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Predisposing chromosome for spinocerebellar ataxia type 6 (SCA6) in Japanese

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The autosomal dominant cerebellar ataxias (ADCAs) are a group of neurodegenerative disorders which can be classified into three major categories on the basis of their clinical features and mode of inheritance.[1] ADCA type III is a pure cerebellar syndrome that is genetically heterogeneous and includes spinocerebellar ataxia type 5 (SCA5),[2] SCA6,[3] SCA10,[4 5] and SCA11.[6] The gene responsible for SCA6 has been identified as coding for the α_{1A} subunit of the P/Q type voltage dependent calcium channel (*CACNA1A*). Moderate CAG expansion in the coding region causes the disorder, with the number of CAG repeats being originally reported as 21-27 in mutant alleles (n=8) and 4-16 in control alleles (n=950).[3] Subsequent studies have indicated that the range of pathological expansion in SCA6 alleles varies from 20[7] to 33.[8] The *CACNA1A* gene was first identified during the search for specific mutations causing familial hemiplegic migraine (FHM) and episodic ataxia type 2 (EA2).[9] The gene product has four transmembrane domains and glutamine repeats are located at the C-terminal side of the intracellular segment. Missense mutations of these transmembrane domains and deletions or splice mutations leading to a truncated protein are responsible for FHM and EA2, respectively. The *CACNA1A* gene is predominantly expressed in Purkinje cells and granule cells of the cerebellum and is essential for the survival and maintenance of normal function by these neurones.[10 11] Biochemical mechanisms leading to the development of SCA6 are not fully understood. However, the fact that slowly progressive ataxia is often observed in EA2 indicates that a small glutamine expansion in the SCA6 gene also disturbs the function of P/Q-type calcium channels, leading to selective neuronal degeneration in the cerebellum.

The pathogenic expansion in SCA6 is relatively small compared with those in other SCAs caused by triplet repeat expansion, but there is still a significant inverse correlation between the age at onset and the number of repeats in SCA6.[8 12–20] Some homozygotes for the SCA6 mutation show a more severe phenotype,[13 15] but others do not.[15 21] Unlike other SCAs with long CAG repeats, the expanded SCA6 allele is known to be relatively stable during meiosis and mitosis, with some exceptions.[7 22] The cardinal feature of SCA6 is slowly progressive ataxia,[3] but exceptions have been reported.[14 19]

The frequency of SCA6 varies between white ethnic subgroups, with a range of 0% to 15.2%.[16 17 19 23–25] In Japan, the frequency varies between regions, ranging from 5.9% to more than 30%.[13 15 20 26 27] In Hokkaido, the northernmost island of Japan, SCA6 accounts for 30% of 161 families with ADCA, the highest frequency reported to date.[28] These findings prompted us to search for a possible founder chromosome in Japanese SCA6, and to determine whether there are any alleles predisposing to the generation of SCA6 mutation.

Material and methods

Twenty one unrelated Japanese SCA6 families were investigated. Twelve non-consanguineous families[8] and one consanguineous family[21] have already been reported elsewhere, while eight families were newly added in this study. Thirteen of 21 families reside in Hokkaido, while the other eight families come from various other areas of Japan. The ancestors of the Hokkaido families moved to this island approximately a century ago from various, random other areas (data not shown). Altogether, 58 subjects were clinically affected, 35 were asymptomatic, and 10 had married into these families. In addition, 25 patients without family members available for testing were recruited from Hokkaido; a family history of ataxia was positive in 18 and negative in seven patients. Among the total of 83 patients, the mean age at onset was 49.6 (SD 11.6) years, ranging from 19 to 75 years.

After informed consent was obtained, high molecular weight DNA was extracted from peripheral white blood cells. According to the method of Zhuchenko *et al*,[3] polymerase chain reaction (PCR) amplification of CAG containing segments in the *CACNA1A* gene was performed using primers S-5-F1 and S-5-R1. S-5-F1 was end labelled with 6-FAM (PE Biosystems). After PCR amplification of genomic DNA using a PE9600 thermal cycler (PE Biosystems), the CAG repeat polymorphism was analysed using an ABI PRISM 377 gene sequencer equipped with GeneScan® software version 2.0 (PE Biosystems). The number of CAG repeats was determined with reference to the product size of the sequenced alleles.

To construct haplotypes carrying the *CACNA1A* gene, D19S840, D19S1150, D19S226, and D19S885 were analysed. These four microsatellites cover a 4 cM interval containing the entire *CACNA1A* genome from the telomeric to the centromeric end.[9] D19S1150 is located in intron 7 of the gene (fig 1). Polymorphism of these microsatellites was analysed using an automated gene sequencer[29] and the alleles of each microsatellite were numbered according to the product size. The CA repeat sequence of D19S1150 was determined using Genome Database information (accession No 1320259). After purification on a Microcon-100 spin column (Amicon), PCR

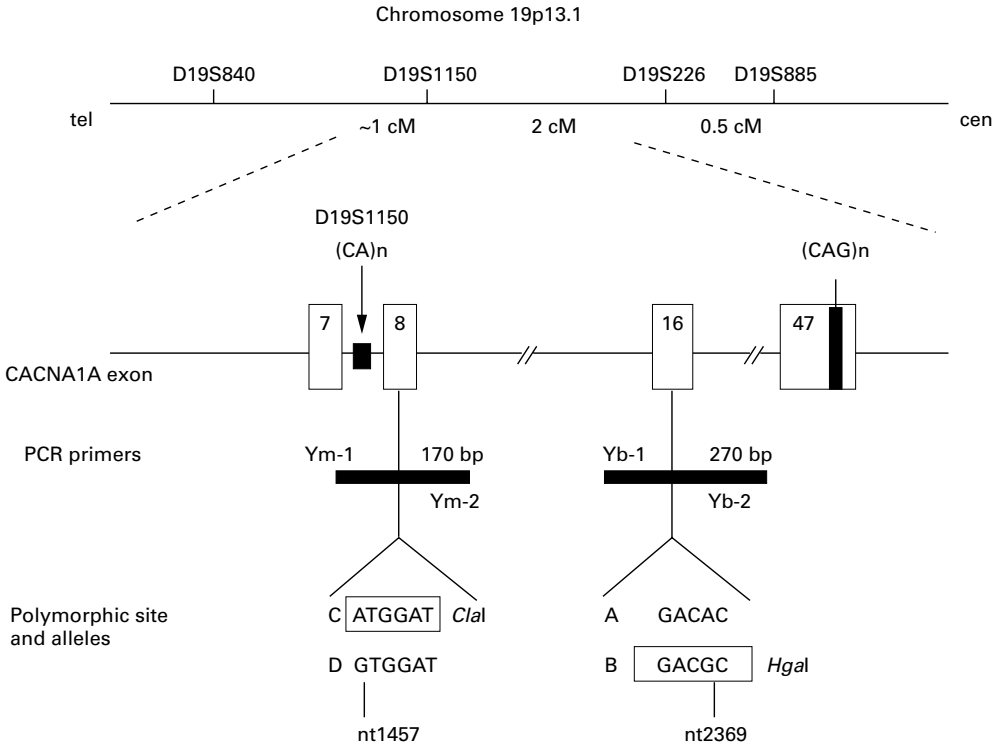

Figure 1 Polymorphic markers and locations examined in this study.

products of homozygotes for D19S1150 were directly sequenced using a BigDye Terminator Cycle Sequencing Kit (PE Biosystems), with p858 FOR as the forward primer.

In addition to these microsatellites, we examined two single nucleotide polymorphisms (SNPs) in the coding region of the gene: one (A/B system) was a G to A substitution at position 2369 in exon 16, and the other (C/D system) was a G to A substitution at position 1457 in exon 8 (fig 1). A pair of primers, Yb-1 (5'-TCCACAGCTGCATCTCC AAG-3') and Yb-2 (5'-ACCCTCCCTTGAG CCCCT-3'), generated a 270 bp fragment covering the site of position 2369 in exon 16 (A/B system). This site was recognised by the *Hga*I restriction enzyme. The SNP at nt 1457 in exon 8 (C/D system) was detected by mismatch PCR. Another primer pair, Ym-1 (5'-ATACTCTGGCTTTTCTATGC-3') and Ym-2 (5'-TTTCATCCTCGGCGAGGATC A CCTCTTCTGCTTTTGAGATCGA-3'), generated a 170 bp fragment that included a *Cla*I restriction site. PCR was done for 30 cycles in a total volume of 20 µl with 1.7 mol/l N,N,N,-trimethyl glycine (Wako) under the same conditions as for amplification of microsatellites, except that denaturation, annealing, and extension were each done for 60 seconds. To facilitate introduction of a restriction site, r*Tth* DNA polymerase® (PE Biosystems) was used with the Ym-1/-2 primer pair. The annealing temperature was set at 58°C for the Yb-1/-2 pair and 52°C for the Ym-1/-2 pair. After digesting the PCR products for one hour at 37°C with 1 U of either *Hga*I or *Cla*I, the alleles at each polymorphic site were determined by agarose gel electrophoresis.

We constructed haplotypes for the mutant SCA6 alleles (SCA6 chromosome) in 21 families based on their family structures and map order of 19p13 markers. Differences in allele frequency between the affected and control haplotypes were analysed using the χ^2 test and $p<0.05$ was considered statistically significant. Unrelated normal Japanese subjects (mostly residents of Hokkaido) served as controls. In addition, three normal subjects who had married into the affected families from outside Hokkaido were included as controls. Both the SCA6 patients and controls were from the same ethnic background.

For phase unknown samples, such as the controls (unrelated normal subjects, n=172) and the SCA6 patients (n=25) for whom family samples were not available, estimation of haplotype frequency was performed by the maximum likelihood method using a simplified version of the GENEF computer program (J-M Lalouel, unpublished data). Procedures for generating the haplotype have been described in full by Jeunemaitre *et al*.[30] Briefly, two polymorphisms were chosen to generate the haplotype, followed by sequential inclusion of one polymorphism at a time. Haplotypes showing a frequency below 1/4N (where N is the sample size) were eliminated during the process, and then the haplotype frequency was re-examined. Simple χ^2 tests of homogeneity were applied for statistical comparison between cases and controls.

Results

Among the 21 families, 58 affected subjects and nine asymptomatic subjects of risk age carried the expanded CAG repeat. The geno-

Table 1 D19S840-D19S1150-C/D-A/B-(CAG)n-D19S226-D19S885 haplotypes of 21 unrelated SCA6 families

	SCA6 haplotype						
	Extragenic (tel)	Intragenic				Extragenic (cen)	
Family Code No	D19S840	D19S1150	C/D	A/B	(CAG)n	D19S226	D19S885
P28	7	5	C	B	33	10	4
P76	7	5	C	B	25	10	2
P70	7	5	C	B	23	10	10
P83	7	5	C	B	23	5	2
P22	7	5	C	B	22	10	11
P27	7	5	C	B	23	10	2
P25	2	5	C	B	23	10	6
P57	2	5	C	B	25	10	2
P78	2	5	C	B	22	10	2
P79	2	5	C	B	24	10	2
P64	2	5	C	B	22	8	6
P60	2	5	C	B	22	11	2
P42	2	5	C	B	22	5	4
P20	2	5	C	B	23	7	2
P63	3	5	C	B	25	6	2
P80	7	1	C	B	22	10	2
P82	2	1	C	B	24	10	2
P81	2	1	C	B	21	5	2
P88	7	1	C	B	24	10	2
P89	ND	5	C	B	22	6	2
P90	2	5	C	B	22	8	ND

ND: phase not determined.

typing data for the 12 previously reported families[8] are included in the present analysis. Among these 21 families, one asymptomatic subject of risk age was homozygous for 21 repeat alleles and the others were all heterozygotes with both expanded and normal alleles. No cases of the unstable transmission of expanded alleles were observed. The 25 other patients without family samples were all heterozygotes for SCA6 mutations. The mean CAG repeat size of the mutant alleles was 23.1 (SD 2.1) (n=93 SCA6 alleles), with the range being 21-33. There was a significant inverse correlation between age at onset and the number of CAG repeats (n=83 patients with known age at onset; $\gamma=-0.706$, $R^2=0.499$, $p<0.0001$, Pearson's product moment method). When polynomial analysis was used, a significant correlation was also obtained ($R^2=0.539$, $p<0.0001$). The number of CAG repeats in unrelated normal alleles ranged from 4~18 (n=388), with a peak of 13 (24.5% of the total); 64.2% of the control alleles had 11-13 repeats and 7.0% had 15 repeats or more.

After construction of the D19S840-D19S1150-C/D-A/B-D19S226-D19S885 haplotype in the affected families, we found that the same haplotype (major haplotype) cosegregated with affected status in each family (table 1). In the D19S1150-C/D-A/B haplotype, either "5-C-B" (17 families, 81%) or "1-C-B" (four families, 19%) was selectively associated with SCA6 chromosomes. Sequencing showed that allele 1 of D19S1150 had $(CA)_6AA(CA)_{17}$, and allele 5 had $(CA)_6AA(CA)_{21}$.

We first compared the allele frequency of each polymorphism for unrelated control chromosomes with SCA6 chromosomes deduced from the affected families. Three intragenic markers, an intronic microsatellite (D19S1150) and two SNPs in exons 8 and 16, showed significant differences in allelic frequency between the affected chromosomes and controls ($p<0.0001$, table 2). Even two extragenic microsatellites, D19S226 and D19S885, showed a significant difference ($p<0.0001$ and $p<0.005$, respectively). These results indicate that there was significant linkage disequilibrium between SCA6 mutations and these markers.

In order to determine the profile of the *CACNA1A* gene haplotype, we then analysed the D19S1150-C/D-A/B polymorphism in controls (172 unrelated normal subjects) and 25 SCA6 patients for whom family data were unavailable. After genotyping, we performed

Table 2 Linkage disequilibrium between SCA6 mutation and 19p13 polymorphic markers from the SCA6 chromosomes of 21 unrelated families

		SCA6 chromosome		Control chromosome	Difference	
Locus	Polymorphism No of alleles	Associated allele	Frequency (No)	Frequency (No)	χ^2	p value
D19S840*	9	2(203 bp)	55% (11)	44.6% (318)	0.47	NS
		7(213 bp)	40% (8)	29.8% (318)	0.63	NS
D19S1150	9	5(158 bp)	81% (17)	16.8% (388)	51.23	p<0.0001
		1(150 bp)	19% (4)	1.8% (388)	22.63	p<0.005†
nt1457 in exon 8‡	2	C	100% (21)	64.5% (344)	11.33	P<0.001
nt2369 in exon 16‡	2	B	100% (21)	71.8% (344)	8.18	P<0.005
D19S226	13	10(245 bp)	57% (12)	14.0% (318)	22.79	p<0.0001†
D19S885*	7	2(175 bp)	70% (14)	32.3% (318)	10.47	p<0.005

*Data from SCA6 chromosomes from 20 unrelated families.
†Fisher's exact probability test.
‡Definitions of the biallelic system are shown in fig 1[f1].
NS: not significant.

Table 3 Estimation of D19A1150- C/D-A/B haplotype frequency in SCA6 patients (n=25) and controls (n=172) using the GENEF computer program

Haplotype	*SCA6 patients*	*Control*
D19A1150- C/D-A/B	(Allele = 50)	(Allele = 332)
1-C-B	0.0515	0
3-C-B	0.0225	0.0687
5-C-B	0.4858	0.1068*
6-C-B	0.0957	0.1282
9-C-B	0.0245	0.0968
10-C-B	0.0200	0.0435
3-D-B	0	0.0223
5-D-B	0	0.0205
6-D-B	0	0.0727
8-D-B	0	0.0281
9-D-B	0	0.1233
1-C-A	0.0285	0
5-C-A	0	0.0304
6-C-A	0.0715	0.0505
8-C-A	0	0.0198
9-C-A	0.020	0.0721
10-C-A	0	0.0248
3-D-A	0.0375	0.0144
5-D-A	0.0542	0.020
6-D-A	0.0328	0.0407
8-D-A	0	0.0123
9-D-A	0.0555	0
10-D-A	0	0.004
C/D-A/B	(Allele = 50)	(Allele = 344)
C-B	0.70	0.4470†
D-B	0	0.2710
C-A	0.12	0.1984
D-A	0.18	0.0836

*χ^2=46.69 (df=1), p<<0.001.
†χ^2=11.14 (df=1), p<0.001.

haplotype estimation by the maximum likelihood method on samples for which the phase was not determined. Estimated haplotype frequencies were compared between SCA6 patients and controls (table 3). The results of this analysis were as follows: (1) the frequencies of 5-C-B and 1-C-B haplotypes were significantly higher in patients than in controls (49% *v* 11%, χ^2=46.69, df=1, p<<0.001 and 5% *v* 0%), indicating that the SCA6 mutant allele in these 25 patients was most likely to carry either haplotype 5-C-B or 1-C-B; (2) the frequency of the C-B haplotype was significantly higher in patients than in controls (70% *v* 45%, χ^2=11.14, df=1, p<0.001); (3) C-B was the most frequent haplotype in controls (45% of 344 alleles); and (4) the D-B haplotype frequency was 27% in controls, but 0% in patients.

Discussion

The present study disclosed several findings about the genetic background of SCA6 in the Japanese. First, study of SCA6 families showed that only two haplotypes, "5-C-B" (81%) and "1-C-B" (19%), were significantly associated with the affected chromosomes (SCA6 chromosomes), and that the allele frequencies of each locus on these chromosomes was significantly different from those of controls. Second, 5-C-B was also the most frequent haplotype in probands (49%, n=50 chromosomes), indicating that one of the two haplotypes in each patient can be expected to be this common haplotype. Third, all of the affected haplotypes carried the C-B haplotype, which was the most frequent haplotype in control chromosomes (45%). The significantly high frequency of the 5-C-B haplotype among the probands implies that their SCA6 mutation also resides on this haplotype, as was found in the affected families. However, since we could not determine directly which chromosomes (haplotypes) were the site of the SCA6 mutation, the possibility that haplotypes other than C-B carry the mutation cannot be completely excluded.

In Hokkaido, the majority of residents including the present subjects are descendants of immigrants from various areas of Japan and share a single ethnic background. Taking these historical data and the results of our genetic analyses into account, there is a possible founder effect in the subjects from Hokkaido and also in those from other areas of Japan. Judging from our data, these results favour the hypothesis that the expanded SCA6 alleles in the Japanese population originated from a chromosome with a C-B haplotype, which is the most frequent haplotype in controls (45% of alleles). The most plausible scenario is as follows. First, the SCA6 mutation occurred on a chromosome with the 5-C-B haplotype. At some point thereafter, the removal of four CA repeats occurred, an event which changed the haplotype from 5-C-B to 1-C-B. This is supported by the finding that the CAG repeat size of mutant SCA6 alleles is more variable on 5-C-B chromosomes than on 1-C-B chromosomes (22-33 *v* 21-24).

In the SCA6 allele, 7 and 11-13 CAG repeats are the predominant alleles in normal populations, regardless of ethnic background. Alleles with 15 CAG repeats or more are quite rare in European/American populations.[3 17 18] However, alleles with 15-19 repeats are not infrequently observed in the Japanese population, having a range of 5.9%[13] to 7.0% (present study, n=388). A recent study indicated that, in dominant SCAs caused by triplet repeat expansion including SCA6, the frequency of large alleles in a normal population is correlated with the relative prevalence in different ethnic groups.[27] These data suggest the possibility that such large alleles are a potential reservoir for full mutant alleles, which may explain the high prevalence of SCA6 in the Japanese. It would be worthwhile to determine whether such intermediate SCA6 alleles in the normal Japanese population have a C-B haplotype.

Recurrent mutations of at risk chromosomes are considered to be potential founders in several CAG triplet disorders. In Huntington's disease (HD), haplotype studies on a cohort of families have shown that only 41% were derived from either one of two common ancestral haplotypes while the rest were from independent mutations.[30] De novo expansions from intermediate alleles have also been reported in HD.[32 33] In Machado-Joseph disease (MJD/SCA3), haplotype analyses using intragenic SNPs have shown several ancestral mutations, and normal chromosomes with intermediate expansions in a prevalent population carry the same haplotype that is shared with the affected chromosomes in that population.[34] On the other hand, in DRPLA, a single predisposing haplotype was selectively associated with the affected chromosome and with normal chromosomes carrying a larger expansion.[35] The frequency of the allele with the pre-

disposing haplotype is considered to be correlated with the prevalence of DRPLA in different ethnic groups. Several different founder haplotypes for SCA6 have been identified in white populations.[36] In addition, de novo expansion from the intermediate alleles has been reported.[7 22] Observation of these three triplet repeat diseases suggests that the number of founder haplotypes is associated with the degree of instability of the predisposing chromosomes, which leads to pathogenic repeat expansion.

Despite extensive ongoing investigation, the molecular mechanism responsible for the instability of expanded repeats remains unknown. Our study showed that the majority of Japanese SCA6 mutations are derived from a C-B haplotype pool. This implies the possibility that some *cis* acting factor plays a role in promoting instability of CAG repeats in the SCA6 gene. A similar mechanism has been postulated through the study of DRPLA.[35] Brock *et al*[37] reported that the expansibility of elongated CAG triplet repeats was strongly correlated with their location within CpG islands and with the GC content in the flanking sequence of CAG repeats. Their study provides insight into the molecular basis of *cis* acting factors, which modify the instability of expanded triplet repeats. However, in SCA6 as well as DRPLA, the molecular mechanism leading to full expansion from a particular predisposing chromosome is not fully understood. To understand the molecular mechanism of SCA6 mutation better, our conclusions need to be confirmed through the study of different ethnic groups.

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Prenatal testing for Huntington's disease: experience within the UK 1994-1998

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Huntington's disease (HD) is an adult onset, autosomal dominant disorder[1] with onset of symptoms usually in the fourth or fifth decade. The classical triad of clinical features, movement disorder, cognitive impairment, and personality and psychiatric disorder, cause serious management problems. There is significant morbidity within the affected families, especially for those who themselves are at risk of developing the disease. HD affects about 5000 people in the UK and about five times that number are considered to be at 50% risk of developing the disease.

Since the mapping of the locus for Huntington's disease on chromosome 4 in 1983,[2] followed by the identification of the gene and its expanded polyglutamine repeat in HD in 1993,[3] it has been possible to offer accurate tests for HD. Prenatal tests and presymptomatic predictive tests for adults at risk for HD are available at genetic centres throughout the world.

There are two common approaches to prenatal testing in HD. Direct testing involves investigating for the presence of the mutation in a pregnancy. This gives an accurate result. If the status of the at risk parent has not been ascertained, then this may produce predictive information about that person.

In exclusion testing, the at risk grandparental chromosome 4 locus is excluded using linkage analysis. This test preserves the 50% risk of the parent, and allows a pregnancy at low risk to continue. In this situation, pregnancies that share the risk of the parent would be terminated. However, should the at risk parent not develop HD, a normal pregnancy would have been lost.

Given the technical feasibility of prenatal mutation testing and the severity of the disorder, it might be expected that prenatal diagnosis would be frequently requested.

Tyler *et al*[4] reviewed a group of referrals for exclusion testing in pregnancy, and surveyed a group of subjects at 50% risk of developing HD about their attitudes to prenatal testing. They concluded that the demand for such testing was likely to be small. We considered it important to assess this demand in relation to that for presymptomatic testing, and since the numbers recorded by individual centres were small, to collect the data on a UK basis.

In Britain, the UK Huntington's Disease Prediction Consortium was created to monitor the use of molecular testing in HD, to evaluate the developing service, and to ensure the highest standards were applied to the procedure.[5] Several studies before the introduction of predictive testing reported the views of those at risk of HD. These showed that between 56% and 80% of at risk subjects would undergo predictive testing once it was available.[6–8] Uptake of such testing has been considerably less than this, 9-15%[1] with some exceptions.[9]

Table 1 Prenatal tests and their outcome (UK) 1994–1998

	1994	*1995*	*1996*	*1997*	*1998*	*Total*
Exclusion tests						
Outcome: low risk	13	4	3	8	6	34
Outcome: high risk	7	5	8	9	6	35
Outcome: uninformative	3	0	0	0	0	3
Terminations	7	5	8	8	6	34
Miscarriage	0	0	0	0	1	1
Total	23	9	11	17	12	72
Direct tests						
Outcome: low risk	8	4	12	10	12	46
Outcome: high risk	6	2	1	8	11	28
Terminations	6	2	1	5	11	25
Total	14	6	13	18	23	74
Total (all tests)	37	15	24	35	35	146