ORIGINAL CONTRIBUTION

Trinucleotide Repeats in 202 Families With Ataxia

A Small Expanded $(CAG)_n$ Allele at the SCA17 *Locus*

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Background: Ten neurodegenerative disorders characterized by spinocerebellar ataxia (SCA) are known to be caused by trinucleotide repeat (TNR) expansions. However, in some instances the molecular diagnosis is considered indeterminate because of the overlap between normal and affected allele ranges. In addition, the mechanism that generates expanded alleles is not completely understood.

Objective: To examine the clinical and molecular characteristics of a large group of Portuguese and Brazilian families with ataxia to improve knowledge of the molecular diagnosis of SCA.

Patients and Methods: We have (1) assessed repeat sizes at all known TNR loci implicated in SCA; (2) determined frequency distributions of normal alleles and expansions; and (3) looked at genotype-phenotype correlations in 202 unrelated Portuguese and Brazilian patients with SCA. Molecular analysis of TNR expansions was performed using polymerase chain reaction amplification.

Results: Patients from 110 unrelated families with SCA showed TNR expansions at 1 of the loci studied. Dominantly transmitted cases had $(CAG)_n$ expansions at the Machado-Joseph disease gene (*MJD1*) (63%), at *SCA2* (3%), the gene for dentatorubropallidoluysian atrophy (*DRPLA*) (2%), SCA6 (1%), or *SCA7* (1%) loci, or $(CTG)_n$ expansions at the *SCA8* (2%) gene, whereas $(GAA)_n$ expansions in the Freidreich ataxia gene (*FRDA*) were found in 64% of families with recessive ataxia. Isolated patients also had TNR expansions at the *MJD1* (6%), *SCA8* (6%), or *FRDA* (8%) genes; in addition, an expanded allele at the TATA-binding protein gene (*TBP*), with 43 CAGs, was present in a patient with ataxia and mental deterioration. Associations between frequencies of SCA2 and SCA6 and a frequency of large normal alleles were found in Portuguese and Brazilian individuals, respectively. Interestingly, no association between the frequencies of DRPLA and large normal alleles was found in the Portuguese group.

Conclusions: Our results show that (1) a significant number of isolated cases of ataxia are due to TNR expansions; (2) expanded *DRPLA* alleles in Portuguese families may have evolved from an ancestral haplotype; and (3) small $(CAG)_n$ expansions at the *TBP* gene may cause SCA17.

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THE SPINOCEREBELLAR ataxias (SCAs) are neurodegenerative disorders that are clinically and genetically heterogeneous. Ten genetically different SCAs are known to be caused by trinucleotide repeat (TNR) expansions. In the dominant SCAs, the mutant proteins show an expanded polyglutamine tract in SCA1, SCA2, Machado-Joseph disease (MJD), SCA6, SCA7, and dentatorubropallidoluysian atrophy (DRPLA),[1-9] whereas SCA8 and SCA12 are caused by untranslated $(CTG)_n$ and $(CAG)_n$ expansions, respectively.[10,11] In Friedreich ataxia (FRDA), the mutant protein is deficient in homozygotes for a $(GAA)_n$ expansion in intron 1 of the *FRDA* gene.[12] Recently, an expanded CAG repeat tract has been found at the TATA-binding protein gene (*TBP*) in an isolated patient with symptoms of ataxia and intellectual deterioration.[13] *TBP* repeat expansions have since been described in 4 Japanese families affected by a new type of ataxia with dementia named SCA17.[14] Matsuura et al[15] found a large expansion of pentanucleotide $(ATTCT)_n$ in intron 9 of the *SCA10* gene in patients with spinocerebellar ataxia type 10, thus being the first to show the existence of a new class of dynamic mutations.

Function of the genes involved is known only for SCA6, SCA12, and SCA17. SCA6 is due to a small CAG expansion (21-33 CAGs) in the coding region of a voltage-gated calcium channel α-subunit gene (*CACNA1A*).[9] SCA12 is caused by a CAG expansion in the 5′-untranslated region of a brain-specific regulatory subunit of the protein phosphatase *PP2A* gene.[11] The re-

For author affiliations, see page 628.

SUBJECTS, MATERIALS, AND METHODS

SUBJECTS

Individuals with SCAs who were referred to UnIGENe, Instituto de Biologia Molecular e Celular (Porto, Portugal) between 1997 and 2000 were included on a consecutive basis. These individuals were referred to this laboratory for the molecular diagnosis of SCAs. Dominant inheritance was assumed based on the presence of at least 1 affected family member in 2 or more successive generations. Recessive transmission was presumed when the patient had a history of consanguinity or siblings affected without (aged) affected parents. An isolated occurrence was assumed in the absence of a family history. We studied 202 unrelated families: 145 lived in Portugal, and 57 in Brazil. Dominant inheritance was apparent in 106 families, whereas recessive transmission was suspected in 33 Portuguese families; 63 individuals had isolated cases of SCA.

METHODS

Peripheral blood was collected from patients and their relatives after written informed consent was obtained. Genomic DNA was isolated from peripheral blood leukocytes using standard techniques.[17]

Molecular analysis of the CAG and CTG repeat loci were performed by polymerase chain reaction (PCR) amplification using the published primer sequences;[1,4,6,8-11,18] the PCR was carried out with 1µM of each primer, 200µM of deoxynucleotides, 1.0mM of magnesium chloride, 10mM of Tris (pH 9.0), 50mM of potassium chloride, 1 U of *Taq* polymerase, and 2% formamide, in a final volume of 25 µL. Samples were processed as previously described.[6,9,19,20] Polymerase chain reaction products were analyzed on 6% polyacrylamide gels. Allele sizes were determined by comparing migration relative to an M13 sequencing ladder. DNA sequencing was performed to accurately assess repeat size and the presence of interruptions. Sequencing reactions were performed using a ThermoSequenase DNA sequencing kit (USB, Cleveland, Ohio) with 5 µL of amplified DNA. Both the CTA and CTG repeats on the *SCA8* gene are polymorphic, and PCR assay determines the combined size of the 2 repeats.

The analysis of the intronic $(GAA)_n$ on the *FRDA* gene was performed by PCR using primers 2500F[12] and 104FGAA[21] for expanded alleles, and primers GAA-R and GAA-F for normal alleles, following the conditions described.[22] Expanded allele sizes were analyzed by comparing migration relative to molecular weight standards. Normal sizes were assessed by analysis of fluorescent-labeled PCR products in an automated DNA sequencer (model 4200; Li-COR, Lincoln, Neb) using 5.5% Long Ranger gels (FMC Bioproducts, Rockland, Me).

STATISTICAL ANALYSES

Possible differences between Portuguese and Brazilian groups in normal repeat frequency distributions were assessed using 2 nonparametric tests: the Kolmogorov-Smirnov 2-sample test and the Mann-Whitney *U* test. Allele frequencies at each locus were estimated by the gene count method, and heterozygosity (*H*) was calculated as

$$H = 1 - \sum_{i=1}^{n} X_i^2,$$

where X_i is the estimated frequency of the *i*th allele at the locus. Statistical analyses of differences in estimates of heterozygosity between Portuguese and Brazilian individuals, as well as differences in the frequency of large normal alleles for each locus, were performed with the Fisher exact test.

cently described SCA17 is caused by a $(CAG)_n$ expansion in the coding region of the transcription factor *TBP* gene.[13]

Associations between prevalence of dominantly inherited SCAs and frequency of large normal $(CAG)_n$ alleles have been found in Japanese and European populations, indicating that these may contribute to the generation of expanded alleles in the SCAs.[16]

In an attempt to improve our understanding of TNR expansions leading to SCA, we have (1) assessed repeat size at the *SCA1*, *SCA2*, *MJD1*, SCA6, *SCA7*, *SCA8*, SCA12, SCA17/*TBP*, *DRPLA*, and *FRDA* loci in a large group of Portuguese and Brazilian patients with ataxia; (2) examined TNR distributions of normal alleles at these loci; (3) determined the frequency of TNR expansions; (4) compared frequencies of large normal alleles with relative frequencies of SCA at each loci; and (5) looked for genotype-phenotype correlations.

RESULTS

TNR DISTRIBUTIONS IN NORMAL ALLELES

The frequency distributions of normal alleles in the dominant SCAs caused by translated TNR expansions in the Brazilian group were not significantly different from those in the Portuguese group ($P>.05$ using the Mann-Whitney *U* test and Kolmogorov-Smirnov 2-sample test) (**Figure 1**). In the case of the *SCA2* locus, the normal $(CAG)_n$ alleles ranged in size from 17 to 31 repeats in the Portuguese group and from 19 to 24 repeats in the Brazilian group; the allele with 22 repeats was the most frequent in both groups. No significant differences were found between the 2 groups at the most recently identified *SCA8*, SCA12, and SCA17/*TBP* ataxia loci (Figure 1). Interestingly, no *SCA8* alleles smaller than 18 repeats and larger than 35 repeats in the Portuguese patients, or larger than 30 repeats in the Brazilian patients, were observed. A third class of extremely large normal alleles at the *SCA8* locus, varying in size from 40 to 91 CTG repeats,[23] was not found in either group; this might be due to the small sample size. At the SCA12 locus, small normal alleles were closely distributed around an allele with 10 CAGs, which represented more than 65% of all normal alleles in each group; the other class of normal alleles comprised CAG repeat sizes of 12 to 18 units. A large normal SCA12 allele containing 28 CAG repeats was also observed in the Portuguese group. The recently described SCA17/*TBP* ataxia locus presented a distribution of normal allele sizes varying from 29 to 40 CAGs in

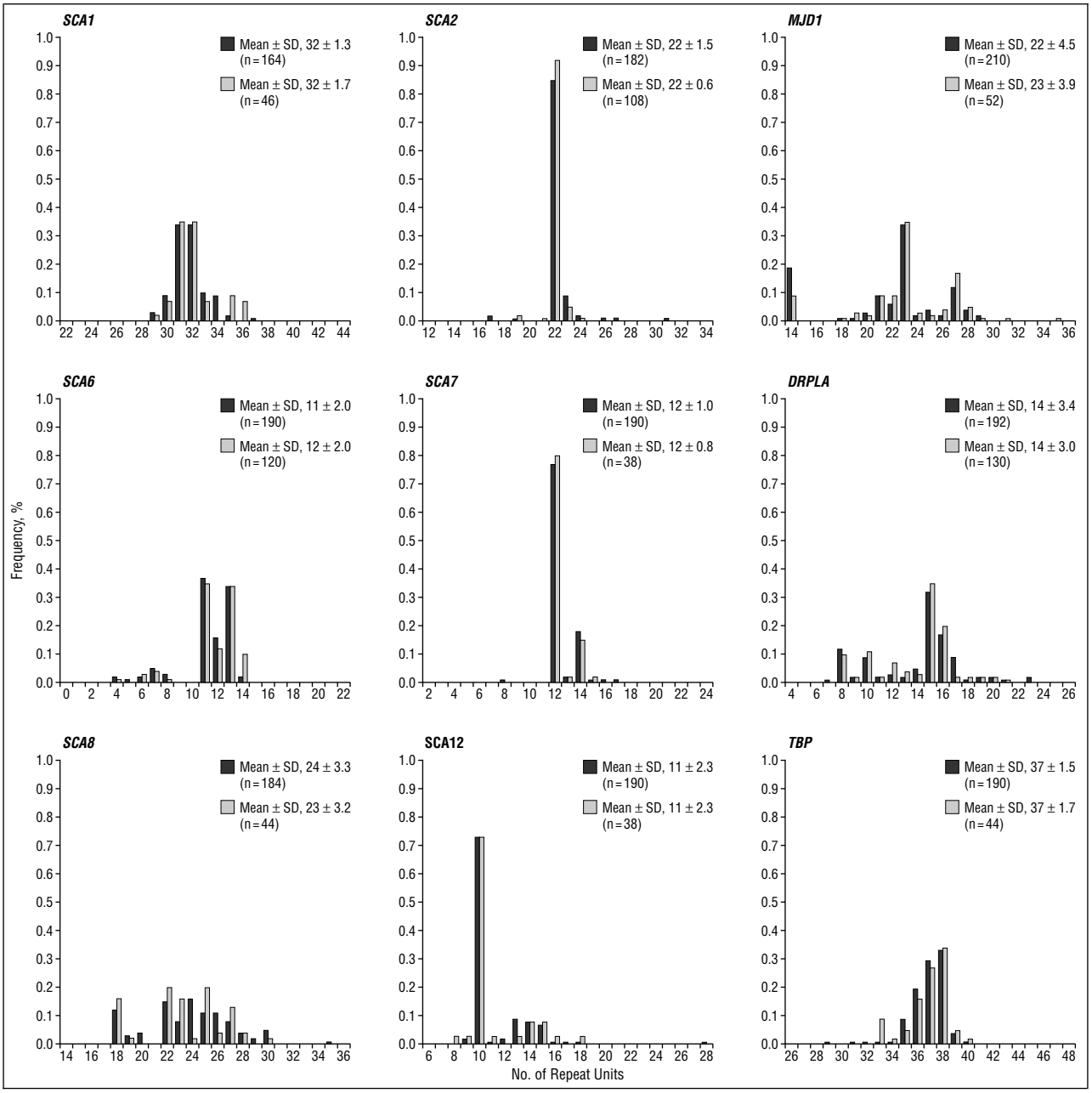

Figure 1. Distribution of triplet repeats in normal alleles at *SCA1, SCA2, MJD1,* SCA6, *SCA7, DRPLA, SCA8,* SCA12, and SCA17/*TBP* loci in studied individuals of Portuguese (black bars) and Brazilian (hatched bars) origin. Vertical axes represent allele frequency, and horizontal axes represent number of repeat units. SCA indicates spinocerebellar ataxia; MJD, Machado-Joseph disease; DRPLA, dentatorubropallidoluysian atrophy; and TBP, TATA-binding protein. SCA6 and SCA12 refer to the loci for the respective diseases, not to the genes themselves.

both groups, with distributions peaking at the allele with 38 CAG units. At the *FRDA* gene, normal alleles can be subdivided into 2 classes depending on their GAA repeat length: short normal alleles, with 5 to 10 GAA triplets, and long normal alleles, with 12 to 60 GAA triplets.[24] At this locus, long normal alleles represented 18% and 22% of Portuguese and Brazilian individuals, respectively (**Figure 2**A). Although the Brazilian group had a slightly larger proportion of long normal alleles, this difference was not significant.

The most polymorphic loci were *DRPLA, MJD1, SCA1, SCA8,* SCA17/*TBP,* and SCA6, whereas *SCA2, SCA7,* and SCA12 were the least polymorphic ones (**Table 1**).

FREQUENCY OF TNR EXPANSIONS

Analysis of loci involved in the SCAs showed that 110 unrelated patients (55%) had ataxia due to a TNR expansion. Analysis of loci with translated $(CAG)_n$ tracts showed that 78 unrelated patients with ataxia had 1 allele in the expanded range. Expansions at the *MJD1* locus were found in 114 individuals, 102 affected and 12 asymptomatic, from 67 families that had ataxia with dominant inheritance (63%), as well as in 4 isolated cases (6%). Six individuals (5 affected) from 3 families with dominant ataxia (3%) had an expanded allele at the *SCA2* gene. An expanded allele at the *DRPLA* locus was found in 5

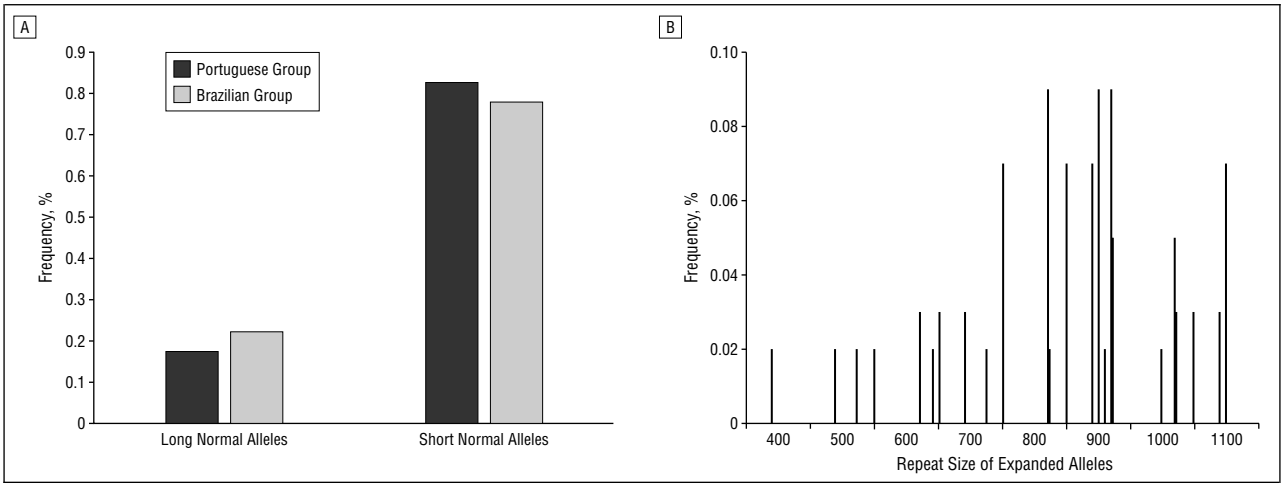

Figure 2. A, Distribution of GAA repeats in normal allele classes at the Friedreich ataxia (*FRDA*) locus by geographic origin of studied individuals. B, Distribution of GAA repeats in expanded alleles at the *FRDA* locus in 58 chromosomes from Portuguese patients.

Table 1. Heterozygosity Values for Dominant Ataxia Loci by Geographic Origin*

	Portuguese Group		Brazilian Group	
Locus	n	Heterozygosity	n	Heterozygosity
SCA1	82	0.77 ± 0.05	23	0.78 ± 0.09
SCA2	91	0.31 ± 0.05	54	0.15 ± 0.05
MJD1	105	0.78 ± 0.04	26	0.73 ± 0.09
SCA6	95	0.60 ± 0.05	60	0.68 ± 0.06
SCA7	95	0.30 ± 0.05	19	0.37 ± 0.11
DRPLA	96	0.81 ± 0.04	65	0.82 ± 0.05
SCA8	92	0.76 ± 0.05	22	0.77 ± 0.09
SCA12	95	0.48 ± 0.05	19	0.53 ± 0.11
TBP	95	0.72 ± 0.05	22	0.64 ± 0.10

*Data are presented as mean ± SD. SCA indicates spinocerebellar ataxia; MJD, Machado-Joseph disease; DRPLA, dentatorubropallidoluysian atrophy; and TBP, TATA-binding protein. SCA6 and SCA12 refer to the loci for the respective diseases, not to the genes themselves.

Table 2. Frequency of SCA TNR Expansions by Mode of Inheritance and Geographic Origin*

TNR Expansions	AD Portuguese/ Brazilian Families, %	AR Portuguese/ Brazilian Families, %	Isolated Cases in Portuguese/ Brazilian Individuals, %
Translated (CAG)n			
SCA2	5/0	. . .	. . .
MJD/SCA3	49/85	. . .	4/13
SCA6	0/2	. . .	. . .
SCA7	0/2	. . .	. . .
DRPLA	3/0	. . .	. . .
Total	**57/89**	. . .	4/13
Untranslated (CTG)n			
SCA8	3/0	. . .	6/6
Intronic (GAA)n			
FRDA	. . .	64/0	9/6

*SCA indicates spinocerebellar ataxia; TNR, trinucleotide repeat; AD, autosomal dominant; AR, autosomal recessive; MJD, Machado-Joseph disease; DRPLA, dentatorubropallidoluysian atrophy; FRDA, Friedreich ataxia; and ellipses, not available. No expansions were found at either the *SCA1* or SCA12 locus in either population.

patients and 1 possibly affected individual, from 2 kindreds with dominant ataxia (2%). One patient from a family that had ataxia with dominant inheritance (1%) had an expanded allele at the SCA6 locus. Three patients from 1 kindred with dominant ataxia (1%) had 1 expanded allele at the *SCA7* gene. Analysis of the untranslated $(CTG)_n$ at the *SCA8* gene showed that 4 patients from 2 unrelated families with dominant ataxia (2%), as well as 4 isolated cases (6%), exhibited 1 expanded allele. Expansions of the intronic $(GAA)_n$ at the *FRDA* gene (Figure 2B) were found in both alleles in 31 patients from 21 families with recessively inherited ataxia (64%) and in 5 isolated cases (8%). **Table 2** shows the frequency of TNR expansions for each SCA by geographic origin.

FREQUENCY OF LARGE NORMAL ALLELES

Evidence from several populations has suggested that the disease prevalence of many of these SCAs may be associated with the presence of large normal alleles at the respective loci.[16] To study the frequency of TNR expansions in our 2 groups relative to the frequency of normal alleles of larger size at the various loci, we used the criteria of Takano et al[16] (**Table 3**). Large normal alleles at the *SCA1* (>34 repeats) and SCA6 (>13 repeats) loci were significantly more frequent in Brazilian individuals than in the Portuguese individuals. Normal alleles in the upper tail of the *SCA2* distribution (>22 repeats) appeared to be overrepresented in the Portuguese group, but the *P* value obtained was of borderline statistical significance.

GENETIC AND CLINICAL FEATURES OF PATIENTS

The clinical phenotypes were very heterogeneous. All of our affected individuals showed clinical symptoms of cerebellar ataxia, with or without other associated features. Epilepsy was present in patients with DRPLA from 1 family, only cognitive impairment was observed in some pa-

Table 3. Frequencies of Large Normal Alleles at the Dominantly Inherited Ataxia Loci in Portuguese and Brazilian Groups*

Locus	Repeat Size	Portuguese Group	Brazilian Group
SCA1	>33	0.11	0.15
	>34	0.02	0.15†
SCA2	>22	0.13	0.06‡
	>23	0.04	0.01
MJD1	>27	0.07	0.07
	>28	0.02	0.03
SCA6	>13	0.02	0.10†
SCA7	>14	0.02	0.02
DRPLA	>16	0.07	0.05
	>17	0.06	0.04
SCA8	>27	0.11	0.07
	>28	0.07	0.02
SCA12	>15	0.03	0.05
TBP	>38	0.05	0.07
	>39	0.01	0.02

*SCA indicates spinocerebellar ataxia; MJD, Machado-Joseph disease; SCA6 and SCA12 refer to the loci for the respective diseases, not to the genes themselves. DRPLA, dentatorubropallidoluysian atrophy; and TBP, TATA-binding protein. SCA6 and SCA12 refer to the loci for the respective diseases, not to the genes themselves.

†$P = .003$.

‡$P = .05$.

tients with DRPLA and SCA8, and visual impairment was seen in affected individuals with SCA7. Two of the 3 families with SCA2 had been thought to have MJD. The families with SCA6, SCA7, and SCA8 and 1 of the 2 kindreds with DRPLA did not have a previous clinical diagnosis of these disorders. Altogether, 13 patients with no family history tested positive for a TNR expansion: 4 isolated patients had a mutation for MJD (2 of them having a clinical diagnosis of possible MJD), 3 of the 5 isolated patients with *FRDA* expansions had a clinical diagnosis of the disease, and the $(CTG)_n$ expansion at the *SCA8* gene was present in 4 isolated cases. **Table 4** shows the mean age at onset and expanded allele size ranges for patients with TNR expansions.

SCA2 ALLELE WITH 32 CAG REPEATS

The analysis of the *SCA2* gene in our patients disclosed an allele with 32 CAGs in a 48-year-old isolated patient who had been clinically diagnosed as having atypical FRDA. Her age at onset of gait ataxia was 2 years; during the following years, she developed a spinocerebellar syndrome. The disease progressed rapidly as time passed, with evidence of neuropathy and a mild cognitive impairment; by age 30 years, she was nonambulatory. We first tested the *FRDA* gene and identified 2 normal alleles; thus, we have proceeded with the analysis of other known SCA mutations and detected an allele with 32 repeats at the *SCA2* gene. Sequence analysis of this allele showed an interrupted CAA repeat configuration: therefore, this is probably not a pathogenic allele. Moreover, a recently performed muscle biopsy showed ragged-red and cytochrome oxidase–negative fibers as well as reduced activity of the mitochondrial respiratory chain on polarography.

Table 4. Repeat Size and Age at Onset of Patients With Expanded TNRs*

Expanded TNR	No. of Patients	Age at Onset, Mean (SD), y	Expanded Allele Size Range	TNR Size in Expanded Alleles, Mean (SD)
SCA2	6	34 (7)	39-46	41 (3)
MJD1	101	34 (13)	65-85	75 (3)
SCA6	1	34	25	25
SCA7	3	32 (14)	43-55	47 (7)
DRPLA	5	37 (11)	58-61	60 (1)
SCA8	7	21 (10)	100-152	119 (20)
TBP	1	52	43	43
FRDA	31	11 (4)	390-1100	858 (167)

*TNR indicates trinucleotide repeat; SCA, spinocerebellar ataxia; MJD, Machado-Joseph disease; DRPLA, dentatorubropallidoluysian atrophy; TBP, TATA-binding protein; and FRDA, Freidreich ataxia. SCA6 refers to the locus for the disease, not to the gene.

SMALL EXPANDED ALLELE AT THE SCA17/*TBP* LOCUS

Besides the mutations described previously, the analysis of the CAG repeat at the *TBP* gene showed an expanded allele with 43 units in a 64-year-old patient with ataxia. Sequence analysis of this allele showed an interrupted repeat configuration of $(CAG)_3(CAA)_3(CAG)_9$ $CAACAGCAA(CAG)_{23}CAACAG$, encoding 43 glutamines. This patient began experiencing symptoms of gait ataxia at age 52 years. During the last 5 years, he exhibited progressive mental deterioration and dementia. The family history indicated that his deceased mother presumably had the same disease. There are no other patients in this family. The normal allele range previously described for *TBP* was 25 to 42 CAG repeats.[13,25-27]

COMMENT

DOMINANT ATAXIA AND *FRDA*

The analysis of TNR sizes at the loci implicated in SCA in a large group of Portuguese and Brazilian patients allowed the identification of an expansion in more than half of them, including a significant number without a family history. This study showed that DRPLA disease, frequent in Asian populations[28] but rare in European individuals,[16] was present in the Portuguese families. On the other hand, no expanded alleles were seen at the SCA12 locus, confirming previous impressions that they are very rare.[11] $(GAA)_n$ expansions at the *FRDA* gene were also frequent among our recessive Portuguese families with ataxia (64%). The genetic basis of approximately 40% of families with ataxia remains unknown.

In most cases of SCAs, phenotypic features are helpful indicators of the underlying genotype. Dementia and seizures associated with symptoms of cerebellar ataxia are characteristic features of DRPLA[29]; however, in 1 of our families with the *DRPLA* mutation, the only patient available had symptoms of cerebellar ataxia alone.

ISOLATED CASES OF TNR EXPANSIONS

We identified the molecular basis in 13 patients with ataxia who had no family history. Eight (13%) did not have a clinical diagnosis of a specific SCA type. A recent study has also shown that 19% of cases of apparently idiopathic ataxia were due to TNR expansions.[30] We propose that all isolated individuals with ataxia of unknown cause should be investigated for TNR expansions at known *SCA* loci.

SMALL CAG EXPANSION AT THE SCA17/*TBP* GENE

The normal CAG size range described for the *TBP* gene (in 2525 chromosomes belonging to individuals of European, Asian, African American, and Hispanic origin) was 25 to 42 repeat units.[13,25-27] Previous findings have implicated a de novo expansion of 63 CAG repeats at the *TBP* gene in a 14-year-old Japanese patient, confined to a wheelchair at age 13 years with symptoms of ataxia and intellectual deterioration.[13] Expansions in the SCA17/*TBP* gene have also been found in 4 Japanese families with ataxia and dementia in which expanded alleles were 47 to 55 CAG units.[14] We found a *TBP* allele with 43 CAG units in a 64-year-old affected individual with mild ataxia and dementia; the late onset of disease as well as the mild clinical symptoms seem to correlate with the small size of the expanded allele (compared with the early onset observed in the patient with 63 glutamines).

LARGE INDETERMINATE ALLELES AT THE *SCA2* GENE

Genetic diagnosis and counseling may sometimes be difficult with these diseases caused by TNR expansions. In SCA1, for instance, there is no gap between normal and pathological alleles, whereas *SCA2* alleles with 32 and 33 CAGs have been considered of indeterminate significance. Normal *SCA1* and *SCA2* CAG repeats are interrupted by CAT or CAA triplets, respectively, whereas pathogenic expansions are pure, uninterrupted CAG repeats.[6,29] Uninterrupted alleles with 32 CAGs[31] and 33 repeat units[32] have been found in young, at-risk subjects in families with SCA2, both resulting from the contraction of alleles with 40 repeats. Recently, another *SCA2* allele with 33 uninterrupted CAG units was found in a patient with a mild balance problem; this patient had an onset at age 60 years.[33] We found an *SCA2* allele with 32 CAGs interrupted by a CAA triplet in a patient with a childhood onset of severe ataxia. The severe phenotype, the altered function of the mitochondrial respiratory chain, and the presence of a CAA interruption suggest that this *SCA2* allele is not the cause of the disease in this case. As in SCA1, in SCA2 there is no gap between normal and pathological alleles.

ASSOCIATIONS BETWEEN PREVALENCE OF SCAs AND LARGE NORMAL ALLELES

Our present findings indicate that for both *SCA2* and SCA6 TNRs, large normal alleles may contribute to the generation of expanded alleles and thus to the relative frequency of these SCAs in the 2 groups. For the remaining loci, the frequency of normal alleles was similar in the 2 groups. Of interest is the fact that no association between the frequency of DRPLA and that of large normal alleles was found in the Portuguese group. This may be owing to the effect of a specific haplotype associated with large normal alleles, shared with patients who have DRPLA, that is prone to further expansion into the disease range. In a group of Japanese individuals, all expanded and intermediate *DRPLA* alleles shared a unique haplotype that is frequent in Asian populations and is usually associated with large normal alleles.[28] The same haplotype has also been found in European patients, although it rarely occurs in normal chromosomes in this population. This suggests that *DRPLA* expanded alleles in Japanese[28] and Portuguese patients may have evolved from a common founder chromosome. Haplotype analyses in our families with DRPLA are now being carried out to test this hypothesis.

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