the expansions of a trinucleotide (CAG) repeat that encodes the polyglutamine tract, uniformly causing the aggregation of polyglutamine-containing causative protein (Ross and Poirier 2004). The expansion of noncoding trinucleotide (CAG or CTG) or pentanucleotide (ATTCT) repeats are involved in SCA8, SCA10, and SCA12 (Holmes et al. 1999; Koob et al. 1999; Matsuura et al. 2000). Very few families are affected by missense mutations in beta-III spectrin (*SPTBN2*) (SCA5 (see Ikeda et al. 2006)), voltage-gated potassium channel KCNC3 (SCA13 (see Waters et al. 2006)), protein kinase C gamma (PKC gamma) (SCA14 (see Chen et al. 2003)), and *FGF14* genes (ADCA with FGF14 mutation (see van Swieten et al. 2003). However, genes or even loci remain unidentified for 20–40% of families with ADCA (Sasaki et al. 2003).

We had previously found that Japanese families with ADCA map to the human chromosome 16q22.1 (16q-ADCA), the gene locus of SCA4 (Flanigan et al. 1996; Hellenbroich et al. 2005; Nagaoka et al. 2000). However, our families show clinically pure cerebellar ataxia without other neurological signs, such as sensory neuropathy or pyramidal tract signs seen in SCA4. All 16q-ADCA patients shared a common haplotype, presumably due to inheritance from a disease chromosome of a founder (Takashima et al. 2001). Our haplotype analysis of 52 families with DNA polymorphic microsatellite markers revealed that they all share a common haplotype for the 400-kb genomic region in 16q22.1 (Ishikawa et al. 2005). Within this region, we found that a heterozygous single nucleotide C-to-T substitution (–16C>T) in the untranslated region of the *puratrophin*-1 gene was entirely segregated with all patients, suggesting a strong association with the disease. This substitution was also found in other cohorts of Japanese families with ataxia (Ouyang et al. 2006; Onodera et al. 2006), while it was not found in Caucasian patients in Europe (Wieczorek et al. 2006). The frequency of 16q-ADCA is considered to be relatively high in Japan, counted as the third or fourth major subtype of ADCA after MJD, SCA6, and DRPLA (Takano et al. 1998; Sasaki et al. 2003; Ohata et al. 2006).

However, one group recently reported an exceptional patient without the –16C>T substitution in the *puratrophin*-1 gene, in a family in which all of the other affected subjects carried the substitution (Ohata et al. 2006). This patient shared the common haplotype in a region centromeric to the substitution in the *puratrophin*-1 gene, suggesting that a true pathogenic mutation may be present in a different gene lying centromeric to the –16C>T substitution in the *puratrophin*-1 gene. Moreover, other patients sharing the common haplotype centromeric to the substitution in the *puratrophin*-1 gene without the substitution might exist.

In this study, we re-examined the haplotype of families showing ataxia in order to clarify a common genomic region shared in all 16q-ADCA patients. Because slippage mutation might cause minor deviations in repeat size for microsatellite markers (Ikeda et al. 2004), single nucleotide polymorphisms (SNPs) detected by ourselves on the disease chromosome were used in the analysis to confirm recombinant regions that are not conserved among families.

Materials and methods

Haplotype analysis

DNA samples from patients showing ataxia referred to our department were examined. After informed consent was obtained, genomic DNA was extracted from peripheral blood lymphocytes or lymphoblastoid cell lines by the use of methods described elsewhere (Ishikawa et al. 1997). All families were excluded for SCA1, SCA2, SCA3/ MJD, SCA6, SCA7, SCA8, SCA12, SCA14, SCA17, and DRPLA by testing for mutations in the disease genes.

Firstly, common haplotypes of the 16q-ADCA families with the –16C>T substitution in the *puratrophin*-1 gene were analyzed. Genotypes were determined for 19 microsatellite markers (D16S3043, D16S3031, D16S3019, CTATT01, TAGA02, GGAA05, D16S397, GGAA10, GATA01, D16S421, TA001, GA001, 17 msm, D16S3107, GGAA01, CTTT01, GT01, D16S3095, D16S512) in 16q22.1 by the use of methods described elsewhere (Ishikawa et al. 2005). Compared to our previous study (Ishikawa et al. 2005), several new markers with high specificity to the 16q-ADCA chromosome were added and the region analyzed was expanded to beyond the previous critical region spanning GATA01 and 17 msm (Ishikawa et al. 2005) in order to determine the maximum genomic region conserved in all of the affected individuals from all of the families. Although the phase of the markers were not confidently determined in families that have only a few examined members, the possibility that they carried the haplotype was indicated in those cases.

Secondly, haplotypes of families without the –16C>T substitution in the *puratrophin*-1 gene were also analyzed to see if they had the common haplotype centromeric to the substitution in the *puratrophin*-1 gene. Their genotypes were determined for 14 markers (D16S3043, D16S3019, CTATT01, TAGA02, GGAA05, D16S397, D16S3086, GATA01, GA001, 17 msm, CTTT01, GT01, D16S3095, D16S512), which are relatively highly specific to the common haplotype in 16q-ADCA.

Single nucleotide polymorphisms

We searched for single nucleotide polymorphisms (SNPs) on the disease chromosome by ourselves because most of

Table 1 The haplotype analysis of 16q22.1-linked autosomal dominant cerebellar ataxia (16q-ADCA) families with the –16CT substitution of the *puratrophin*-1 gene. The gray squares indicate that the alleles are one repeat-unit different fromthe common allele of 16q-ADCA and the black squares indicate alleles with two or more repeat-unitdifferences. One repeat-unit difference was seen for markers D16S397, GGAA10,GATA01, and TA001, close to the *puratrophin*-1 gene in several families, and greaterrepeat-units differences were observed for GGAA05 and other centromeric markers. Similarly, greater repeat-units differences were observed for 17msm and markers lyingtelomeric to 17msm. n.e.=not examined

Marker	most common haplotype	family No. frequency in control (%)	P2	P4	P14	T2	T3	T4	T5	T6	T7	T12	T15	T19	T21	T25	T26	T28	T30	T37	T42	T43	T44	T46
D16S3043	1	25.8	1	1/6	7	5	8	1	1/8	1	1/5	5	1/7	n.e.	1/8	n.e.	n.e.	1	n.e.	1	n.e.	1/5	1/5	1
D16S3031	9	68.1	9	9	9	9	9	9	10	1	10	9	9/10	9	9	9	9	1/9	9	9	9	9	9	9
D16S3019	4	41.4	4	4	4	4	4	4/5	3/4	3/4	3/4	1/4	4	n.e.	4	n.e.	n.e.	4/7	3/4	2/4	n.e.	3/4	3/4	1
CTATT01	1	32.4	1	2/4	1	1	1	1/4	1	1	1	1	1/3	n.e.	1/3	n.e.	n.e.	1/3	n.e.	1	n.e.	1/2	1	0/3
TAGA02	4	38.3	4	6	4	4	4	4/6	4/6	4/5	4	2/4	4/5	n.e.	4/5	n.e.	n.e.	4/5	4	3/4	n.e.	5/6	4/5	2/6
GGAA05	1	1.4	1	6	1	1	5	1	1	1	2	1	2/4	1/3	1/5	5	1/2	1/7	1/5	1/3	1/3	1/5	2/4	3/6
D16S397	1	47.1	n.e.	1/2	1	1	1	0/4	1/2	1/3	1/3	2/3	1	n.e.	1	n.e.	n.e.	1/4	-3/0	-3/1	n.e.	-3/1	1	-3/1
GGAA10	3	13.2	3	3	3	3	4	3/5	3	3	3	3	3	3/6	2/3	2/4	3/7	3/5	3/8	4/6	3	3/6	3/5	3/7
GATA01	2	44.1	2	2/3	2	2	2	3	3	3	2	2	1/2	2/3	1/3	3/4	1/3	1/3	1	2	3	1/3	2/3	2/3
D16S421	3	75.7	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3/4	3	3	3
puratrophin-1(C/T)	T	0.0	T	T	T	T	T	T	T	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	23.8	1	1	1	1	1	1	1	1	1	1	1	1/9	1/7	2/8	1/6	1/10	1/9	1/9	1	1/9	1/9	1/5
GA001	4	0.1	4	4	4	4	4	4	4	4/7	4/7	4	4	1/4	4/8	4/5	4/7	4/7	4/6	4/11	4/5	4/7	4/5	4/7
17msm	2	8.3	2	2	2	2	2	2	2	2	2	5	2	2/5	2/5	2/4	2/4	2/4	2/4	2/5	2/4	2/4	4/6	2/4
D16S3107	7	13.9	7	7	7	7	7	7	6	7	7	7	5	6	5/6	3/7	6/10	6	7	5/7	3/7	6/7	4/7	6/7
GGAA01	6	18.8	6	6	6	6	6	6	6	7	6	6	1/6	3/6	2/6	2/6	4/6	6/7	6	6	6	6/7	6/7	5/6
CTTT01	8	28.2	8	8	8	8	9/10	8	5	9	8	10	8	9/10	3/9	9/10	8/10	8	8/11	6/9	8/15	9/10	1/7	3/8
GT01	6	15.8	6	6	6	6	6	6	7	6	6	4	6	2/6	1/6	4/6	4/6	2/6	5/6	4/6	6	3/6	3/4	4/6
D16S3095	1	9.7	2	3	1	0	1	1	2	3	2	1/2	1	1	1/2	1/2	1/2	1/3	1	1	1/2	1/3	2	1
D16S512	1	32.3	n.e.	2/4	1	4	1/5	4	2/4	1/5	1/5	1/5	4	n.e.	4/5	n.e.	n.e.	5	1	1/2	n.e.	2/4	4	4/5

the SNPs obtained from public databases were not present on the disease allele or did not have enough specificity to the disease chromosome. SNPs were revealed by direct sequencing of the genomic DNA from a homozygous patient who carries the common haplotypes between D16S3031 and GT01 in both of the chromosomes. Primers were designed to amplify about 800 bp from genomic DNA (primer sequences are available on request), and polymerase chain reaction (PCR) and sequencing were performed with the same methods as previously described (Ishikawa et al. 2005). Comparing the sequenced data and the annotated databases with use of DNASIS (Hitachi) software revealed many SNPs. With the sequenced data of the control genomic DNA, SNPs with high specificity to the 16q-ADCAs were chosen. With these SNPs, 16q-ADCA families were analyzed to reveal the borders of the maximally conserved genomic region.

Results

Haplotype analysis of 16q-ADCA with the –16C>T substitution in the *puratrophin*-1 gene

One hundred and twenty-five patients from 64 families were diagnosed as 16q-ADCA based on the clinical features and the presence of the –16C>T substitution in the *puratrophin*-1 gene. The families included 52 families that we had previously reported (Ishikawa et al. 2005) and 12 new families that had not been reported elsewhere. They all share similar haplotypes around the *puratrophin*-1 gene. The most common haplotype among these families are shown in the left column in Table 1. Twenty-two families out of the 64 families showed different alleles at least for one of the DNA markers as shown in Table 1. The remaining 42 families, which are not listed in Table 1, harbored or had the possibility to harbor the common haplotype.

There was one repeat-unit difference from the common alleles for D16S397, GGAA10, GATA01, and TA001 close to the *puratrophin*-1 gene in 13 out of 22 families. For centromeric DNA markers from the *puratrophin*-1 gene, such as GGAA05, TAGA02, D16S3031, and D16S3043, eight families (P4, P14, T2, T3, T6, T12, T25, T46) harbored alleles with greater differences in repeat number (more than two repeat-units). Furthermore, families P4 and T46 carried different alleles in three consecutive markers, GGAA05, TAGA02, and CTATT01.

Similarly, for telomeric DNA markers such as 17 msm, D16S3107, CTTT01, and GT01, greater differences were seen in three families (T12, T15, T44). Especially, families T12 and T44 harbored different alleles for markers 17 msm, CTTT01, and GT01, which were highly specific to the common haplotype.

The presence of large differences in repeat number and successively different alleles would indicate that the families were sharing the common chromosomal region,

inherited from a founder, between markers GGAA05 and 17 msm.

Haplotype analysis of families without identifiable genetic mutations

Twenty-three patients from 22 families presenting pure cerebellar ataxia did not carry identifiable genetic mutations. Nine families showed autosomal dominant inheritance, and the other families had no apparent family history. Their haplotypes are shown in Table 2. Although no family carried entirely identical alleles to the common haplotype consecutively for the markers telomeric to the *puratrophin*-1 gene, one family (U09) harbored the identical alleles for the markers between D16S3043 and GATA01 centromeric to the *puratrophin*-1 gene. It suggested the possibility that the U09 family have the common haplotype of 16q-ADCA in the region centromeric to the −16C>T substitution in the *puratrophin*-1 gene.

Haplotype analysis with SNPs

Four markers, GGAA05, D16S397, GGAA10, and GATA01 centromeric to the *puratrophin*-1 gene, showing different alleles in Table 1 suggested that ancestral chromosomal recombination might have occurred around the markers. Family U09 and the family reported by Ohata et al. 2006) also suggested ancestral chromosomal recombination around the substitution in the *puratrophin*-1 gene. Therefore, we searched the SNPs around these four markers and the *puratrophin*-1 gene. Five SNPs were identified around the marker GGAA05, one SNP around D16S397, four SNPs around GGAA10, one SNP around GATA01, and two SNPs around the *puratrophin*-1 gene (Table 3). SNP05 and SNP06 showed high specificity to the disease chromosome because they were absent in 200 control chromosomes.

Eighteen families showed different alleles for GGAA05, D16S397, GGAA10, or GATA01 (Table 1). Among them, sufficient amounts of DNA samples were not available in four families (T25, T26, T30, T42). The remaining 14 families were analyzed as shown in Table 4. While 13 out of the 14 families carried all of the same SNPs, family T46 did not carry SNP01, SNP02, SNP03, and SNP04. This confirmed that the genomic region between SNP01 and SNP04 of family T46 was a recombinant region, which was not conserved in all families.

These SNPs were also analyzed for the U09 family suspected of having the common haplotype of 16q-ADCA (Table 4). The family had all 13 SNPs, including SNP05 and SNP06, which are highly specific to the disease chromosome. This strongly suggested that family U09 shared the 16q-ADCA common haplotype centromeric to the −16C>T substitution in the *puratrophin*-1 gene.

Discussion

16q-ADCA is one of the most common ataxic diseases in Japan. We previously showed that 52 families shared the common haplotype in the genomic 400-kb region between the markers GATA01 and 17 msm by analysis with

Table 2 The haplotype analysis of families without identifiable genetic mutation. The black squares indicate that the families carry the identical alleles to the common alleles of 16q-ADCA, and the gray squares indicate alleles with one repeat-unit difference. Only family U09 harbored the identical alleles consecutively for the markers from D16S3043 to GATA01, suggesting that this family may harbor the common haplotype of 16q-ADCA. n.c.=notclear. A.D.=autosomal dominant inheritance was suspected

	Family No.	U01	U02	U03	U04	U05	U06	U07	U08	U09	U10	U11	U12	U13	U14	U15	U16	U17	U18	U19	U20	U21	U22
	Family history	n.c.	n.c.	n.c.	A.D.	n.c.	n.c.	n.c.	A.D.	n.c.	n.c.	A.D.	A.D.	A.D.	n.c.	A.D.	A.D.	A.D.	A.D.	n.c.	n.c.	n.c.	n.c.
Marker	most common haplotype	frequency in control (%)																					
D16S3043	1	25.0	1/6	4/8	1/2	1/7	1	4/6	5	5	1/7	1/7	4/5	4/5	1/6	1/5	5	1/6	5/7	1/6	1	1	1/5	1/8
D16S3019	4	41.4		1/4	4	1	3/4	5	3/4	4	4	3/4	1	2	4	4/5	4/5	3/4	1	2	1	1	1/3	1/2
CTATT01	1	32.4	3/4	1/4	1/3	3	2/4	1/2	3	3	1/4	1/3	1/3	1/3	4	1	1/3	2/5	1	2/3	1/3	1/2	1	1/3
TAGA02	4	10.3	3/5	6	2/4	4/5	5/6	4/6	5/6	5/6	4/5	5	5	6/7	5	2/6	3/5	4/5	6	4/5	4/6	3/6	2/6	5/6
GGAA05	1	1.4	3	4/5	4/5	4	5	3/4	4/6	4	1/3	3	2/4	4	4	5	4/5	2/6	4	4/5	4/6	5/6	4/6	4/5
D16S397	1	47.1	-1/1	1/3	2/3	4/6	4	1	3	4	1	3	1/4	1	1	1/2	1/4	3/5	1/4	2/6	1	-3/3	1/3	-3/6
D16S3086	2	65.7	2	2/3	3/4	3/4	3	2	3	3	2	3	2/4	2	2	2	2/3	2/3				3	2/3	3/4
GATA01	2	44.1	2	1/2	1/3	2/4	3	3	1/3	3	1/2	2/3	2	2/3	2	2/3	2/3	2	1	0/2	3/4	2	1	0/2
puratrophin-1 (C/T)	T	0.0	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
GA001	4	0.1	1/8	6/8	8	8/9	8	7/11	8/9	1	5/7	7/10	6/7	5/8	5	5/8	5/9	1/7	6/7	5/7	6/9	6/8	7/9	7/8
17msm	2	8.3	3/4	4	4/5	4/5	5	2/6	5	2	5	3/4	4	3/4	4	1/3	2/4	7	5	2/6	4	2/4	4/5	2/5
CTTT01	8	28.2	5/7	8/10	6/10	6/9	4/5	7/10	5/6	9	5/10	6/7	9	7/9	5/6	7/8	7/10	5/11	5/7	7/10	8/10	4/7	6/8	7/9
GT01	6	15.8	2/4	4/6	2/6	2/4	2/5	1/2	2/3	7	2/4	2/6	1/4	4/6	3/4	4	2/6	3/4	4/7	2/6	3/6	4/6	5/8	2
D16S3095	1	9.7	2	1/3	2/3	2/5	1/3	2/3	2/4	6	2	2/3	2/4	1/2	3	2/3	1/2	1/3	3/4	3/4	1/2	2/6	2/4	2/4
D16S512	1	32.3	2/4	1/5	4/5	2/4	2/4	4	2/4	1	2	2/4	1/4	4/5	4	2/4	4	2/3	1/4	2	4/5	4/5	4/5	4

Table 3 Single nucleotide polymorphisms (SNPs) on the disease chromosome of 16q22.1-linked autosomal dominant cerebellar ataxia (16q-ADCA). We identified thirteen SNPs by ourselves. SNP05 and SNP06 were absent in control chromosomes (n=200) and are thought to be highly specific to the disease chromosome

SNP/marker		Position on Chr 16	SNP change on 16q-ADCA	Frequency in control (%)
	GGAA05	64,938,933		
SNP01		64,972,150	A → G	27.8
SNP02		64,977,170	A → C	22.2
SNP03		64,977,733	T → C	30.0
SNP04		64,982,678	C → T	27.8
SNP05		65,049,292	G → A	0.0
	D16S397	65,295,770		
SNP06		65,337,827	A → G	0.0
SNP07		65,449,825	C → T	56.3
SNP08		65,451,833	T → A	45.5
	GGAA10	65,452,426		
SNP09		65,457,741	T → A	42.4
SNP10		65,458,302	T → C	45.5
SNP11		65,669,454	T → C	30.3
	GATA01	65,700,022		
SNP12		65,771,917	G → A	18.2
SNP13		65,793,152	C → T	8.7
puratrophin-1 (C/T)		65,871,434	C → T	0.0

microsatellite markers. Within this region, we had found that the single nucleotide –16C>T substitution in the *puratrophin*-1 gene was strongly associated with the disease (Ishikawa et al. 2005). Since then, a number of patients with the substitution and the common haplotype were reported in various areas of Japan. However, a report of the one exceptional patient without the substitution in the family in which all other affected subjects carried the substitution (Ohata et al. 2006) raised the possibility that a true pathogenic mutation may be present in a different gene. This exceptional patient indicated that the mutation might be lying centromeric to the substitution in the *puratrophin*-1 gene, where the patient shared the common haplotype with other affected individuals in the family.

Here, we re-examined the 16q-ADCA families with the –16C>T substitution in the *puratrophin*-1 gene with microsatellite markers and found four possible centromeric borders of the disease locus (GATA01, D16S397, GGAA10, GGAA05), based on the difference of alleles. We searched for informative SNPs around the markers capable of distinguishing the chromosomes derived from a founder and analyzed haplotypes with the SNPs. Because all of the examined families carried SNPs around the markers GATA01, D16S397, and GGAA10, ancestral chromosomal recombination around the markers was not confirmed. The differences in alleles for these markers was only one repeat-unit, suggesting that the allele differences

Table 4 The haplotype analysis with single nucleotide polymorphisms (SNPs). Fourteen familiesof 16q-ADCA with different alleles for microsatellite markers and family U09 are shown.The gray squares indicate that the family carried the SNPs common to 16q-ADCA. Family T46 did not carry the common SNPs from SNP01 to SNP04. This is consistent with the findingon microsatellite markers (Table 1), further suggesting that the centromeric border of thedisease locus is SNP04. Family U09 carried all of the 13 SNPs. This would also supportthe theory that family U09 shares the 16q-ADCA common haplotype centromeric to the substitution inthe *puratrophin*-1 gene

SNP	SNP change on 16q-ADCA	frequency in control (%)	family No.													
			P4	T3	T4	T5	T6	T7	T12	T15	T21	T28	T37	T43	T44	T46	U09
SNP01	A → G	27.8	G/A	G/A	G/A	G	G	G/A	G	G/A	G/A	G/A	G	G/A	G	A	G/A
SNP02	A → C	22.2	C/A	C/A	C/A	C	C	C/A	C	C/A	C/A	C/A	C	C/A	C/A	A	C/A
SNP03	T → C	30.0	C/T	C/T	C/T	C	C	C/T	C	C/T	C/T	C/T	C	C/T	C	T	C/T
SNP04	C → T	27.8	T/C	T	T/C	T	T	T/C	T	T/C	T/C	T	T	T/C	T	C	C/T
SNP05	G → A	0.0	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G
SNP06	A → G	0.0	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A	G/A
SNP07	C → T	56.3	T	T/C	T/C	T	T	T/C	T/C	T	T	T/C	T/C	T/C	T	T/C	T/C
SNP08	T → A	45.5	A	A/T	A/T	A	A	A/T	A/T	A	A	A/T	A/T	A/T	A	A/T	A
SNP09	T → A	42.4	A	A/T	A/T	A	A	A/T	A/T	A	A	A/T	A/T	A/T	A	A/T	A
SNP10	T → C	45.5	C	C/T	C/T	C	C	C/T	C/T	C	C	C/T	C/T	C/T	C/T	C/T	C
SNP11	T → C	30.3	C	C/T	C/T	C/T	C	C/T	C	C	C/T	C/T	C/T	C/T	C	C/T	C/T
SNP12	G → A	18.2	A	A/G	A/G	A/G	A/G	A/G	A/G	A	A/G	A/G	A/G	A/G	A	A/G	A/G
SNP13	C → T	8.7	T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	C/T	T	C/T	C/T
puratrophin-1(C/T)	C → T	0.0	T	T	T	T	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	T/C	C

in GATA01, D16S397, and GGAA10 might have resulted not from recombination events, but from the microsatellite slippage mutation (Ikeda et al. 2004). On the other hand, four families (P4, T3, T25, T46) showed great allele differences in GGAA05 and one family (T46) did not carry four SNPs, confirming that family T46 did not share the genomic region centromeric to GGAA05 with the other 16q-ADCA families. This strongly indicates that the centromeric border of the disease locus of 16q-ADCA could be placed at SNP04.

The U09 family had the identical alleles for all markers and SNPs in the region centromeric to the –16C>T substitution in the *puratrophin*-1 gene. It is impossible to conclude that the family has the common haplotype of 16q-ADCA because only one examined family member was available for the present genetic analysis. However, carrying the rare alleles for GGAA05 and infrequent SNPs, both highly specific to the disease chromosome, strongly suggests that the U09 family shares a part of the 16q-ADCA common haplotype. The patient in the U09 family developed pure cerebellar ataxia later in life without apparent family history. Because 16q-ADCA patients were found among sporadic cases (Ouyang et al. 2006), these clinical features of the U09 family are consistent with those of 16q-ADCA. Importantly, this family had not been reported previously and, therefore, would be the second case of 16q-ADCA without the substitution in the *puratrophin*-1 gene following the family reported by Ohata et al. (2006). These cases indicate that the telomeric end of the disease locus could be placed at the –16C>T substitution in the *puratrophin*-1 gene.

Haplotype analysis of a number of 16q-ADCA families with microsatellite markers and SNPs in this study suggests

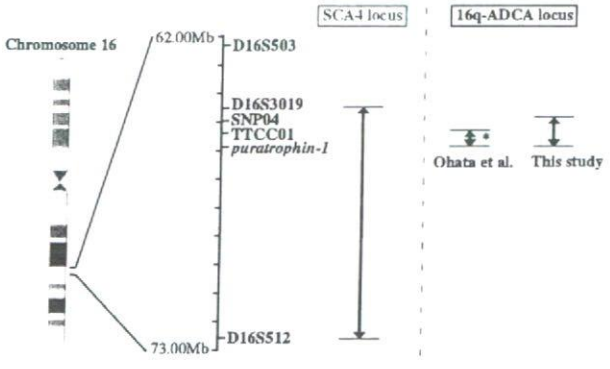

Fig. 1 A summary of critical intervals for 16q-ADCA and SCA4. Our study could define the disease locus of 16q-ADCA to a 900-kb genomic region between SNP04 and the –16C>T substitution in the *puratrophin*-1 gene. This region is completely inside the candidate locus of SCA4 (Flanigan et al. 1996).The haplotype region (*asterisk*) between TTCC01 and the *puratrophin*-1 gene shown by Ohata et al. (2006) is also shown, together with an alternative critical region between D16S503 and the *puratrophin*-1 gene (see text for details)

that the gene locus of 16q-ADCA could be re-assigned to a 900-kb genomic region between SNP04 and the substitution in the *puratrophin*-1 gene (Fig. 1). This region partly overlaps with, but is not the same as, the candidate region previously set by Ohata et al. (2006). They showed that three large 16q-ADCA families shared a common haplotype between D16S3086 and D16S412, and suggested the possibility that real pathogenic mutation would exist in the region between TTCC01 and the –16C>T substitution in the *puratrophin*-1 gene. However, the allele difference for TTCC01 in their families was only one repeat-unit, and all of their patients shared identical allele for TAGA02, lying centromeric to TTCC01. Since the possibility of slippage mutation remains as an explanation for the allele difference seen in TTCC01, as we observed for GATA01, D16S397, and GGAA10, it would be cautious to place the centromeric border at the marker TTCC01. Given that the allele differences in TTCC01 is due to slippage mutation, the centromeric border in their families would be alternatively set at D16S503, since an obligate recombination was seen between D16S503 and TAGA02. It would be, thus, important to analyze GGAA05 and specific SNPs in their families to see to what extent their patients harbor conserved haplotypes.

Although we found a patient without the –16C>T substitution in the *puratrophin*-1 gene, the substitution was present in all patients except the one in the U09 family (i.e., 125/126=99.2% sensitivity; 100% specificity) and, thus, the *puratrophin*-1 genetic change still remains to be a useful marker. Molecular diagnosis with multiple microsatellite markers and SNPs will help to identify 16q-ADCA patients more accurately. Through the present study, we showed that the truly pathogenic mutation would lie in a 900-kb genomic region between SNP04 and the –16C>T substitution in the *puratrophin*-1 gene. Further investigations for finding a genetic mutation within the critical region are needed to elucidate the molecular pathogenesis of 16q-ADCA.

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J Hum Genet (2008) 53:287–295
DOI 10.1007/s10038-007-0242-2

ORIGINAL ARTICLE

Direct and accurate measurement of CAG repeat configuration in the *ataxin-1* (*ATXN-1*) gene by "dual-fluorescence labeled PCR-restriction fragment length analysis"

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Received: 30 September 2007 / Accepted: 15 December 2007 / Published online: 27 February 2008

Abstract Spinocerebellar ataxia type 1 (SCA1; OMIM: #164400) is an autosomal dominant cerebellar ataxia caused by an expansion of CAG repeat, which encodes polyglutamine, in the ataxin-1 (*ATXN1*) gene. Length of polyglutamine in the ATXN1 protein is the critical determinant of pathogenesis of this disease. Molecular diagnosis of SCA1 is usually undertaken by assessing the length of CAG repeat configuration using primers spanning this configuration. However, this conventional method may potentially lead to misdiagnosis in assessing polyglutamine-encoding CAG repeat length, since CAT interruptions may be present within the CAG repeat configuration, not only in normal controls but also in neurologically symptomatic subjects. We developed a new method for assessing actual CAG repeat numbers not interrupted by CAT sequences. Polymerase chain reaction using a primer pair labeled with two different fluorescences followed by restriction enzyme digestion with SfaNI which recognizes the sequence "GCATC$(N)_5$", lengths of actual CAG repeats that encode polyglutamine were directly detected. We named this method "*dual fluorescence labeled PCR-restriction fragment length analysis*". We found that numbers of actual CAG repeat encoding polyglutamine do not overlap between our cohorts of normal chromosomes ($n = 385$) and SCA1 chromosomes ($n = 5$). We conclude that the present method is a useful way for molecular diagnosis of SCA1.

Keywords SCA1 · PCR · RFLP (restriction fragment length polymorphism) · Molecular diagnosis · Polyglutamine disease · Ataxia · Mutation detection

Analysis of CAG repeats in the *ataxin-1* gene.

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Introduction

Spinocerebellar ataxia type 1 (SCA1) (OMIM: #164400) is caused by an expansion of trinucleotide (CAG) repeat that encodes polyglutamine tract in the *ataxin-1* (*ATXN1*) gene (OMIM: *601556) lying in the short arm of human chromosome 6 (6p23) (Chung et al. 1993; Orr et al. 1993; Banfi et al. 1994). In normal chromosomes, this CAG repeat shows repeat-length polymorphism ranging in size between 19 and 39 repeats. In contrast, the length of expanded CAG repeat in the SCA1 disease chromosomes ranges from 39 up to 81 repeats. The length of expansion is inversely correlated with age-of-onset of disease, suggesting a direct role of CAG repeat/polyglutamine length in the pathogenesis of SCA1 (Chung et al. 1993; Orr et al. 1993; Banfi et al. 1994).

The *ataxin-1* (*ATXN-1*) gene encodes a protein called ataxin-1 (ATXN-1), which is a 816-amino acid protein with a molecular mass of 87 kDa. The polyglutamine tract lies at its amino (N)-terminal region. In addition to the

polyglutamine tract, there are some other important domains in ATXN-1, such as nuclear localization signal and the AXH domains. Although the functions of ATXN-1 are not fully understood, recent investigation suggests that the AXH domain in the ATXN-1 interacts with Gfi-1/Senseless protein depending on the length of the polyglutamine tract, resulting in reduction of the Gfi-1 level, which in turn may contribute to neurodegeneration (Tsuda et al. 2005).

The molecular diagnosis of SCA1 is usually undertaken by PCR amplification using primer pairs spanning the CAG repeat (Matilla et al. 1993; Orr et al. 1993; Goldfarb et al.1996). However, this conventional method may contain caveats mainly stemming from the presence of trinucleotide CAT interruption sequence(s) within the CAG repeat tract. Most (98%) of the normal alleles contain one to three CAT interruptions, resulting in a much shorter polyglutamine tract when it is translated. On the other hand, expanded CAG repeats are characterized by continuous, "pure" CAG stretches, resulting in pure polyglutamine expansions (Chung et al. 1993). This fact would imply that the diagnosis of SCA1 is important to assess, not merely by the length of "CAG repeat configuration" (i.e., total length of CAG and CAT repeats), but by determining the length of pure CAG repeat encoding polyglutamine. Indeed, some exceptional cases have been previously reported. An allele with 44 repeats, which indicates "expansion" in conventional method, has been reported in an asymptomatic subject (Quan et al. 1995). This subject harbored CAG repeat configuration of $(CAG)_{12}$CATCAGCAT $(CAG)_{12}$CATCAGCAT $(CAG)_{14}$, showing that this allele encodes for polyglutamine tract with normal length due to four CAT interruptions. Another example has been reported in an expanded allele with 58 repeats interrupted with two CAT sequences as, 5′-$(CAG)_{45}$CATCAGCAT$(CAG)_{10}$-3′ (Matsuyama et al. 1999). Particularly, special caution would be needed when assessing the CAG repeat length near the border of normal and expanded repeats, since the upper limit of normal repeat and the shortest expansion overlap at 38 repeats. While "pure" 38 CAG stretch is pathogenic, the 38 CAG repeat with CAT interruption is not pathogenic due to the much shorter polyglutamine stretch (Ranum et al. 1994). From these observations, it would be better if one could directly assess the actual CAG repeat length not interrupted by CAT sequences, since the length of polyglutamine tract in the ATXN-1 is the only basic defect that leads to pathogenesis.

In this study, we developed a new method that would allow one to detect the actual number of CAG repeat length not interrupted by CAT sequence(s). We show here that, by using our new method, one can directly assess 5′- and 3′-CAG repeat numbers disrupted by CAT sequence(s) which may be contained in the CAG repeat configuration. We propose that this new method, "dual fluorescence labeled PCR-restriction fragment length analysis", is a direct way to accurately diagnose SCA1. We not only introduce this new method, but we also show that actual "pure" CAG repeat numbers do not overlap between normal Japanese and SCA1 subjects by using this method. We also describe a unique family with short, but pathogenic, CAG repeats detected with our new method.

Materials and methods

Overall design of the dual fluorescence labeled PCR-restriction fragment length analysis

As previously described by others (Chung et al. 1993), every CAT interruption within "the CAG repeat configuration" (i.e., total length of CAG and CAT repeats) is theoretically recognized and digested with the restriction enzyme, "SfaNI", which recognizes 5′-GCATC$(N)_5$-3′. There are no other consensus SfaNI sites outside the CAG repeat configuration when the genome is amplified using appropriate primers. Using this advantage, we designed a comparison of fragment lengths of the PCR product both with and without the SfaNI digestion (Fig. 1).

When there are no CAT interruptions within the CAG repeat configuration, the fragment length after SfaNI digestion will theoretically be the same as that without digestion (Fig. 1A).

When there is one CAT interruption, the length of the PCR product without the digestion would be calculated as the sum of lengths of two different fragments yielded after the digestion: the sum of the lengths of "the 5′-digested fragment", "the 3′-digested fragment", and 3 (Fig. 1B). The 5′- and 3′- SfaNI digested fragments could be differentiated if the sense and anti-sense primers are labeled with different fluorescent dyes.

When there are two CAT interruptions, the length of the PCR product without the digestion would be calculated as the sum of lengths of three different fragments yielded after the digestion: "5′-digested fragment", "GCATC$(N)_5$", and "the 3′-digested fragment" (Fig. 1C). Conversely, the size difference between fragment length without the digestion and the sum of "5′-digested fragment" and "the 3′-digested fragment", would be the length of "GCATC$(N)_5$".

CAG repeat configurations containing more than two CAT interruptions were not seen in our cohort of samples (data shown in Results). We therefore artificially generated such clones using site-directed mutagenesis (Invitrogen, Calif., USA). When there are more than two CAT interruptions, the length of PCR fragments without the enzyme digestion "minus" the sum of lengths of the 5′- and 3′-

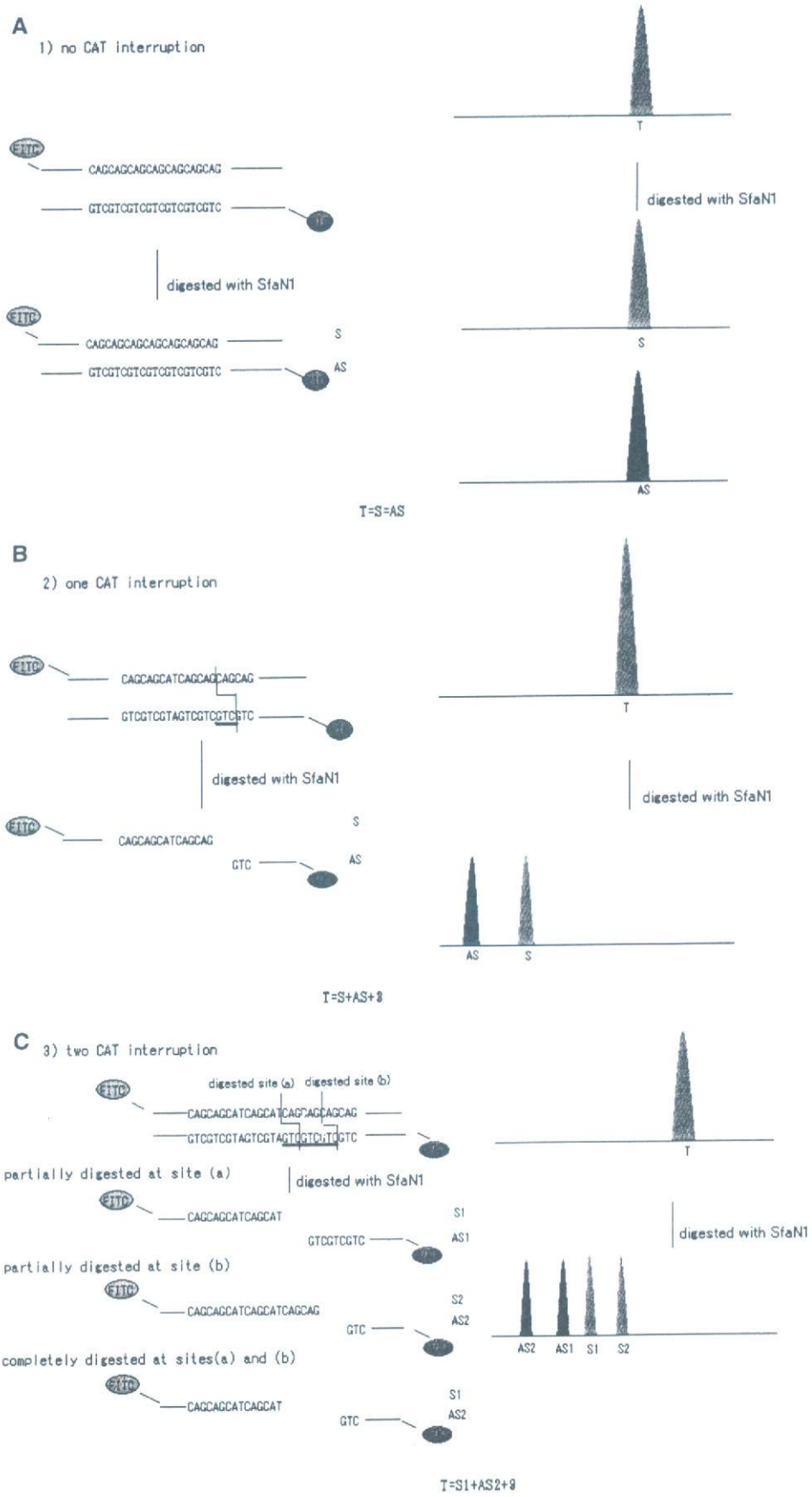

Fig. 1 A basic concept of the dual-fluorescence labeled PCR-restriction fragment length analysis. *T* Length of PCR product containing whole CAG repeat configuration. *S* Fragment length of 5′ sense-strand PCR product, labeled by FITC (fluorescence isothiocyanate) yielded after restriction enzyme SfaNI, digestion. *AS* Fragment length of 3′ anti-sense-strand PCR product labeled by VIC yielded after restriction enzyme SfaNI, digestion. **A** In case of no CAT interruption in the CAG repeat configuration, PCR fragment without SfaNI (*T*), 5′ sense-strand PCR product labeled by FITC yielded after SfaNI digestion (*S*), and the 3′ anti-sense-strand PCR product labeled by VIC yielded after SfaNI digestion (*AS*) all coincide in length on ABI PRISM 3100-Avant System. **B** In case one, CAT interruption is present within the CAG repeat configuration. The length of PCR fragment without SfaNI (*T*) is calculated as the sum of (5′ sense-strand PCR product labeled by FITC yielded after SfaNI digestion: *S*), (3′ anti-sense-strand PCR product labeled by VIC yielded after SfaNI digestion: *AS*), and 3 base-pair (bp) due to trinucleotide, GTC, on anti-sense strand (*underlined*). $T = S + AS + 3$ (bp). **C** In case two, CAT interruptions are present within the CAG repeat configuration. Two different SfaNI-digestion sites (*a* and *b*) are predicted (*right panel*). Theoretically, four types of fragments would be yielded due to combination of complete and partial digestions (two FITC-labeled fragments *S1*; ad *S2*, and two VIC-labeled fragments, *AS1* and *AS2*). The length of PCR fragment without SfaNI (*T*) could be calculated as the sum of $S1 + AS2 + 9$ bp (corresponding nine-nucleotide "GTCGTCGTC", *underlined* in *right panel*)

SfaNI digested fragments would be the differences harboring CAT interruptions.

Thus, it seemed theoretically possible to detect both 5′- and 3′-CAG repeat sequences disrupted by CAT interruption. We tested our hypothesis first by examining 10 plasmid clones containing *ATXN1* CAG repeat configurations obtained from five individuals (Table 1).

DNA samples and materials

One hundred and ninety-one control individuals and two SCA1 subjects were first analyzed. The control group comprised of 60 neurologically normal subjects, 50 subjects with cerebrovascular diseases, in whom no family history of ataxia was present, and 81 individuals with dominantly inherited ataxias previously excluded for SCA1 by conventional diagnostic method. Two subjects with typical clinical features of SCA1 had been molecularly confirmed as having this disease by the conventional method. Peripheral blood samples were obtained after informed consent, and DNA was extracted as reported elsewhere (Ishikawa et al. 1997). The study was approved by the Institutional Review Board of Tokyo Medical and Dental University.

Detection of CAG repeat/CAT interruption with automated fluorescence sequencer

For amplifying the CAG repeat configuration, a primer set "CAG-a and CAG-b" (Chung et al.1993) was used, although the sense primer was labeled with FITC (fluorescent isothiocyanate) at its 5′-end (CAG-b: 5′-[FITC] CCAGACGCCGGGACACAAGGCTGAG-3′), and the anti-sense primer was 5′-end labeled with VIC (CAG-a: 5′-[VIC]-CCGGAGCCCTGCTGAGGTG-3′). PCR was performed in an ordinary condition in a final volume of 25 μl, containing 50 ng of genomic DNA, 4.0 pmol of each primer, 1 μl of 10% dimethylsulfoxide (DMSO), 2 mM each deoxynucleosides (dNTPs), and 1.0 unit of Gold Tag DNA polymerase (Takara, Japan). Thermal setting was initial denature at 95°C for 4 min, 3 cycles of 95°C for 1 min, 70°C for 30 s, 72°C for 30 s; subsequent 3 cycles of 95°C for 1 min, 68°C for 30 s, 72°C for 30 s; 3 cycles of 95°C for 1 min, 66°C for 30 s, 72°C for 30 s; 3 cycles of 95°C for 1 min, 64°C for 30 s, 72°C for 30 s; final 20 cycles of 95°C for 1 min, 62°C for 30 s, 72°C for 30 s, and final extension at 72°C for 10 min. For each reaction, 10 μl of PCR product was digested with SfaNI (New England BioLabs, USA) in a final volume of 15 μl containing optimal reaction condition (10 mM sodium chloride, 5 mM Tris–hydrochloride, 1 mM Mg Cl_2, 0.1 mM dithiothreitol (DTT; pH 7.9) incubated at 37°C, as recommended by the supplier (Chung et al. 1993). PCR products both with and without the SfaNI digestion were diluted with the same solution (Hi-Di Formamide and Gene Scan-500 LIZ Size Standard), and then heated at 95°C for 2 min for denature, immediately cooled within ice, and loaded on ABI PRISM 3100-Avant System (Applied Biosystems). The electrophoresis was undertaken at 50°C, with 15 volts.

Table 1 The sequence configurations of 10 alleles derived from five control individuals and subcloned into pCR-TOPO plasmid vector

Case 1	Allele 1	(CAG)22
	Allele 2	(CAG)13CATCAGCAT (CAG)10
Case 2	Allele 1	(CAG)13CATCAGCAT (CAG)10
	Allele 2	(CAG)11CAT (CAG)16
Case 3	Allele 1	(CAG)11CAT (CAG)16
	Allele 2	(CAG)11CAT (CAG)16
Case 4	Allele 1	(CAG)13CATCAGCAT (CAG)10
	Allele 2	(CAG)13CATCAGCAT (CAG)10
Case 5	Allele 1	(CAG)11CAT (CAG)16
	Allele 2	(CAG)16CATCAGCAT (CAG)10

Evaluation of dual fluorescence labeled PCR-restriction fragment length analysis

We first randomly chose five individuals with different CAG repeat configurations (Table 1). On each subject, genomic DNA was amplified with CAG-a and -b primers, and PCR product was subcloned into PCR-TOPO (Invitrogen, USA). Then, 10 clones from each individual were sequenced with universal and reverse primers as previously described (Li et al. 2003). These five individuals harbored any of the CAG repeat configurations without CAT interruption, or 1–2 CAT interruption(s). Then, the dual fluorescent-labeled PCR-restriction fragment length analysis was performed on these clones to check the consistency.

Results

Evaluation of dual fluorescence labeled PCR-restriction fragment length analysis

To evaluate consistency of our new method, we first examined on 10 plasmid clones which contained CAG repeat configuration in the *ATXN1* gene (Table 1). On the plasmid clones without CAT interruption within the CAG repeat configuration (e.g., Case 1, Allele 1), there were no differences in fragment lengths on ABI 3100-Avant System between data with and without the SfaNI restriction enzyme digestion (Fig. 1A), as has been hypothesized.

When plasmids with one CAT interruption were examined (e.g., Table 1, Case 2, Allele 2), the fragment length of the PCR product amplified by the two primers should be the sum of the lengths of "5′-digested fragment labeled with FITC", that of "3′-digested fragment labeled with VIC", and 3. The SfaNI digestion yielded was two fragments exactly corresponding the 5′-digested fragment labeled with FITC and the 3′-digested fragment labeled with VIC (Fig. 1B).

If the CAG repeat configuration contained two CAT interruptions, the PCR product should be separated into three fragments: "5′-digested fragment labeled with FITC", "3′-digested fragment labeled with VIC", and the "internal fragment limited by the two SfaNI-recognition sites". Since this internal fragment is not labeled, this fragment will not be detected. However, the length of the "internal fragment limited by the two SfaNI-recognition sites" could be calculated by the following formula: (the length of internal sequence between the two CAT interruptions) = (the length of PCR product without SfaNI digestion) – (the sum of lengths of "5′-digested fragment labeled with FITC" and "3′-digested fragment labeled with VIC"). This was indeed confirmed by the experiment on plasmids with two CAT interruptions (e.g., Table 1, Case 4, Allele 1) with the dual fluorescence labeled PCR-restriction fragment length analysis (Fig. 1C).

These examinations not only confirmed our hypothesis, but also suggested that the new method is able to directly assess nucleotide sequences of CAG repeat configuration. If the length-difference between the fragments yielded with and without the SfaNI restriction enzyme digestion was nine nucleotide long, the nucleotide excised by the SfaNI was always as "5′-CATCAGCAT-3′", confirmed by experiments on plasmid clones. Similarly, if the length-difference was 12 nucleotides, the internal sequence was always as "5′-CAT$(CAG)_2$CAT-3′". If the lengths was 15 nucleotides, the internal sequence was always as "5′-CAT$(CAG)_3$CAT-3′".

We did not find DNA samples with more than two CAT interruptions with the CAG repeat configuration in our cohort of 191 control subjects (data described later). To check the usefulness of our new method for alleles with more than two CAT interruptions, a CAG repeat configuration with three CAT interruptions was artificially generated from a plasmid clone, Case 5 Allele 2 (Table 1), by using site-directed mutagenesis. The exact sequence of this configuration containing three CAT interruption was "5′-$(CAG)_9$CAT$(CAG)_6$CATCAGCAT$(CAG)_{10}$-3′". When digested with SfaNI, three fragments corresponding 5′-completely digested fragment labeled with FITC, 3′-completely digested fragment labeled with VIC, and 3′-partially digested fragment labeled with VIC, were detected (Fig. 2). The combined length of the 5′-completely digested fragment labeled with FITC (S1) and the 3′-completely digested fragment labeled with VIC (AS2) was 18 base-pairs (bp) shorter than the length of total CAG repeat configuration, consistent with the fact that six CAG repeats were lying between two CAT interruption sequences.

Based on these observations, we confirmed that the dual fluorescence labeled PCR-restriction fragment length analysis appeared to be a useful and rapid way to directly assess CAG repeat configuration.

Results on 191 control individuals and two clinically typical SCA1 subjects

We next examined 191 control Japanese individuals and two SCA1 subjects by the dual fluorescence labeled PCR-restriction fragment length analysis. The two most frequent CAG repeat configurations were those with 26 and 28 combined CAG and CAT repeats (Fig. 3). The frequencies of these alleles in normal Japanese chromosomes were 27.1% for 28 CAG/CAT repeat-units, and 24.0% for 26 CAG/CAT repeat-units. The range of CAG repeat configuration was from 17 up to 40 repeat-units in control groups. Two SCA1 subjects harbored 46 and 54 repeat-units on their SCA1 chromosomes.

When 386 chromosomes from 193 individuals were examined by the dual fluorescence labeled PCR-restriction fragment length analysis, we found that 140 chromosomes had one CAT interruption with the CAG repeat configuration, counting 36.4% of our cohort of Japanese control chromosomes (Table 2). On the other hand, there were 243 chromosomes with two CAT interruptions, counting 62.9% of 386 chromosomes. Table 3 shows exact sequences of CAG repeat configurations that contain either 1 or 2 CAT interruption found in this study. There were seven chromosomes which did not contain CAT interruptions. Two of these were from SCA1 subjects with pure CAG expansions (46 and 54 CAG repeat-units) and typical clinical features. The remaining five chromosomes were observed from control subjects without ataxia. The frequency of normal CAG repeat length with pure stretch was calculated as 1.3%. The relation between the length of CAG repeat configuration (i.e., combined CAG and CAT repeats) and presence/absence of CAT interruptions is summarized in Table 2 (Note: this table includes three atypical SCA1 subjects, as described later. Therefore, the total number of chromosomes becomes 390, the number of normal chromosomes is 385, and the number of SCA1 chromosomes is five). Of note is that we did not find any alleles with three CAT interruptions.

When the SfNaI digestion was performed in the dual fluorescence labeled PCR-restriction fragment length

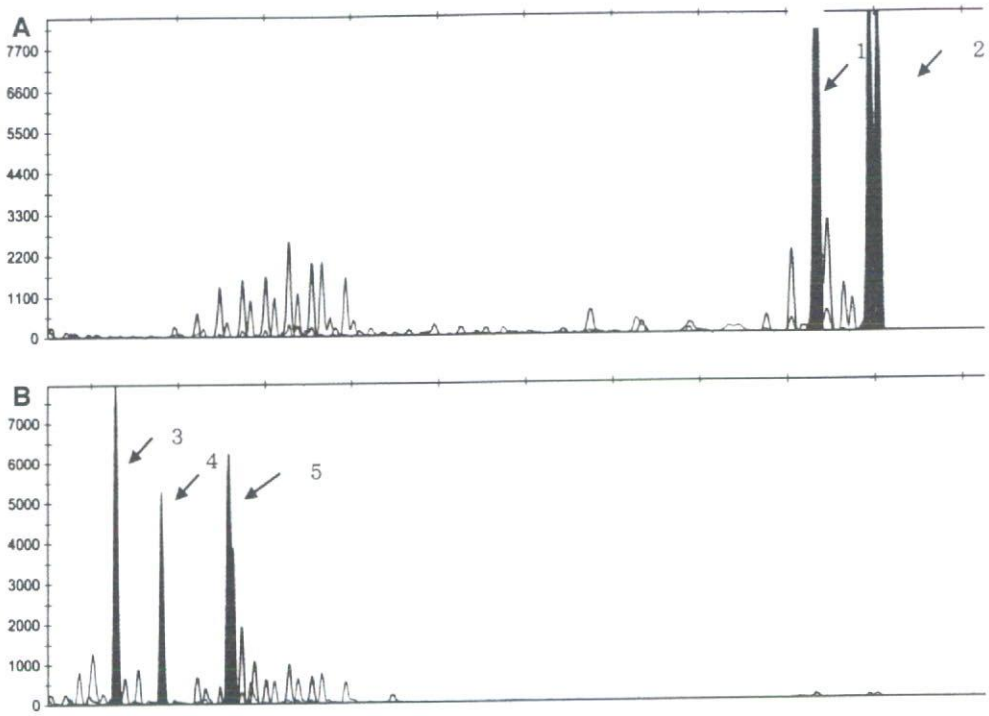

Fig. 2 The electrophoresis pattern of the dual-fluorescence labeled PCR-restriction fragment length analysis on a plasmid clone containing three CAT interruptions. **A** The fragment analysis on ABI 3100 without SfaNI digestion. FITC-labeled PCR product (*arrow #1*) and VIC-labeled PCR product (*arrow #2*) which are actually the same size in length, differ in size for 5.8 base-pairs (bp) on this analyzing system. This 5.8-bp gap between the FITC and VIC fragments was always constantly seen on ABI 3100. **B** The fragment analysis after SfaNI digestion. Three major peaks corresponding to a completely digested VIC-labeled 3′ anti-sense-strand PCR fragment (*arrow #3*; designated, AS2), a partially digested, VIC-labeled 3′ anti-sense-strand PCR fragment (*arrow #4*; designated, AS1), and a completely digested, FITC-labeled 5′ sense-strand PCR product (*arrow #5*; designated, S1), are demonstrated. When a total length of PCR fragment containing whole CAG repeat configuration is designated "T", T = AS1 + AS2 + S1 + 18 (bp) was confirmed both by fragment length analysis and actual sequence analysis. Although presence of a partially digested FITC-labeled 5′ anti-sense-strand PCR fragment (designated, S2) has been considered on hypothesis, it was never observed on ABI3100 sequencer. Therefore, we considered that FITC-labeled PCR product directly reflects a completely digested fragment (S1)

analysis, we were able to determine where CAT interruptions were present in the CAG repeat configuration (Fig. 3B). The CAG repeat length in the FITC-labeled 5′ fragment ranged in length from 10 up to 20 repeats in control chromosomes. This would indicate that the normal CAG repeat-unit lying in the 5′-region of the CAT interruption ranges between 10 and 20. Similarly, the CAG repeat length in the VIC-labeled 3′ fragment ranged in length from 7 to 23 repeats (Table 3).

Identification of a family with 40 CAG repeats with interruption

In our series of examining 193 individuals by the dual fluorescence labeled PCR-restriction fragment length analysis, we encountered an individual who harbored an allele with very small CAG repeat expansion. The size of this allele was 40 combined CAG and CAT repeats, which could be diagnosed as SCA1 by the classical criteria. By the dual fluorescent labeled PCR-restriction fragment length analysis, however, the repeat configuration was suggested to have two CAT interruptions with 27 CAG repeats in the 5′-end, and 10 CAG repeats in the 3′-end ("5′-CAG_{27}CATCAGCATCAG_{10}-3′"). When the PCR product was sub-cloned into PCR-TOPO and sequenced, the allele with 40 CAG repeat configuration was confirmed to harbor two CAT interruptions as expected from the dual fluorescent labeled PCR-restriction fragment length analysis. From the distribution pattern of actual CAG repeat number found in control Japanese (Fig. 3B), we considered that this particular individual had an abnormal SCA1 allele.

This patient was a 47-year-old male subject showing gait disturbance due to marked spastic paraparesis and mild truncal ataxia. He first noticed difficulties in walking at the age of 43. He had two elder siblings with similar neurological symptoms. The patient's mother, who died at the age of 75, also showed progressive gait disturbance beginning from her fourth decade. Although a DNA sample of this mother was not available for examination, DNA samples of the patient's siblings were tested and were both confirmed to have the same allele with "5′-

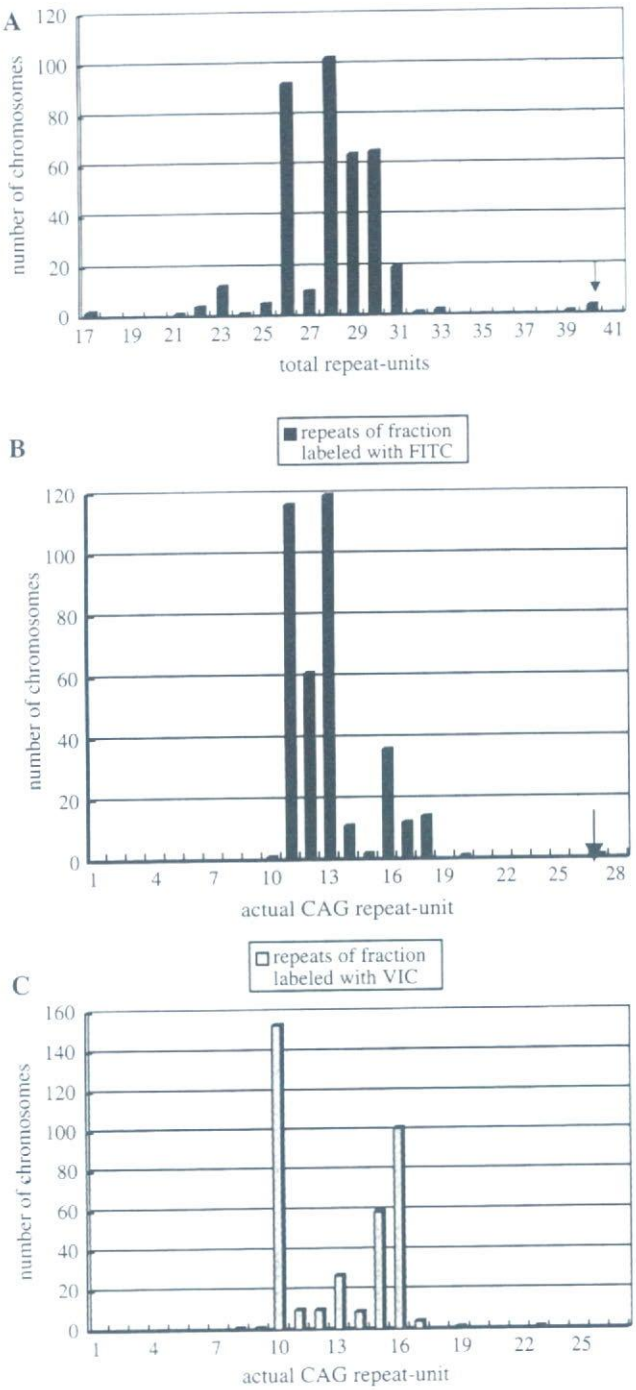

Fig. 3 The analysis of the SCA1 CAG repeats in control Japanese individuals. **A** The distribution of CAG repeat configuration (i.e., combined CAG repeat and CAT interruptions). In the present cohort of 193 Japanese subjects, the CAG repeat number which may contain CAT interruptions ranged from 17 to 40 repeat-unit. **B** The distribution of actual (pure) CAG repeat number encoding polyglutamine in the region 5′ (*upstream*) of the first CAT interruption. Pure CAG repeat in the 5′-region ranges from 10 to 20 repeat-units, and two major peaks are seen at 11 and 13 CAG repeats. Notice that a chromosome with 27 pure CAG repeat was an exceptionally large repeat. **C** The distribution of actual (pure) CAG repeat number encoding polyglutamine in the region 3′ (*downstream*) of the last CAT interruption. Pure CAG repeat in the 3′-region ranges from 7 to 23 repeat-units, and two major peaks are seen at 10 and 16 CAG repeats

Table 2 The distribution of CAT interruption among various CAG repeats in the present study

Length of allele	Pure CAG repeats	One CAT interruption	Two CAT interruption
17	2		
21		1	
22	2	1	1
23	1	10	1
24			1
25		2	3
26		13	81
27		8	2
28		88	15
29		7	58
30		7	58
31		1	18
32		1	
33			2
39		1	
40			3
46	1		
54	1		
Total	7	140	243

$CAG_{27}CATCAGCATCAG_{10}$-3′". Magnetic resonance imaging of the brain and the cervical, thoracic and lumbar spinal cord of these three subjects revealed mild cerebellar atrophy without obvious spinal cord or brainstem atrophy, which is compatible with SCA1 (Burk et al.1996). We also tested for mutations in *spastin* gene (Proukakis et al.2003), the most common gene identified for autosomal dominant spastic paraplegia (Svenson et al.2001). However, there was no mutation at least in the coding region of this gene (data shown upon request). From these observations, we conclude that spastic ataxia phenotype in these patients carrying 40 CAG repeat configuration ("5′-CAG_{27}CAT-$CAGCATCAG_{10}$-3′") may be caused by a mild CAG repeat expansion in the *ATXN1* gene.

Discussion

The main fruit of this study is the development of a new diagnostic method that would allow ones to detect actual CAG repeat numbers and the number of CAT interruptions in the CAG repeat configuration. The conventional method using a primer pair flanking CAG repeat configuration

Table 3 CAG repeat configurations with CAT interruptions in the present study

Length of allele	Number of alleles	The sequence configurations with one CAT interruption	Number of alleles	The sequence configurations with two CAT interruption
21	1	5′-$CAG_{11}CATCAG_9$-3′		
22	1		1	5′-$CAG_{11}CATCAGCATCAG_8$-3′
23	10	5′-$CAG_{11}CATCAG_{11}$-3′	1	5′-$CAG_{10}CATCAGCATCAG_{10}$-3′
24			1	5′-$CAG_{11}CATCAGCATCAG_{10}$-3′
25	2	5′-$CAG_{11}CATCAG_{16}$-3′	3	5′-$CAG_{12}CATCAGCATCAG_{10}$-3′
26	13	5′-$CAG_{13}CATCAG_{12}$-3′	81	5′-$CAG_{13}CATCAGCATCAG_{10}$-3′
27	8	5′-$CAG_{11}CATCAG_7$-3′	2	5′-$CAG_{14}CATCAGCATCAG_{10}$-3′
28	80	5′-$CAG_{11}CATCAG_{16}$-3′	15	5′-$CAG_{14}CATCAGCATCAG_{11}$-3′
	8	5′-$CAG_{12}CATCAG_{15}$-3′		
29	3	5′-$CAG_{13}CATCAG_{15}$-3′	19	5′-$CAG_{13}CATCAGCATCAG_{13}$-3′
	2	5′-$CAG_{12}CATCAG_{16}$-3′	38	5′-$CAG_{16}CATCAGCATCAG_{10}$-3′
	1	5′-$CAG_{14}CATCAG_{14}$-3′	1	5′-$CAG_{12}CATCAGCATCAG_{14}$-3′
	1	5′-$CAG_{18}CATCAG_{10}$-3′		
30	5	5′-$CAG_{13}CATCAG_{16}$-3′	42	5′-$CAG_{15}CATCAGCATCAG_{12}$-3′
	2	5′-$CAG_{12}CATCAG_{19}$-3′	6	5′-$CAG_{14}CATCAGCATCAG_{13}$-3′
			10	5′-$CAG_{17}CATCAGCATCAG_{10}$-3′
31	1	5′-$CAG_{18}CATCAG_{12}$-3′	14	5′-$CAG_{18}CATCAGCATCAG_{10}$-3′
			1	5′-$CAG_{15}CATCAGCATCAG_{13}$-3′
			2	5′-$CAG_{16}CATCAGCATCAG_{12}$-3
			1	5′-$CAG_{17}CATCAGCATCAG_{11}$-3′
32	1	5′-$CAG_{12}CATCAG_{19}$-3′		
33			1	5′-$CAG_{20}CATCAGCATCAG_{10}$-3′
			1	5′-$CAG_{16}CATCAGCATCAG_{14}$-3
39	1	5′-$CAG_{15}CATCAG_{23}$-3′		
40			3	5′-$CAG_{27}CATCAGCATCAG_{10}$-3′
Total	140		243	

allowed one to measure total CAG and CAT repeat-units (Goldfarb et al. 1996; Jodice et al. 1997; Matsuyama et al. 1999; Pujana et al. 1999; Zhulke et al. 2002).

Sobczak and Krzyzosiak (2004) developed a new method, "SSCP-duplex analysis", and showed exact CAG repeat configurations in 50 Polish individuals. Hellenbroich and his colleague developed a method that would detect actual CAG repeat by using non-labeled primers and SfaNI digestion (Zhulke et al. 2002). They studied in their German population and found rare alleles with mild CAG repeat expansion. However, their method would not discriminate 5′-end SfaNI-digested fragment with 3′-end SfaNI-digested fragment, and would need another step to disclose true CAG repeat configuration. In contrast, our method could allow one to discriminate both 5′- and 3′-fragments by labeling sense and anti-sense primers with different fluorescent dyes. Therefore, it would be much convenient and accurate to determine actual CAG repeat-unit encoding polyglutamine tract by employing our dual fluorescence labeled PCR-restriction fragment length analysis on fluorescence sequencers.

By using this new method, we have shown CAG repeat configurations in 385 control Japanese chromosomes and five SCA1 patients chromosomes. As has been reported in many studies, most of the normal chromosomes (98.7%) contain at least one CAT interruption in the CAG repeat configuration (Chung et al. 1993). However, we also found that 5 out of 385 (1.3%) control Japanese chromosomes do not contain the interruption. Since presence of CAT interruptions is considered to stabilize the CAG repeat length, presence of normal alleles without the CAT interruption may have some effect in the emergence of CAG repeat expansion through transmission. Comparing repeat numbers of CAG repeat configuration with and without CAT interruptions, alleles with interruption tended to have longer CAG repeat. In other words, it was not clear whether pure CAG repeat expansion occurs from the normal CAG repeat without CAT interruption.

In conclusion, the present method could be a convenient way to detect the accurate number of CAG repeat-unit encoding polyglutamine tract. The usefulness would be

particularly important for CAG repeats with borderline length.

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J Hum Genet (2006) 51:461–466
DOI 10.1007/s10038-006-0385-6

ORIGINAL ARTICLE

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

Received: 27 October 2005 / Accepted: 24 January 2006 / Published online: 14 April 2006

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

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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-*

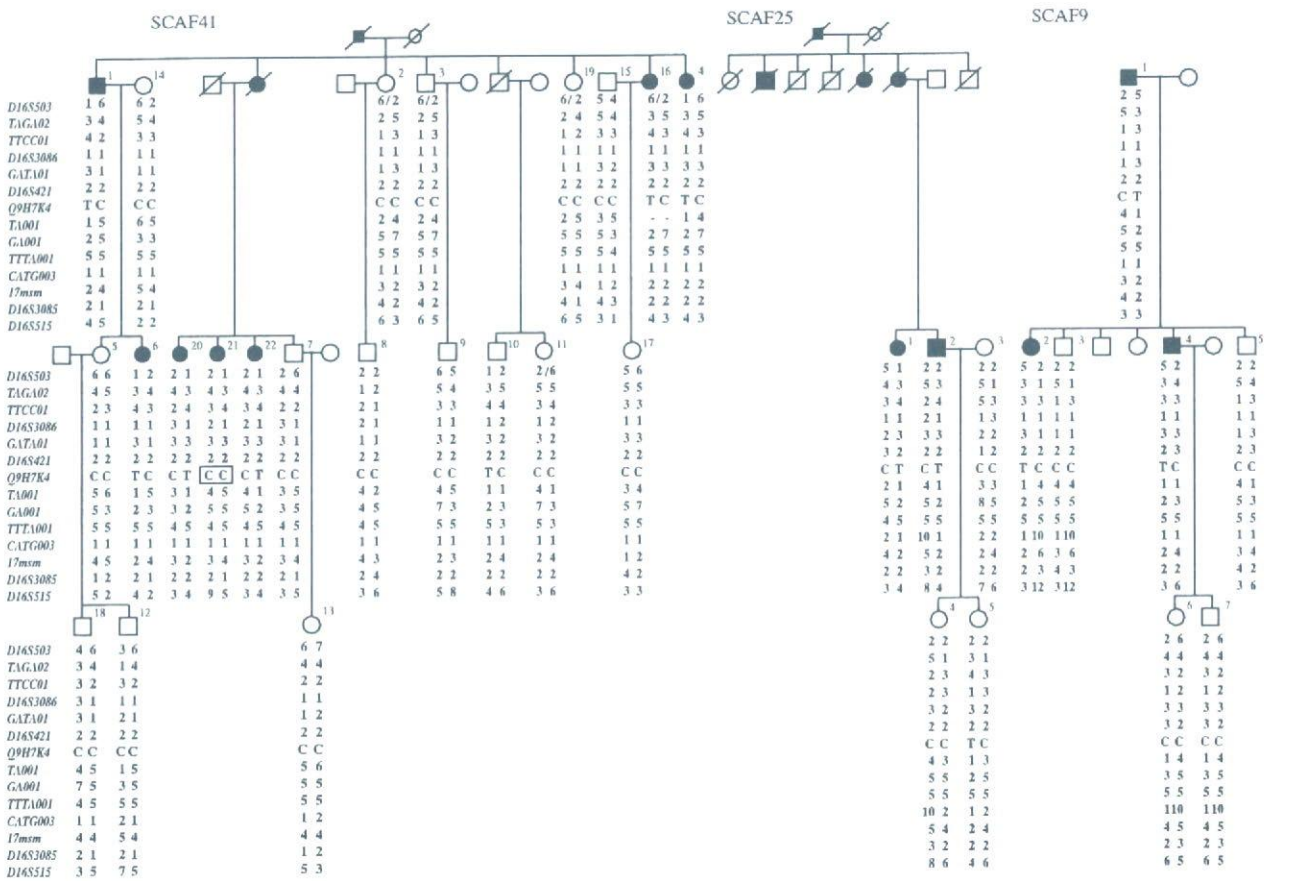

Fig. 1 Haplotype analysis in three large ADCA families, SCAF41, SCAF25, and SCAF9. Thirteen polymorphic markers mapped to 16q21–16q23 and the −16C > T substitution in *puratrophin-1 (Q9K7H4)* are shown. Twelve affected and two unaffected individuals had a common haplotype (1-3-2) at *D16S3086–GATA01–D16S421*. 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	1/2	1	1	1	1	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	2/4	2	2	2	2	2	2	2	2	2	2	2	2	2	2/3	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	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	5/4	5	5	5/4	5	5/4	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	5/6	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	1/6	1	1	1	1	1	1/10	1	1/2	1/6	1	1	1	1	1/2	1	1	1/2	1	1/4	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	2/3	2	2/1	2	2	2	2	2	2	2	2/3	2	2	2	2	2/4	2	2	2/1	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	39	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	4/11	6	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.

Acknowledgements We express gratitude to the patients, their families, and clinicians for participating in this study. This work was supported by a grant from the Research Committee of Ataxic Disease, Research on Specific Disease, the Ministry of Health, Labour, and Welfare of Japan.

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