasciculus gracilis and the anterior and posterior spinocerebellar tracts. Neuronal loss in the dorsal root ganglia of the 7th thoracic spine was not apparent, with only a few Nageotte's nodules. Other findings are summarized in Tables 2 and 3.

Immunohistochemical analysis for calbindin-D28k showed that the Purkinje cells had undergone various morphological changes. Some Purkinje cells with a plump cell body were a bizarre shape, with abnormal dendritic structures stemming from the cell body (Fig. 3A). Some of these changes were reminiscent of the "somatic sprouting" seen in kinky hair disease. In many Purkinje cells with rather shrunken cell bodies, several patterns of changes were recognized in the vicinity of their cell bodies. The first pattern was the presence of granular calbindin-D28k-immunoreactive structures that were probably a remnant of the Purkinje cell dendrites stemming from the cell body (Fig. 3B). The second pattern was a diffuse calbindin-D28k-immunoreactive area, probably corresponding with the amorphous structure seen on HE staining (Fig. 3C). The third pattern was an absence of calbindin-D28k-immunoreactivity despite the presence of amorphous material (Fig. 3D). In some Purkinje cells, combinations of these changes were observed, indicating that these patterns represent consecutive morphological changes in Purkinje cells.

On ubiquitin immunohistochemistry, granular immunoreactive structures also were recognized within the amorphous material (Fig. 3E). There were no obvious nuclear inclusions as seen in polyglutamine disease. Synaptophysin immunohistochemistry showed an increase in the immunoreactivity surrounding the Purkinje cell body (Fig. 3F). This clearly indicates that the amorphous structure surrounding the Purkinje cell is composed not only of Purkinje cell dendrites, as seen by calbindin-D28k immunohistochemistry, but also of an increase in presynaptic terminals from certain neurons innervating the Purkinje cells. The immunoreactivity for synaptophysin was preserved in the glomerulus of the granule cell layer and in the molecular layer. Immunohistochemistry for phosphorylated neurofilament (SMI31.) showed SMI31-immunoreactive basket cells' axons attached to the outer rim of the amorphous structure enclosing the shrunken cell body of the Purkinje cells (Fig. 3G). Astrocytic processes also were seen in the rim of the amorphous material (Fig. 3H).

Our neuropathological study revealed that 16q-linked ADCA type III shows the characteristic neuropathological feature of Purkinje cells surrounded by eosinophilic amorphous material, which could be recognized by routine HE staining. As far as we know, such a change has not been described in detail and seems to be characteristic of this disease.

Although the precise mechanism underlying the formation of the amorphous material is not clear, we hypothesize that there are three important components in the formation of the amorphous material. First is the Purkinje cell dendrites, shown by the presence of calbindin-D28k-immunoreactive granules/dendrites, probably in the early

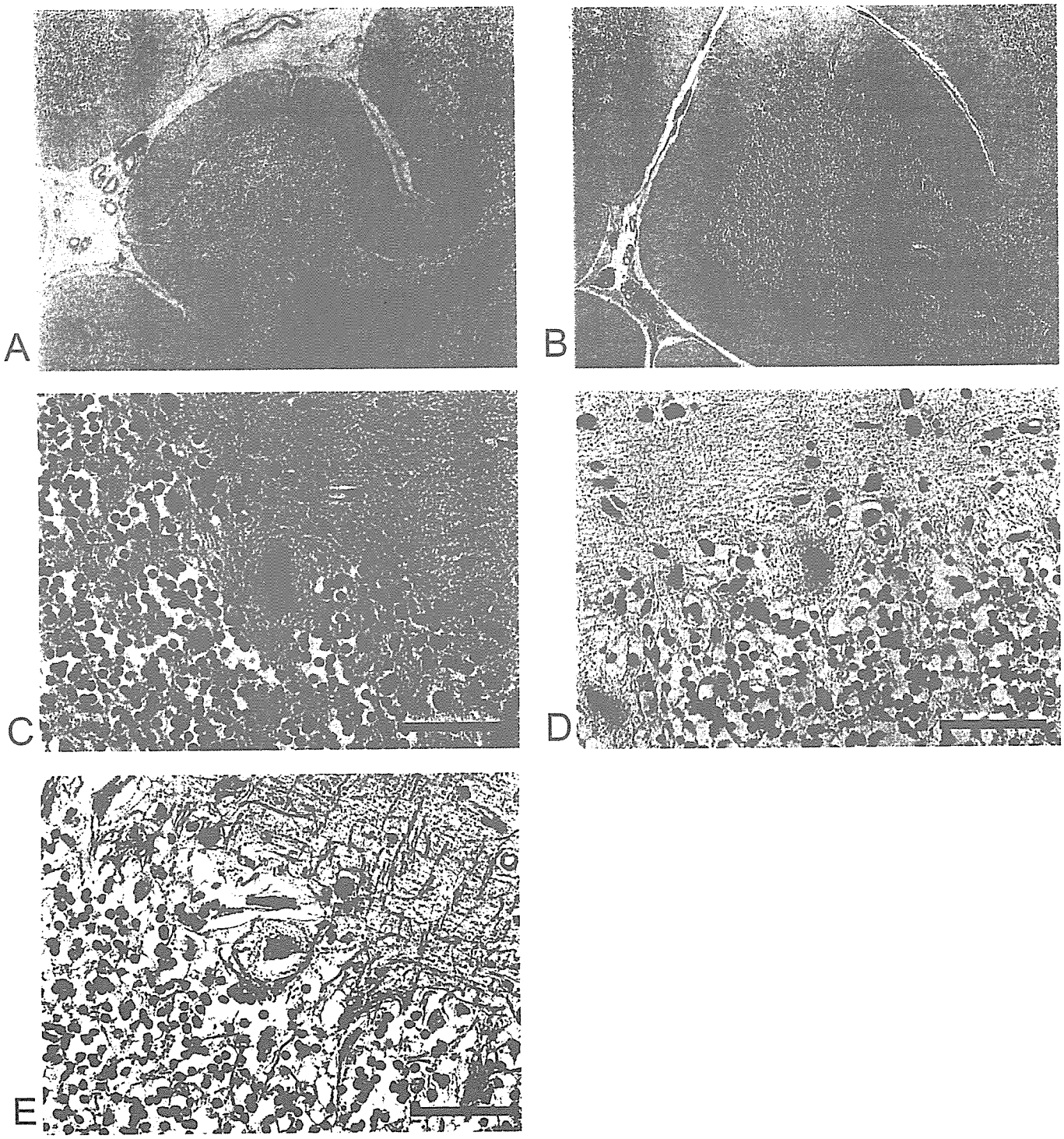

Fig. 2 Histological finding of a patient with 16q-linked autosomal dominant cerebellar ataxia type III. (A) A low magnification view of the cerebellar vermis where a severe Purkinje cell dropout and a moderate granule cell loss are seen. (HE, ×20). (B) In the cerebellar hemisphere, Purkinje cells are moderately lost, whereas granule cells are well preserved in number (HE, ×20). (C) An atrophic Purkinje cell body is surrounded by eosinophilic amorphous material (HE, bar indicates 50 μm). (D) The amorphous material surrounding the Purkinje cell body appears pale on the KB stain (bar indicates 50 μm). (E) The amorphous material is not stained with modified Bielschowsky's method (bar indicates 50 μm).

Table 2 Neuropathological features

Location	Degree of neuronal loss	Other remarkable changes	Gliosis	White matter changes
Cerebrum				
Cerebral cortex				
Superior frontal gyrus	0	–	0	/
Precentral gyrus	0	–	0	/
Parietal lobule	0	–	0	/
Visual cortex	0	–	0	/
Parahippocampal gyrus	0	–	0	/
Middle temporal gyrus	0	–	0	/
Superior temporal gyrus	0	–	0	/
Inferior temporal gyrus	0	–	0	/
Cingulate gyrus	0	–	0	/
Cerebral gray matter	0	–	0	/
Caudate nucleus	0	–	0	/
Putamen	0	–	0	/
Globus pallidus (external and internal)	0	–	0	/
Thalamus	0	–	0	/
Claustrum	0	–	0	/
Hippocumpus (CA1–CA4)	0	Moderate number of NFTs	0	/
Hippocampus, dentate gyrus	0	–	0	/
Cerebral white matter	/	–	0	0
Brainstem				
Substantia nigra, pars reticulata and compacta	0	–	0	/
Red nucleus	0	–	0	/
Periaqueductal gray	0	–	Mild	/
Oculomotor nucleus	0	–	0	/
Basal pontine nuclei	0	Lacunar infarct	Mild	/
Paramedian pontine reticular formation	0	–	0	/
Cranial nuclei:V, VI, VII	0	–	0	/
Inferior olivary nucleus	0	–	Mild	/
Vestibular and cochlear nuclei, trapezoid bodies	0	–	0	/
Cranial nuclei: IX, X, XI, XII	0	–	0	/
Pyramidal tract	/	–	0	0
Cerebellar peduncle (superior)	/	–	0	0
Cerebellar peduncle (middle)	/	–	0	0
Cerebellar peduncle (inferior)	/	–	0	0
Nucleus gracilis	0	Moderate number of spheroids	Mild	Mild myelin pallor
Nucleus cuneatus	0	–	0	0
Dentate nucleus	0		Moderate	
Fastigial nucleus	0		0	
Spinal cord (thoracic and lumbar spine)				
Anterior horn	0		0	/
Posterior horn	0		Mild	/
Lateral horn and Clarke's column	0		0	/
Funiculus, (anterior)	/		0	0
Funiculus (posterior: fasciculus gracilis)	/		Mild	Mild myelin pallor
Pyramidal tract	/		0	0
Anterior and posterior spinocerebellar tract	/		Mild	Mild myelin pallor
Anterior root of spinal nerve (4th lumbar)	/		/	0
Posterior root of spinal nerve (4th lumbar)	/		/	0
Dorsal root ganglia (7th thoracic spine)	Not apparent	A few Nageotte's nodules	0	

The degree of neuronal loss was rated in four steps: 0, mild, moderate and severe. /, sites that do not contain white matter.

stage of amorphous material formation. The presence of ubiquitin-immunoreactive granules, mimicking calbindin-immunoreactive granules, may suggest that a certain protein degradation system is taking place in the amorphous material. The second component is the presynaptic terminals innervated by certain neurons, which is evidenced by synaptophysin immunoreactivity. The third component is the astroglial processes. Further study using a larger number of patients will be needed to clarify the present hypothesis. Such effort will lead to the establishment of the

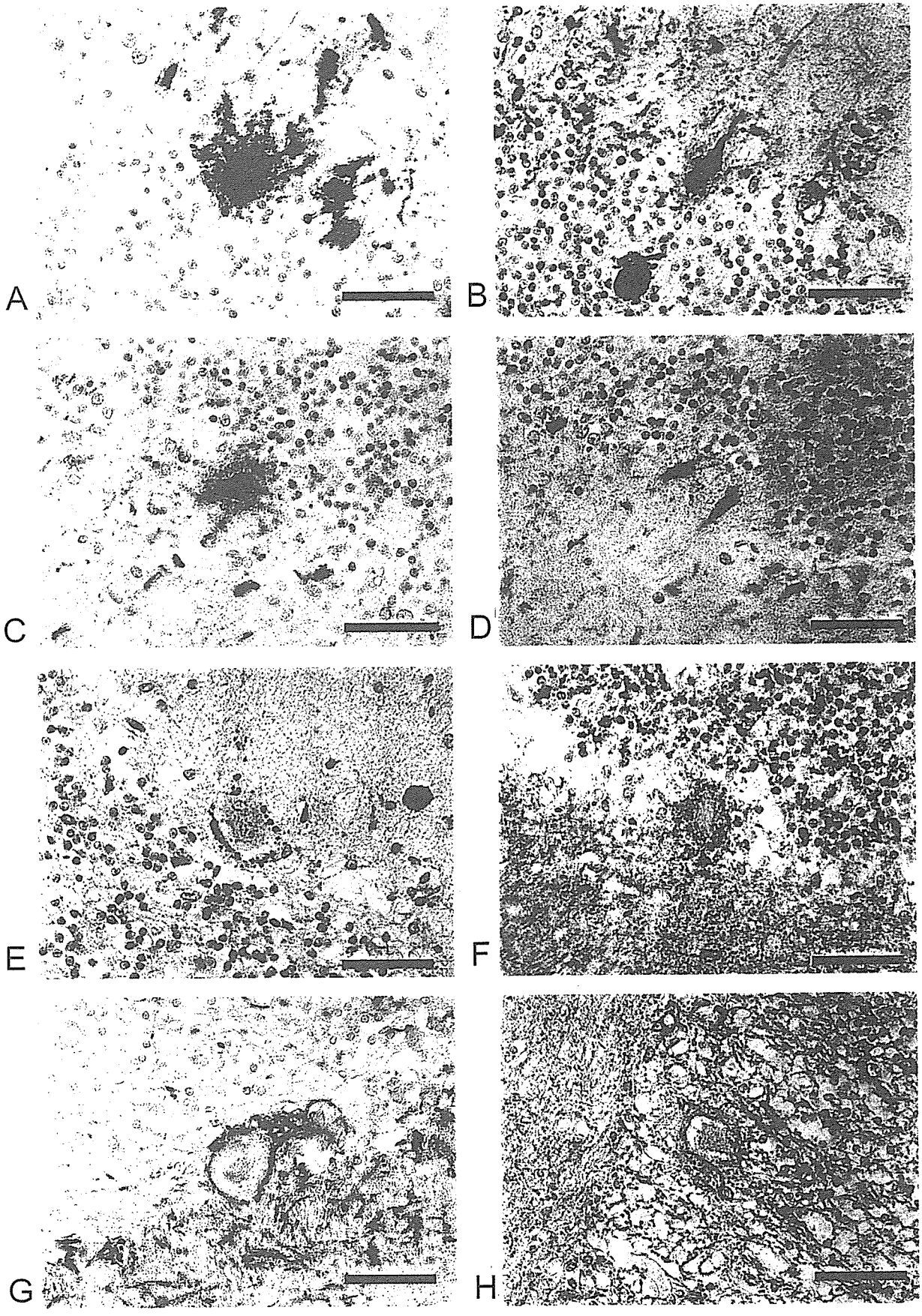

Fig. 3 Immunohistochemistry of the Purkinje cells of a patient with 16q-linked autosomal dominant cerebellar ataxia type III. (A) A Purkinje cell is a bizarre shape, with an abnormal dendritic structure stemming from its plump cell body (calbindin-D28k immunohistochemistry, bar indicates 50 μm). (B) Granular immunoreactive structures are seen nearby a shrunken Purkinje cell body (calbindin-D28k immunohistochemistry, bar indicates 50 μm). (C) A diffuse calbindin-D28k immunoreactive area is seen surrounding the Purkinje cell body (calbindin-D28k immunohistochemistry, bar indicates 50 μm). (D) Two Purkinje cells are surrounded by amorphous material without obvious calbindin-D28k immunoreactivity (calbindin-D28k immunohistochemistry, bar indicates 50 μm). (E) Ubiquitin immunoreactive granules are seen within amorphous material (ubiquitin immunohistochemistry, bar indicates 50 μm). (F) Synaptophysin immunohistochemistry shows increased immunoreactivity at the amorphous material (bar indicates 50 μm). (G) Immunohistochemistry against phosphorylated neurofilament shows immunoreactivity at the outer rim of the amorphous material (bar indicates 50 μm). (H) Astroglial processes are occasionally seen within the amorphous material (GFAP immunohistochemistry, bar indicates 50 μm).

Table 3 Neuropathological features specifically in relation to the Purkinje cells and granule cells

Location	Degree of neuronal loss		Other remarkable findings	Gliosis	White matter changes
	Purkinje cells	Granule cells			
Cerebellum					
Cerebellar cortex					
Culmen and declive	Severe	Moderate	Reduced thickness of the molecular layer	Prominent	Mild myelin pallor
Nodulus	Moderate	Mild	–	Mild	None
Uvula	Moderate	Mild	–	Mild	None
Lobules (quadrangularis and simplex)	Moderate	Mild	–	Mild	None
Lobules (semilunaris caudalis and gracilis)	Mild	0 to mild	–	Mild	None
Flocculus	Mild	0 to mild	–	Mild	None
Tonsils	Mild	0 to mild	–	Mild	None
Cerebellar white matter	None	None	–	Moderate	Mild myelin pallor

The degree of neuronal loss was rated in four steps: 0, mild, moderate and severe.

clinical and neuropathological features of a newly identified ADCA that is common in Japan.

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16q-linked autosomal dominant cerebellar ataxia: A clinical and genetic study

Y. Ouyang [a], K. Sakoe [a], H. Shimazaki [a], M. Namekawa [a], T. Ogawa [a], Y. Ando [a], T. Kawakami [a], J. Kaneko [b], Y. Hasegawa [c], K. Yoshizawa [d], T. Amino [e], K. Ishikawa [e], H. Mizusawa [e], I. Nakano [a], Y. Takiyama [a,*]

[a] *Department of Neurology, Jichi Medical School, Tochigi 329-0498, Japan*
[b] *Kaneko Clinic, Gunma 373-0841, Japan*
[c] *Department of Neurology, Showa University School of Medicine, Tokyo 142-8555, Japan*
[d] *Department of Neurology, National Mito General Hospital, Ibaraki 311-3193, Japan*
[e] *Department of Neurology and Neurological Science, Graduate School, Tokyo Medical and Dental University, Tokyo 113-8519, Japan*

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Abstract

The autosomal dominant cerebellar ataxias (ADCAs) comprise a genetically and clinically heterogenous group of neurodegenerative disorders. Very recently, a C-to-T single nucleotide substitution in the *puratrophin-1* gene was found to be strongly associated with a form of ADCA linked to chromosome 16q22.1 (16q-linked ADCA; OMIM 600223). We found the C-to-T substitution in the *puratrophin-1* gene in 20 patients with ataxia (16 heterozygotes and four homozygotes) and four asymptomatic carriers in 9 of 24 families with an unknown type of ADCA. We also found two cases with 16q-linked ADCA among 43 sporadic patients with late-onset cortical cerebellar atrophy (LCCA). The mean age at onset in the 22 patients was 61.8 years, and that of homozygous patients was lower than that of heterozygous ones in one family. Neurological examination revealed that the majority of our patients showed exaggerated deep tendon reflexes in addition to the cardinal symptom of cerebellar ataxia (100%), and 37.5% of them had sensorineural hearing impairment, whereas sensory axonal neuropathy was absent. The frequency of 16q-linked ADCA was about 1/10 of our series of 110 ADCA families, making it the third most frequent ADCA in Japan.

Keywords: 16q-linked ADCA; *Puratrophin-1* gene; Heterozygote; Homozygote; Haplotype analysis

1. Introduction

Autosomal dominant cerebellar ataxias (ADCAs) comprise a genetically and clinically heterogeneous group of neurodegenerative disorders characterized by progressive cerebellar ataxia that can be variably associated with other neurological features [1]. ADCAs are now classified on the basis of the causative genes or gene loci. To date, at least 26 subtypes of ADCA have been identified including spinocerebellar ataxia (SCA) type 1, 2, Machado-Joseph disease (MJD/SCA3), 4–8, 10–19/22, 21, 23, 25–28, and dentatorubral and pallidoluysian atrophy (DRPLA) [2,3].

Among these subtypes, SCA4 was mapped to chromosome 16q22.1 in a Scandinavian family residing in Utah and Wyoming in 1996 [4]. This family showed prominent sensory axonal neuropathy and pyramidal tract signs in addition to cerebellar ataxia. In 2003, a German family characterized by cerebellar ataxia and sensory axonal neuropathy was assigned to the same locus as SCA4 [5].

Meanwhile, the gene locus responsible for six Japanese families with ADCA was mapped to the same region as SCA4 in 2000 [6]. Although SCA4 and this form of ADCA might be allelic, the clinical features of the Japanese families

* Corresponding author. Tel.: +81 285 58 7352; fax: +81 285 44 5118.
E-mail address: ytakiya@jichi.ac.jp (Y. Takiyama).

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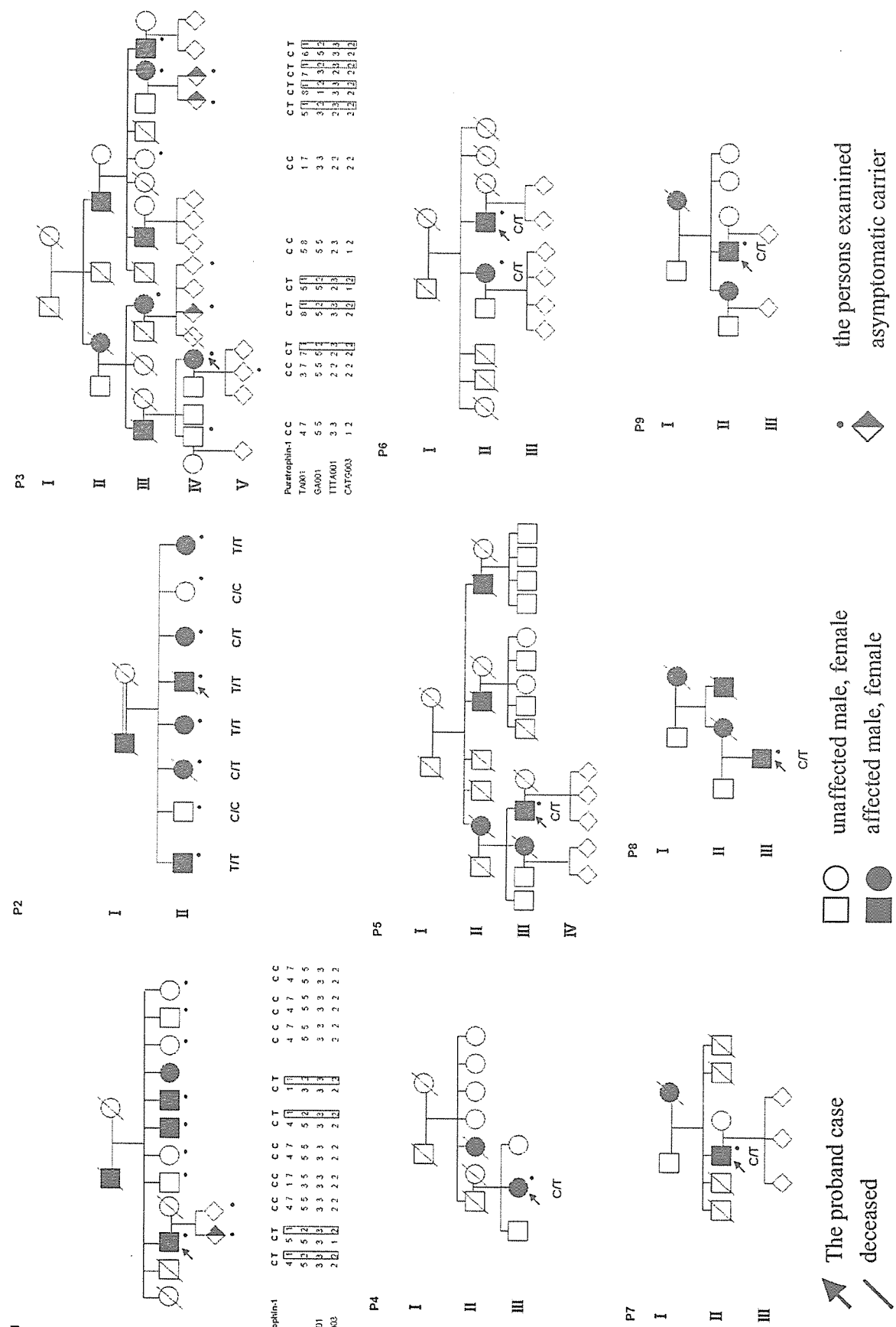

Fig. 1. The pedigrees of nine Japanese families with 16q-linked ADCA. In pedigrees 1 and 3, the gender is concealed in those individuals, including asymptomatic ones, denoted by diamonds to maintain the anonymity of the families.

were somewhat different from those in the case of SCA4, i.e., pure cerebellar ataxia without obvious evidence of extracerebellar neurological dysfunction. Therefore, the term "16q-linked ADCA" instead of "SCA4" was used to describe these Japanese families [7]. It is considered that 16q-linked ADCA shows prominent cerebellar ataxia with a later age at onset (>55 years) than that in SCA4 [8]. Very recently, a heterozygous C-to-T single nucleotide substitution in the 5′ untranslated region (UTR) of the *puratrophin-1* gene was found to be strongly associated with 16q-linked ADCA [9]. Thereafter, a substantial number of patients with this mutation showed progressive sensorineural hearing impairment in addition to cerebellar ataxia [10]. The clinical spectrum and the prevalence of 16q-ADCA, however, remain unclear.

We report here the clinical and molecular features of 20 patients including four homozygotes and four asymptomatic carriers in nine families, and two apparently sporadic patients with 16q-linked ADCA. Furthermore, we describe the frequency of 16q-linked ADCA in our series of 110 Japanese families with ADCA.

2. Subjects and methods

2.1. Clinical study

Clinical data were collected for 20 patients, four asymptomatic carriers in nine Japanese ADCA families, and two sporadic patients with a C-to-T substitution in the *puratrophin-1* gene (16q-linked ADCA). Fig. 1 shows the pedigrees of the nine families. Pedigrees 1 and 2 were partially described in the previous reports [6,8,9]. In pedigree 2, the parents (generation I) were first cousins, and thus consanguinity was present. In addition to neurological examination, brain MRI ($n=15$), peripheral nerve conduction studies ($n=8$), and audiograms ($n=8$) were performed in the patients as much as possible.

2.2. Molecular analysis

Blood samples were obtained with informed consent from 190 patients in 110 Japanese families with ADCA seen in the past 14 years (from 1992 to 2005). Genomic DNA was extracted from peripheral blood leukocytes. Screening for CAG repeat expansion for SCA1, SCA2, MJD/SCA3, SCA6, SCA7, SCA8, SCA12, SCA17, and DRPLA was performed by PCR as described elsewhere [11–19]. In this study, the SCA10, SCA14, and FGF mutations were not analyzed.

The C-to-T substitution in the *puratrophin-1* gene were analyzed in 33 patients, 16 at risk individuals, and 5 normal spouses in 24 of 110 families after exclusion of SCA1, SCA2, MJD/SCA3, SCA6, SCA7, SCA8, SCA12, SCA17, and DRPLA gene mutations (unknown ADCA families), and 43 sporadic patients with LCCA (late-onset cortical cerebellar ataxia without apparent extracerebellar signs or genetic inheritance). Using the primer pair of UK1-E1F1 (5′-CAGCGCGTTCACACTGAGA-3′) and UK1-E1R1 (5′-GGCCCTTTCTGACAGGACTGA-3′), exon 1 flanking the C-to-T change in the 5′ UTR of the *puratrophin-1* gene was amplified by PCR from 200 ng of genomic DNA [9], and then sequenced directly with an ABI PRISM 310 genetic analyzer; analysis was performed with Sequencing Analysis software, ver. 3.4.1 (Applied Biosystems). The PCR products of exon 1 with the primers were digested with *Eco*NI at 37 °C, subjected to electrophoresis on 2% agarose gels, and then stained with ethidium bromide. In addition, we performed haplotype analysis for the family members in pedigrees 1 and 3 using chromosome 16q markers TA001, GA001, TTTA001 and CATG003 [9].

This study was approved by the Medical Ethical Committee of Jichi Medical School.

3. Results

3.1. Clinical study

We found 20 patients (16 heterozygotes and four homozygotes) with 16q-linked ADCA and four asymptomatic carriers (two with and two without clinical signs) in 9 of 24 families with an unknown type of ADCA (Fig. 1). Furthermore, we found two sporadic patients with 16q-linked ADCA among 43 with LCCA.

Table 1
Clinical features in the patients with 16q-linked ADCA

Number of patients	22 (Male 13, Female 9)
Age at examination (years)	
Range	61–88
Mean	74.5
Age at onset (years)	
Range	50–83
Mean	61.8
Disease duration (years)	
Range	1–13
Mean	12.5
Initial symptoms (%)	
Unsteadiness of gait	77.3
Dysarthria	13.6
Tremor	9.1
Clinical features (%)	
Cerebellar	
Ataxic gait	100
Dysarthria	100
Nystagmus	77.3
Pyramidal	
Spasticity	13.6
Brisk DTRs	54.5
Babinski signs	0
Peripheral	
Depressed DTRs	13.6
Decreased vibration sense	13.6
Hearing impairment	37.5[a]
Tremor	13.6

[a] Audiograms revealed hearing impairment in three of the eight patients examined.

Table 1 summarizes the clinical features in the 22 patients with 16q-linked ADCA. The age at onset in the patients ranged from 50 to 83 years, the mean age at onset being 61.8 years. In pedigree 2, the mean age at onset in homozygous patients ($n=4$) was 55.6 years and that in heterozygous ones ($n=2$) was 68.5 years, showing an earlier age at onset in the former than in the latter. In pedigrees 1 and 3, anticipation was not noted.

The cardinal clinical feature was cerebellar ataxia including ataxic gait (100%), dysarthria (100%), and nystagmus (77.3%). Fifteen patients showed lateral gaze nystagmus, and two showed down-beat nystagmus. Oscillopsia was noted in one patient with down-beat nystagmus. Although external ophthalmoparesis was not evident, 13.6% of the patients complained of diplopia. Brisk deep tendon reflexes were found in the majority of the patients (54.5%), but Babinski signs were absent. In pedigree 3, three of the four patients examined showed moderate spasticity of the lower extremities in addition to brisk deep tendon reflexes. Meanwhile, 13.6% of the patients showed depressed deep tendon reflexes and depressed vibration sense in the toes. Audiograms revealed hearing impairment in three (37.5%) of the eight patients examined. Tremor was noted in 13.6% of the patients. Unfortunately, since we examined each homozygous or heterozygous patient in pedigree 2 only one time, we could not compare the disease course progression in them. However, there seemed to be no apparent differences in clinical phenotype between them. Among the four asymptomatic carriers, two individuals (mean, 46.0 years old) showed transient nystagmus and mild hyperreflexia.

Brain MRI ($n=15$) revealed cerebellar atrophy whereas the brainstem was of normal size and shape. Brain MRI of a homozygous (disease duration, 20 years) and a heterozygous patient (disease duration, 22 years) showed cerebellar atrophy of the same degree (Fig. 2). The results of a motor and sensory nerve conduction study ($n=8$) including two patients with depressed deep tendon reflexes or depressed vibration sense were normal, there being no sensory axonal neuropathy.

3.2. Molecular study

Fig. 1 shows the results of a heterozygous or homozygous C-to-T substitution of exon 1 in the *puratrophin-1* gene. Fig.

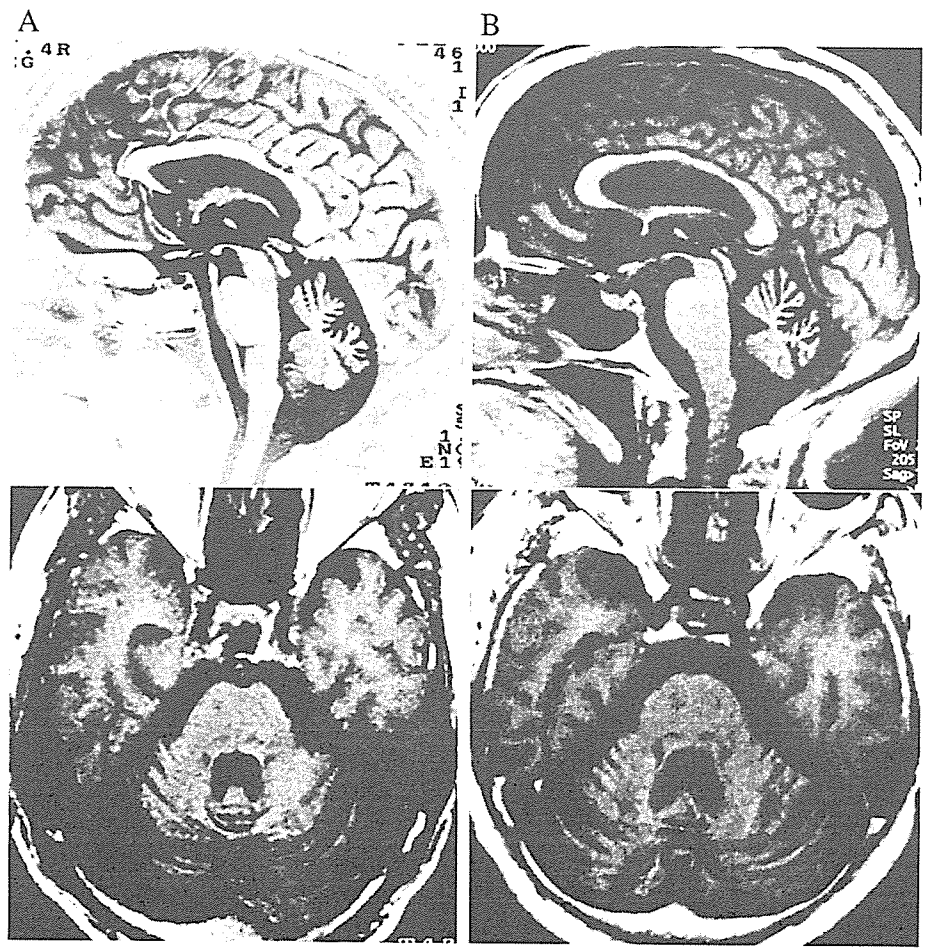

Fig. 2. (A) Brain MRI in a homozygous patient (disease duration, 20 years). Top: Reversed T2-weighted, sagittal slice. Bottom: T1-weighted, axial slice. (B) Brain MRI in a heterozygous patient (disease duration, 22 years). Top: T1-weighted, sagittal slice. Bottom: T1-weighted, axial slice. Both patients showed cerebellar atrophy of the same degree.

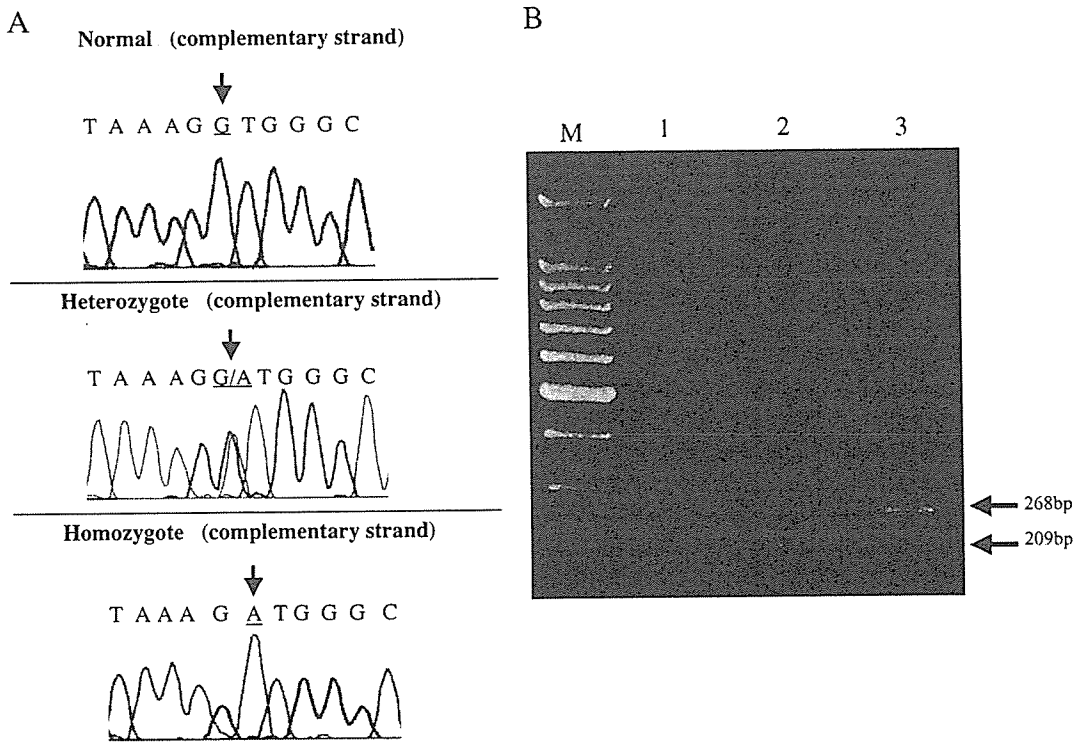

Fig. 3. (A) Nucleotide sequences of exon 1 in the *Puratrophin-1* gene. A G-to-A change (complementary strand) in a patient with a heterozygous or a homozygous state, and the normal sequence in a normal individual are shown. (B) The PCR products after *Eco*NI digestion. Lane M, 100 bp size markers; lane 1, a normal individual; lane 2, a patient with a heterozygous C-to-T change; lane 3, a patient with a homozygous C-to-T change. The wild-type *Eco*NI-digested PCR products gave three bands (209, 92, and 59 bp). Meanwhile, the *Eco*NI-digested PCR products with a heterozygous C-to-T change gave four bands (268, 209, 92, and 59 bp), and those with a homozygous C-to-T change gave two bands (268 and 92 bp). Two *Eco*NI-digested bands (92 and 59 bp) cannot be seen.

3A shows the results of GeneScan analysis of the nucleotide sequence flanking the C-to-T change (complementary strand) in the *puratrophin-1* gene in a heterozygous or homozygous patient and a normal individual in the same family. Fig. 3B shows the results as to the *Eco*NI-digested PCR products in the heterozygous or homozygous patient and the normal individual.

We constructed haplotypes for pedigrees 1 and 3, the two families large enough for haplotype analysis, and the results revealed that all patients in the two families were segregated with the haplotype 1-2-3-2 for the chromosome 16q markers TA001, GA001, TTTA001 and CATG003 (Fig. 1). The genotypes of the remaining patients and carriers were also identical with the haplotype 1-2-3-2 (data not shown). Furthermore, the specific allele, 2, of GA001 was only seen in all 22 patients and the four carriers with 16q-linked ADCA. The results indicate that the GA001 marker is very specific for the diagnosis of patients with16q-linked ADCA.

Table 2
Frequencies of various subtypes of ADCA in 110 Japanese families

	Number of families	%	Number of patients	%
MJD/SCA3	36	32.7	79	41.6
SCA6	27	24.5	44	23.1
16q-linked	9	8.2	20	10.5
DRPLA	9	8.2	11	5.8
SCA2	7	6.4	12	6.3
SCA1	6	5.5	6	3.2
SCA8	1	0.9	1	0.5
Unknown	15	13.6	17	9.0
Total	110	100	190	100

Approximately 80% of the 110 families were from the Kanto region, in a central region of the mainland of Japan. Five of the nine families with 16q-linked ADCA were from the Kanto region, whereas the remaining families were from the other regions of Japan.

3.3. Frequency of 16q-linked ADCA

The frequencies of various subtypes of ADCA in Japan are shown in Table 2. The results showed that the frequency of 16q-linked ADCA families is 8.2%, this being lower than those of MJD/SCA3 (32.7%) and SCA6 (24.5%) ones, and thus it is the third most frequent ADCA together with DRPLA in Japan. Similarly, concerning the number of patients, 16q-linked ADCA was the third-most frequent next to MJD/SCA3 and SCA6.

4. Discussion

In the present study, we found 22 Japanese patients with 16q-linked ADCA, and revealed some characteristic clinical features of this disease in comparison with those found in

earlier studies on 16q-linked ADCA in Japan, and SCA4 in Utah and Germany, most of which involved linkage analyses [4–10,20,21]. First, the mean age at onset in our patients was 61.8 years, this being later than those in two earlier reports on Japanese families with 16q-linked ADCA (mean, 55.9 and 56.7 years old) [6,20]. Moreover, the mean age at onset in our patients was much later than that in the SCA4 patients in Utah and Germany (mean, 39.3 and 38.3 years old) [4,5]. The age at onset in our patients with 16q-linked ADCA is much later than that in the patients with SCA6 (mean, 45.0 years old) [6], which indicates late-onset pure cerebellar ataxia. Therefore, 16q-linked ADCA appears to exhibit the oldest age at onset among the ADCA subtypes with assigned loci [9]. Second, we found that although cerebellar ataxia was the most common and predominant feature in 16q-linked ADCA, 54.5% of our patients showed exaggerated deep tendon reflexes. Furthermore, moderate spasticity in the lower limbs was noted in three of the four patients examined in pedigree 3. Thus, although we observed no Babinski signs in our patients, possible pyramidal tract signs can accompany cerebellar ataxia in 16q-linked ADCA, as described for SCA4 [4,5]. Since spasticity in the lower limbs was noted only in one pedigree, the presence of some modifying genetic factors for this phenotype is suggested. Meanwhile, the sensory axonal neuropathy described in SCA4 [4,5] was absent in our patients, as in the earlier reports of 16q-linked ADCA [6–10,20,21]. Sensorineural hearing impairment was considered to be another important clinical feature of the disease [9,10], and 6 (42.8%) of 14 families were reported to have this condition in addition to age-related hearing loss [9]. In our study, audiograms revealed that 37.5% of the patients examined had hearing impairment. However, since we examined only eight patients by means of audiograms, further examinations including audiograms and brainstem auditory evoked potential measurement will be necessary to clarify whether or not hearing impairment is associated with 16q-linked ADCA. Third, we found two asymptomatic carriers with transient nystagmus and mild hyperreflexia, suggesting they are early clinical signs of this disease.

It is noteworthy that we found two sporadic patients with 16q-linked ADCA who had been diagnosed as having LCCA. The parents of the two sporadic patients were all normal until death in their 40s and at 73, 74, and 94 years old, and there were no individuals with cerebellar ataxia in their families. Since the age at onset in our patients with 16q-linked ADCA is very late, the parents who could have harbored a C-to-T mutation in the *puratrophin-1* gene appeared to be neurologically free until their death. Otherwise, incomplete penetrance can be suspected in 16q-linked ADCA. Thus, there is a possibility that a patient with this disease can be misdiagnosed as having sporadic LCCA, and we should analyze the *puratrophin-1* gene even in an apparently sporadic case with cerebellar ataxia.

In pedigree 2, four of the six patients were homozygous for the C-to-T substitution in the *puratrophin-1* gene. Comparing the mean age at onset in homozygotes with that in heterozygotes in this pedigree, the former was earlier than the latter. Unfortunately, we could not accurately compare the phenotypic severity during the disease course in them. Since the number of observation is low, we should be prudent in interpretation for a gene dosage effect in 16q-linked ADCA. In SCA6, although a gene dosage effect is considered [22–24], the increase in the severity of symptoms with homozygosity is not as great as that observed in MJD/SCA3 [13]. Similarly, a gene dosage effect in 16q-linked ADCA, if one exists, might be mild and similar to that in SCA6. Further studies are required to clarify whether a gene dosage effect indeed exists in 16q-linked ADCA or not, because the brain MRI findings revealed similar atrophy of the cerebellum in a homozygous patient and a heterozygous one.

Our study revealed that 16q-linked ADCA was the third-most frequent subtype of ADCA next to MJD/SCA3 and SCA6 in 110 Japanese families with ADCA. Although SCA6, MJD/SCA3, and DRPLA are considered to be the most prevalent subtypes of ADCA in Japan despite considerable variation in the frequency of each subtype among districts [25], our study showed that 16q-linked ADCA is also frequently seen among Japanese patients with ADCA, and thus this disease may be widespread in Japan. Meanwhile, 13.6% of our ADCA families still remained to be caused by an unknown molecular basis. The clinical features of these families showed adult-onset cerebellar ataxia with or without extracerebellar neurological dysfunction. Although a linkage analysis could not be performed on these families because of a small number of the family members, we should elucidate the molecular etiology of these ADCA families in the near future.

We confirmed that a C-to-T single nucleotide substitution in the 5′ UTR of exon 1 in the *puratrophin-1* gene is strongly associated with a distinct form of ataxia. This substitution appears to be the mutation that causes 16q-linked ADCA for the following reasons. First, this change was completely segregated with the disease in 52 Japanese ADCA families, whereas such a change was not seen in 1000 control chromosomes [9]. Second, the C-to-T change resulted in reduced expression in the in vitro luciferase assay, which was consistent with the tendency for reduction in mRNA expression in the cerebellum in 16q-linked ADCA [9]. Third, *puratrophin-1* was aggregated in the major target neurons, i.e., Purkinje cells, in 16q-linked ADCA [9]. In the present study, we also confirmed that allele 2 of GA001 was only seen in all affected and asymptomatic carriers with the C-to-T substitution. Since allele 2 ("allele 4" in the previous report) has been seen in all affected individuals in all 52 families with 16q-linked ADCA, but in only 1 in 1000 control chromosomes, GA001 shows strong linkage disequilibrium [9]. Although we could perform haplotype analysis in only two families, the haplotype of "1-2-3-2" (TA001-GA001-TTTA001-CATG003) was common in the two families, suggesting a founder effect in 16q-linked ADCA. Similarly, a strong founder effect has been observed for 16q-linked ADCA in Japan [7, 9].

Finally, it is interesting as to whether 16q-linked ADCA and SCA4 are allelic or not [26]. Since the possible pyramidal tract signs with cerebellar ataxia seen in our patients are common in 16q-linked ADCA and SCA4 despite the absence of sensory axonal neuropathy in the former, the two disorders might be allelic. There is a possibility that patients with 16q-linked ADCA will hereafter be found throughout the world. Further investigations are necessary to clarify the molecular mechanisms underlying 16q-linked ADCA and SCA4.

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ORIGINAL ARTICLE

Takako Ohata · Kunihiro Yoshida · Haruya Sakai
Haruka Hamanoue · Takeshi Mizuguchi
Yusaku Shimizu · Tomomi Okano · Fumio Takada
Kinya Ishikawa · Hidehiro Mizusawa
Ko-ichiro Yoshiura · Yoshimitsu Fukushima
Shu-ichi Ikeda · Naomichi Matsumoto

A −16C>T substitution in the 5′ UTR of the *puratrophin-1* gene is prevalent in autosomal dominant cerebellar ataxia in Nagano

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Abstract The molecular bases of autosomal dominant cerebellar ataxia (ADCA) have been increasingly elucidated, but 17–50% of ADCA families still remain genetically undefined in Japan. In this study we investigated 67 genetically undefined ADCA families from the Nagano prefecture, and found that 63 patients from 51 families possessed the −16C > T change in the *puratrophin-1* gene, which was recently found to be pathogenic for 16q22-linked ADCA. Most patients shared a common haplotype around the *puratrophin-1* gene. All patients with the −16C > T change had pure cerebellar ataxia with middle-aged or later onset. Only one patient in a large, −16C > T positive family did not have this change, but still shared a narrowed haplotype with, and was clinically indistinguishable from, the other affected family members. In Nagano, 16q22-linked ADCA appears to be much more prevalent than either SCA6 or dentatorubral-pallidoluysian atrophy (DRPLA), and may explain the high frequency of spinocerebellar ataxia.

Keywords Autosomal dominant cerebellar ataxia · 16q22-linked ADCA · *puratrophin-1* · Nagano

Takako Ohata and Kunihiro Yoshida have equally contributed to this work

T. Ohata · K. Yoshida · H. Sakai · H. Hamanoue
T. Mizuguchi · N. Matsumoto
Department of Human Genetics, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan

T. Ohata · F. Takada
Department of Medical Genetics, Kitasato University Graduate School of Medical Sciences, 1-15-1 Kitasato, Sagamihara 228-8555, Japan

K. Yoshida (✉) · Y. Shimizu · T. Okano · S. Ikeda
Third Department of Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan
E-mail: kyoshida@hsp.md.shinshu-u.ac.jp
Tel.: +81-263-372673
Fax: +81-263-373427

K. Yoshida · Y. Fukushima
Division of Clinical and Molecular Genetics, Shinshu University Hospital, 3-1-1 Asahi, Matsumoto 390-8621, Japan

K. Ishikawa · H. Mizusawa
Department of Neurology and Neurological Science, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

K. Yoshiura
Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

Introduction

Autosomal dominant cerebellar ataxia (ADCA) is genetically heterogeneous (Margolis 2003; Schols et al. 2004). The most updated GeneTests (8 November 2005) and HUGO Gene Nomenclature Committee (25 November 2005) cover at least 27 different ADCA subtypes including SCA28. Among these, a coding CAG (or CAA, both coding glutamine) repeat expansion has been found in seven subtypes: SCA1, 2, 3/Machado-Joseph disease (MJD), 6, 7, and 17, and dentatorubral-pallidoluysian atrophy (DRPLA) (Banfi et al. 1994; Kawaguchi et al. 1994; Nagafuchi et al. 1994; Imbert et al. 1996; Pulst et al. 1996; Sanpei et al. 1996; David et al. 1997; Zhuchenko et al. 1997; Koide et al. 1999; Nakamura et al. 2001). A non-coding repeat expansion occurs in three subtypes: SCA8, 10, and 12 (Holmes et al. 1999; Koob et al. 1999; Matsuura et al. 2000; Fujigasaki et al. 2001), and a missense mutation in two of them: SCA14 and 27 (Chen et al. 2003; van Swieten et al. 2003). Several reports regarding 16q22-linked ADCA have been released (Nagaoka et al. 2000; Takashima et al. 2001; Li et al. 2003; Hirano et al. 2004), and a single nucleotide substitution (−16C > T) in the 5′

UTR in the *puratrophin-1* gene was recently identified in all patients from 52 unrelated Japanese families sharing a common haplotype at 16q22.1 (Ishikawa et al. 2005).

In Japan, the incidence of spinocerebellar degeneration/ataxia (SCD/SCA) including multiple-system atrophy (MSA) is 15.68 in 100,000. Although SCA6, SCA3/MJD, and DRPLA are the three most prevalent subtypes, their frequencies quite differ from region to region (Maruyama et al. 2002; Sasaki et al. 2003). We previously showed that the incidence of SCA, excluding MSA, was higher (22 in 100,000) in Nagano than in other parts of Japan. In 86 unrelated ADCA families from Nagano, SCA6 (19%) and DRPLA (10%) were common, while SCA3/MJD (3%), SCA1 (2%), and SCA2 (1%) were infrequent (Shimizu et al. 2004). More importantly, the majority of families (65%) were genetically undefined; such families make up 17–50% of the ADCA families in other parts of Japan (Maruyama et al. 2002; Sasaki et al. 2003; Shimizu et al. 2004). A common haplotype of 16q22-linked ADCA reported by Li et al. (2003) was not confirmed in our series (Shimizu et al. 2004).

We hypothesized that there may be distinct ADCA subtypes in Nagano because it is relatively isolated by steep mountains. A genome-wide linkage study was performed in undefined ADCA families to identify possibly new ADCA loci. The −16C > T substitution in *puratrophin-1* was also investigated.

Materials and methods

Subjects

A total of 105 individuals (83 affected and 22 unaffected) from 67 ADCA families originating from the Nagano prefecture were recruited to this study. All affected individuals were examined by at least one experienced neurologist according to the standard clinical criteria. Dominant inheritance was presumed when affected individuals were recognized in at least two generations. Three families (SCAF9, SCAF25, and SCAF41) with several affected members were used for linkage studies. Genomic DNA was isolated from peripheral leukocytes using a PUREGENE DNA purification kit (Gentra Systems, Minneapolis, MN, USA). SCA 1, 2, 3/MJD, 6, 7, 12, and 17, and DRPLA were ruled out after confirming the (CAG)n length by PCR as previously described (Shimizu et al. 2004). This research protocol was approved independently by the Ethical Committee of Shinshu University School of Medicine and by the Committee for Ethical Issues at Yokohama City University School of Medicine.

Linkage analysis

A large family, SCAF41, consisting of 7 affected and 15 unaffected members, was analyzed using 400 polymorphic markers (ABI PRISM Linkage Mapping Set version 2.5-MD10; Applied Biosystems, Foster City, CA, USA). Furthermore, an additional 21 polymorphic markers mapped to 16q21–16q23.1 (*D16S3111*, *D16S3050*, *D16S3021*, *D16S3043*, *D16S3019*, *TAGA02*, *TTCC01*, *D16S3086*, *GATA01*, *D16S421*, *TA001*, *GA001*, *TTTA001*, *CATG003*, *17msm*, *D16S3085*, *D16S3025*, *CTTT01*, *D16S3067*, *GT01*, and *D16S3018*) were used for the study of three families, SCAF9, SCAF25, and SCAF41. Primer sequences are described elsewhere (Hirano et al. 2004; Ishikawa et al. 2005). PCR was cycled 40 times at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s in a 10-μl mixture containing 10 ng genomic DNA, 0.5 μM of each primer, 0.2 mM each of dNTP, 10× PCR buffer (TaKaRa, Ohtsu, Japan), and 0.25 U of Takara Ex Taq DNA polymerase (TaKaRa). PCR products were analyzed by an ABI 3100 Genetic analyzer (Applied Biosystems), and their product sizes were determined using the GeneMapper Software version 3.5 (Applied Biosystems). Two-point linkage analysis was carried out using the LINKAGE Program Package (FASTLINK software, version 5.1). The allele frequencies of the markers were set as equal when they were unknown. The disease gene frequency was assumed to be 0.00001. The possibly affected individuals were scored as unknown. LOD scores were corrected by age-dependent penetrance established based on the cumulative age at onset (penetrance 0 for persons aged 39 years and younger, 0.08 for those aged 40–49 years, 0.37 for those aged 50–59 years, 0.79 for those aged 60–69 years, and 0.99 for those aged 70 years or older).

Analysis of a single nucleotide substitution (−16C > T) in the 5′ UTR of *puratrophin-1*

Primer sequences have been described elsewhere (Ishikawa et al. 2005). PCR was cycled 35 times at 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s in a 20-μl mixture containing 30 ng genomic DNA, 0.5 μM of each primer, 0.2 mM each of dNTP, 10× PCR buffer (TaKaRa), and 0.25 U of Takara Ex Taq DNA polymerase (TaKaRa). PCR products were purified with ExoSAP-IT (USB, Cleveland, OH, USA) and sequenced by a standard protocol using BigDye terminator (Applied Biosystems) on an ABI PRISM 3100 Genetic analyzer (Applied Biosystems). Nucleotide substitution was confirmed using the SeqScape software version 2.0 (Applied Biosystems), and by *Eco*NI RFLP designed by Ishikawa et al. (2005). All patients were genotyped for at least nine markers: *16S3086*, *GATA01*, *D16S421*, *TA001*, *GA001*, *TTTA001*, *CATG003*, *17msm*, and *D16S3085*, to confirm haplotypes.

Results

Genome-wide linkage analysis using 400 markers in SCAF41 did not give any locations of maximum LOD scores of three or more. Although several locations with

relatively high scores were identified, including *D1S2785* on 1q43 (LOD, 1.18 [$\theta=0$]), *D8S549* on 8p22 (LOD, 1.43 [$\theta=0$]), *D16S515* on 16q23.1 (LOD, 1.03 [$\theta=0$]); thus, the initial screening failed to reveal a specific locus for the disease.

During this study, the single nucleotide substitution (−16C > T) in the 5′ UTR of the *puratrophin-1* gene was identified as a possible pathological change for 16q22-linked ADCA (Ishikawa et al. 2005). We found this substitution in 11 out of 12 affected and 2 out of 22 unaffected individuals in SCAF9, SCAF25, SCAF41 (Fig. 1), and 16q22-focused linkage analysis in these three families using additional 21 markers gave a maximum LOD score at *TAGA02* of 2.42 ($\theta=0$). Haplotype analysis demonstrated a common haplotype (1-3-2-T-1-2-5-1-2-2 at *D16S3086 –GATA01 –D16S421* – [−16C/T of *puratrophin-1* {*Q9H7K4*}] *–TA001 –GA001 –TTTA001 –CATG003 –17msm –D16S3085*) in all affected members except SCAF41-21 and two young unaffected members, SCAF41-10 (40 years old) and SCAF25-5 (41 years old), who may be obligate carriers. It is noteworthy that SCAF41-21 (59 years old) did not have the −16C > T change, but shared only a narrowed haplotype (1-3-2 at *D16S3086 –GATA01 –D16S421*) as it is assumed that a recombination happened between *D16S421* and *Q9H7K4*. While her clinical symptoms were still mild, a slowly progressive gait ataxia and clumsiness in the hands were evident.

Further analysis of nine markers (*D16S3086*, *GATA01*, *D16S421*, *TA001*, *GA001*, *TTTA001*, *CATG003*, *17msm*, and *D16S3085*), as well as the −16C > T change, was performed in the other 71 patients from 64 families (Fig. 2). We found that 53 patients from 48 families carried the −16C > T substitution and their phenotypes were compatible with pure cerebellar ataxia. Their average age of disease onset was 60.2 ± 9.3 years (mean ± 1 SD), while the average age of onset for 18 patients from 16 families without the substitution was 37.9 ± 20.8 years and most of them showed juvenile-onset cerebellar ataxia, or extracerebellar neurological symptoms such as parkinsonism, dementia, and/or involuntary movements. Genetic anticipation was observed in two families. Five out of 16 families without the −16C > T change showed late-onset, pure cerebellar ataxia indistinguishable from that of typical patients with the −16C > T change in the *pura-*

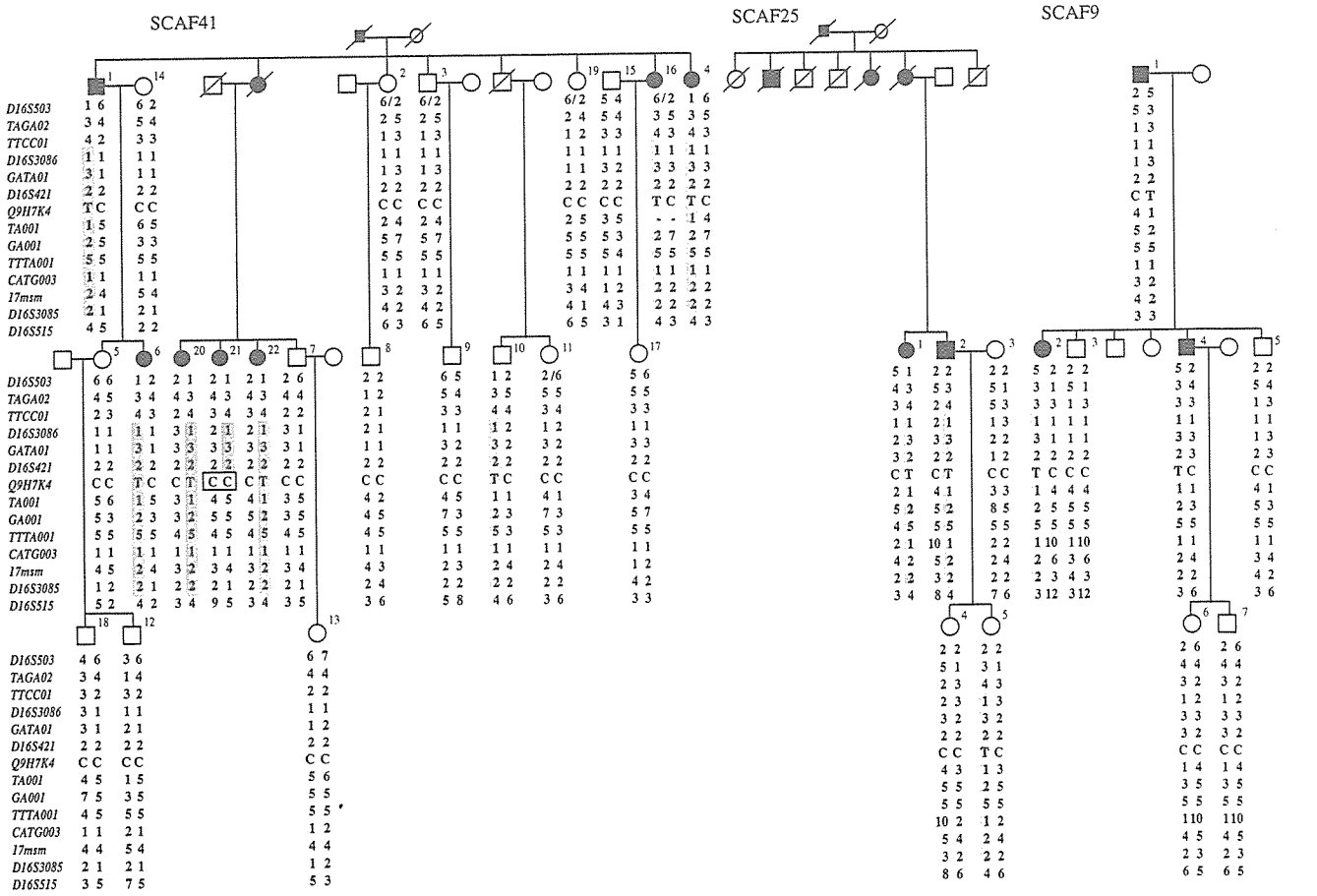

Fig. 1 Haplotype analysis in three large ADCA families, SCAF41, SCAF25, and SCAF9. Thirteen polymorphic markers mapped to 16q21–16q23 and the −16C > T substitution in *puratrophin-1 (Q9K7H4)* are shown. Twelve affected and two unaffected individuals had a common haplotype (1-3-2) at *D16S3086–GA-TA01–D16S421*. The −16C > T substitution was found in 11 out of 12 affected and 2 out of 22 unaffected individuals

A: Patients with -16C>T substitution

patient ID	1	2	5	7	8	9	10	12	15	16	17	18	21	22	24	25	26	29	31	32	33	35	36	37	38	39	40	41	43	44	45	47	48	49	50	51	55	56	57	59	60	61	63	64	67	68	69	70	71	72	73	74	75
family ID	11	13	18	20	21	22	24	33	38	40	42	46	49	50	57	58	59	61	64	65	67	69	70	71	73	74	75	76	79	80	81	82	84	85	86	87	83	91	92	94	95	96	99	100	29	29	105	106	100	15	15	15	15
D16S3086	1	1/2	1	1	1	1	1	1/3	1	1/3	1	1	1/5	1/2	1	1/2	1/3	1	1	1/2	1	1/3	1/3	1	1/2	1/2	1/3	1	1/2	1	1/2	1	1	1	1	1	1	1	1/2	1/2	1	1	1	1/2	1/2	1	1	1	1	1/2	1/2	1/4	1/4	1
GATA01	3/2	2	2	2	2/5	2/1	3/1	3/2	3	3/2	3/1	2	2/1	2/1	2	3/2	2	3	2	3/1	3	3/2	3	3/2	3	3/1	3/2	3/2	3/1	3	3/2	3/1	3	2/1	2	2	2/1	2/5	2/1	2/1	2	3	3/1	3	3/1	3	2/1	2/1	3	3/1	3	3	3/1
D16D421	2	2/3	2	2	2	2/6	2/4	2	2	2	2/4	2/3	2	2	2/1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2/4	2	2	2	2	2	2	2	2	2	2	2	2	2	2/3	2	2	2	2	2	2	2
Q9H7K4	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c	t/c
TA001	1	1/8	1/6	1/5	1	1/5	1/4	1/3	1/9	1/4	1/4	1/10	1/12	1/12	1/2	1/3	1/6	1	1	1/3	1/13	1/4	1/4	1	1/3	1/2	1/4	1/4	1/3	1/4	6/14	1/5	1/5	1/5	1/5	1/5	1/4	1/3	1/4	1/4	1/3	1/13	1/13	6/14	1/5	1/2	1/4	1/4	6/14	1/7	1/4	1/4	1/2
GA001	2/3	2/5	2/1	2/3	2/3	2/3	2/6	2/3	2/1	2/6	2/6	2/5	2/6	2/5	2/5	2/4	2/1	2/3	2/3	2/5	2/5	2/7	2/6	2/3	2/3	2/6	2/7	2/5	2/5	2/9	2/6	2/5	2/5	2/5	2/3	2/3	2/5	2/1	2/5	2/5	2/4	2/5	2/5	2/5	2/5	2/3	2/5	2/5	2/5	2/6	2/6	2/6
TTTA001	5	5	5	5/4	5	5/4	5/4	5/4	5/4	5/4	5/4	5	5/4	5/4	5	5	5	5	5/4	5	5	5	5/4	5	5	5	5	5	5/4	5/6	5/2	5	5	5	5/4	5	5	5/4	5	5	5	5	5/4	5	5	5	5	5	5	5	5/6	5/6	5/4
CATG003	1/3	1/2	1	1/2	1	1	1/10	1/2	1/7	1	1/10	1	1	1/10	1/2	1	1	1	1/6	1	1	1	1	1	1	1/10	1	1/2	1/6	1	1	1	1	1/2	1	1/2	1	1/5	1/5	1	1	1/6	1/2	1	1/2	1	1/2	1	1	1	1/10
17msm	2/4	2/6	2/6	2/3	2/5	2	2/5	2/3	2/5	2/4	2/5	2/4	2/5	2/5	2/3	2/4	2/5	2/4	2/5	2/6	2/1	2/4	2/4	2/5	2/3	2/4	2/4	2/4	2/6	2	2/9	2/5	2/4	2	2/3	2/3	2/5	2/7	2/4	2/4	2/4	2/4	2/7	2	2/4	2/3	2/5	2/3	2	2/4	2/4	2/4	2/4
D16S3085	2	2	2	2	2	2	2/3	2	2	2	2/3	2	2	2/3	2	2	2	2	2	2/3	2	2	2	2	2	2/3	2	2	2/3	2	2	2/1	2	2	2	2/3	2	2	2	2	2	2/3	2	2	2	2/4	2	2	2/1	2	2	2/3
age of onset	61	62	46	45	66	76	N	N	57	N	N	62	57	62	61	50	69	58	54	58	50	75	53	61	54	60	59	77	78	60	73	75	77	70	N	72	68	56	50	50	61	56	69	74	56	54	68	48	70	44	50	45	N

B: Patients without -16C>T substitution

patient ID	3	4	6	11	13	19	20	28	34	42	46	52	53	54	58	62	65	66
family ID	14	16	19	19	34	47	48	60	68	78	19	88	89	90	93	98	102	103
D16S3086	2	1	1	1	1	1	1/3	1/3	1/2	1	1/2	1/3	1/2	1	1/2	1/3	1/3	1
GATA01	2/1	2	3	2/3	2/3	2	1	2/3	2/6	1/6	2/3	2/3	2/3	3	2	3	3	2/3
D16D421	2/5	2	2	2/3	2/3	2	2	2/3	2	2	2/3	2	2/4	2	2/3	2	2	2
Q9H7K4	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c	c/c
TA001	2/5	4/6	1	1/2	2	4/116	2/5	3/5	3/5	1/13	4	5	1/4	4	1/4	2/4	8/13	
GA001	5/6	3	3	3/5	5	3	5	1/6	3/5	5	3/5	5	5	3/9	5	3/6	5/6	4/5
TTTA001	5/2	5/4	5	5	5	5	5	5/4	5/4	5	5	5	5/2	5/4	5	5/4	5/4	5/2
CATG003	3/4	1	1/3	1/3	1/2	1/2	4	1	1/6	1	1/3	1/10	2/4	1	1/3	1	1/2	1
17msm	2/4	1/5	4/5	2/5	3	4	4	4/8	3/4	5	4/5	5	4/10	2/5	3/5	4/5	2/4	4/11
D16S3085	2/1	2	2	2	2	2	2	2	2/4	2/4	2	2/3	2	2	2/3	2	2	2
age of onset	22	22	20	N	46	58	N	68	71	52	5	24	17	57	N	N	48	44

Fig. 2 Genotype of 71 patients from the other 64 families. The −16C>T substitutions in *puratrophin-1 (Q9K7H4)* was observed in 53 patients from 48 families (**A**), but not in 18 patients from 16 families (**B**). A haplotype, 2-1-2-5-1-2-2 of seven markers (*D16S421 −TA001−GA001−TTTA001−CATG003−17msm−D16S3085*) was shared by 50 patients with the −16C>T change and another haplotype, 2-6/14-2-5-1-2-2 for the same markers was shared by three patients (patient IDs, 45, 64, and 71). *N* unknown

trophin-1 gene. Additionally, four negative controls, SCA6 patients, also did not have the −16C>T change (data not shown). Among the 51 families with the −16C>T change, 49 of them shared a common haplotype around the *puratrophin-1* gene, 2-1-2-5-1-2-2 for seven markers (*D16S421 −TA001 −GA001 −TTTA001 −CATG003 −17msm −D16S3085*), and two families showed a slightly different haplotype, 2-6/14-2-5-1-2-2.

Discussion

The −16C>T change in the 5′ UTR of *puratrophin-1* was found in 51 out of 67 ADCA families (76%) from the Nagano prefecture, in which SCA1, 2, 3/MJD, 6, 7, 12, 17, and DRPLA had been previously ruled out. Among 106 ADCA families genetically analyzed to date (unpublished observation), the frequency of 16q22-linked ADCA (51 out of 106, 48%) is much higher than that of either SCA6 (18 out of 106, 17%) or DRPLA (9 out of 106, 8%). Thus, an accumulation of 16q22-linked ADCA families leads to a high prevalence of SCD in Nagano. Almost all patients shared the haplotype 2-1-2-5-1-2-2 for markers *D16S421 −TA001 −GA001 −TTTA001 −CATG003 −17msm −D16S3085*. For five of these markers, *D16S421 −TA001 −GA001 −TTTA001 −CATG003*, our haplotype 2-1-2-5-1 was identical to the haplotype 3-1-4-4-4 reported by Ishikawa et al. (2005), based on the data of four patients from two families that were analyzed independently by both groups. Sixty-four patients from 51 families with the −16C>T substitution showed pure cerebellar ataxia with middle-aged or later onset (Harding's ADCAIII; Harding 1993), while most of the patients without the substitution showed clinical phenotypes characterized by juvenile-onset cerebellar ataxia, additional extracerebellar neurological symptoms, and/or genetic anticipation. Previously, we could not confirm the common haplotype of 16q22-linked ataxia reported by Li et al. (2003; Shimizu et al. 2004), being inconsistent with the current data. This is partly explained by the fact that the focused region presented here was much narrower than the previously haplotyped region.

The −16C>T substitution in the 5′ UTR of *puratrophin-1*, a region of the gene presumed to be regulatory, is unique as a disease-causing change for ADCA. To date, pathological single nucleotide substitutions have been found only in SCA14 or SCA27 (Chen et al. 2003; van Swieten et al. 2003), both of which are missense mutations. It has been speculated that the −16C>T change might decrease mRNA expression of *puratrophin-1*, and cause aggregation of puratrophin-1 protein in Purkinje cells in affected cerebellum (Ishikawa et al. 2005).

Ishikawa et al. (2005) found the −16C>T substitution in the *puratrophin-1* gene in all affected individuals from 52 unrelated Japanese families. However, in this study there was one exceptional patient without this substitu-

tion in a family in which all other affected individuals carried the change, a finding confirmed by two independent examiners. This patient showed clinical features that did not differ significantly from the other affected members in her family. At present, it is unclear whether she may be a phenocopy or whether the real pathogenic mutation may exist in other regions within the shared haplotype between *TTCC01* and *Q9H7K4*. Careful observation of her clinical course and more comprehensive genetic analyses of her family are needed. The pathological consequence of the −16C > T substitution in the *puratrophin-1* gene should be further investigated.

In conclusion, we have found that the −16C > T substitution in the 5′ UTR of *puratrophin-1* was very prevalent in ADCA families in Nagano, where the frequency of 16q22-linked ADCA is much higher than that of SCA6, DRPLA, and SCA3/MJD, the most common subtypes in Japan. An accumulation of 16q22-linked ADCA families may be the main reason for the high incidence of SCD in Nagano. Further studies are needed to elucidate the clinical details and molecular pathogenesis of 16q22-linked ADCA.

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