Association of Moderate Polyglutamine Tract Expansions in the Slow Calcium-Activated Potassium Channel Type 3 With Ataxia

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Background: The small-conductance calcium-activated potassium channel gene (hSKCa3) contains 2 CAG repeats, 1 of which is highly polymorphic. Although this repeat is not pathologically expanded in patients with schizophrenia, some studies have suggested an allelic association with schizophrenia. CAG expansions in other genes such as the α1 subunit of a brain-specific P/Q-type calcium channel gene cause spinocerebellar ataxia type 6, whereas the length of the CAG repeat in the RAI1 gene modifies the age of onset of spinocerebellar ataxia type 2.

Objectives: To evaluate expansions in the hSKCa3 polyglutamine domain as causative for ataxia, and to study the association between the length of the polyglutamine repeat and the presence of ataxia.

Methods: We analyzed this repeat in 122 patients with autosomal dominant cerebellar ataxia, or sporadic ataxia, and compared allele distribution with 750 alleles seen in 2 healthy control groups and 172 alleles in patients with Parkinson disease.

Results: The distribution of alleles in ataxia patients and controls was significantly different by Wilcoxon rank test (P < .001). Twenty-two or more polyglutamine tracts were more common in ataxia patients compared with controls by χ² analysis (P < .001).

Conclusion: Longer stretches of polyglutamines in a human potassium channel are not causative for ataxia, but they are associated with the presence of ataxia. There is no association with the presence of Parkinson disease.

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Despite the identification of 6 spinocerebellar ataxia (SCA) genes, 20% to 50% of autosomal dominant cerebellar ataxia (ADCA) mutations are still unaccounted for. The molecular basis of late-onset sporadic ataxias, sometimes also referred to as olivoponto-cerebellar atrophies, has remained largely unexplained because mutations in known genes are very rare in patients without a positive family history and involve mutations in the SCA1, SCA2, or Friedreich ataxia gene. Only expansions in the SCA6 repeat are found in appreciable numbers in patients with sporadic ataxia. In one study, a third of patients with a repeat expansion in the SCA6 gene had no family history of the disorder.

The SCA6 mutation is distinct in several respects. The polyglutamine tract is located in the α1 subunit of a brain-specific P/Q-type calcium channel. Nonsense and missense mutations in this channel occur as well, and are associated with episodic ataxia type 2 and familial hemiplegic migraine, respectively. The range of expansion seen on chromosomes of affected patients with SCA6 is 21 to 27 repeats—smaller than in any of the other genes. Expanded repeats do not exhibit the marked meiotic instability that is typical of other CAG-repeat diseases.

Recently, a novel, small-conductance calcium-activated potassium channel gene (hSKCa3) was identified that has 2 CAG repeats in its coding region. The first of these repeats is not very polymorphic. It ranges in size from 5 to 12 repeats, with 12 being the most common; the second repeat is highly variable, with alleles containing 7 to 28 repeats and the modal containing 19 repeats. The hSKCa3 gene was initially mapped to a region of human chromosome 22, which is thought to contain genes predisposing individuals to schizophrenia. Owing to this map position, the allele distribution in healthy controls and patients with schizophrenia was extensively studied. Allele distribution seemed to be differentially distributed between the 2 samples, with patients with schizophrenia having slightly larger alleles. However, no allele class was
MATERIALS AND METHODS

GENOTYPE ANALYSIS

Genotyping was performed according to the method used for the European part of the study described in Chandy et al. European primers were used to amplify both repeats. The following nested primers were designed to amplify each repeat independently. For the first repeat "forward":

3A 5'-GGGTGGGACTGGATGAA-3'

and "reverse"

3B 5'-GCTGAAGCTGGGAGCAGT-3'
generated a 170-base pair (bp) fragment when 12 repeats are present. For the second repeat "forward":

2A 5'-GCCCTACCTCCCGAGCT-3'

and "reverse"

2B 5'-GGACGGGCTGGCTCTGGA-3'
generated a 110-bp fragment at 14 repeats. Polymerase chain reaction conditions for both primers were identical. Initial denaturation at 95°C for 5 minutes, 35 cycles at 95°C for 1 minute and 30 seconds, 62°C for 30 seconds, 72°C for 45 seconds, and a final extension at 72°C for 5 minutes. Polymerase chain reaction products were analyzed using a 6% polyacrylamide gel with an M13 ladder as a size marker. Samples were also selected from all gels and run on a single gel as an additional check that all allele sizes were consistently scored between runs.

The sizes of the resulting polymerase chain reaction products were then analyzed as described earlier. Twenty samples ranging in repeat size from 5 to 12 for the first repeat, and 10 to 23 for the second repeat were sequenced in an ABI373 DNA sequencer (PE Corp, Foster City, Calif) to serve as size controls.

To address the possibility that the presence of multiple affected individuals from single nuclear families may produce false-positive results, a single affected individual per family was chosen at random.

STATISTICAL ANALYSIS

Data were analyzed by Wilcoxon rank test and by performing an overall \( \chi^2 \) test with the appropriate degrees of freedom. Comparisons were made for allelic distribution and for distribution of genotypes.

With regard to chromosome localization, mapping of hSKCa3 was performed using the GeneBridge 4 panel, consisting of 93 radiation hybrid clones (Research Genetics, Huntsville, Ala) and primers 2A and 2B as previously described. Results were submitted to the Whitehead Institute Genome Center server (http://www.genome.wi.mit.edu).

RESULTS

MAPPING OF hSKCa3

We performed radiation hybrid mapping using the GeneBridge 4 panel. Results indicated that hSKCa3 maps to chromosome 1q21 with a lod score of 1.46. The gene resides between markers D1S305 and IB1251, at a distance of 2.63 centimorgan from D1S305.

ALLELIC ASSOCIATION

We examined 24 patients representing 20 families with ADCA, as well as 98 patients who did not have an apparent family history of ataxia. None of these patients were positive for a mutation in SCA1, SCA2, SCA3, SCA6, or SCA7. The hSKCa3 gene encodes a protein of 731 amino acids containing 2 adjacent polyglutamine arrays in its N-terminal domain, separated by 25 amino acids. The 5' CAG repeat was not highly polymorphic, with a major allele of 12 repeats accounting for 97.9% of alleles. Alleles with 5, 7, and 10 repeats made up 1.5%, 0.1%, and 0.5%, respectively.

The 3' CAG repeat was highly polymorphic. The allele distribution for the 3' CAG repeat in patients with ataxia is shown in Figure 1. The most common allele contained 19 repeats, followed in frequency by alleles of 18 and 20 repeats. A bimodal character was evident in the distributions, with a smaller peak present at 12 to 15 repeats, as has also been reported in 2 previous studies. Only 1 of 122 patients had an allele size that was in a range not previously reported for healthy individuals or patients with a history of psychiatric illness. This sporadic ataxia patient had 1 allele with 28 repeats and a second allele with 19 repeats. A search for additional patients with ataxia who carry alleles of this size range or larger yielded negative results, except for 1 patient with a known SCA6 mutation who had an hSKCa3 allele of 27 repeats.

Although these observations did not suggest a direct pathogenic role for long hSKCa3 alleles, analysis of allele distribution in patients with ataxia suggested that long alleles were more common than in previously reported control groups. Whereas alleles with 22 repeats were much rarer than alleles with 21 repeats in previously reported healthy controls and psychiatric patients, in patients with ataxia, alleles with 22 repeats were actually more common than alleles with 21 repeats.

To verify these observations, we examined 2 independent control groups. The first consisted of 228 chro-
mosomes from spouses of patients with Parkinson disease (PD) in California. The second control group consisted of 522 chromosomes from healthy octogenarians from central Europe. Allele distributions for these groups are shown in Figure 1. There were no significant differences in the allele distributions between the 2 control groups by Wilcoxon rank test or $\chi^2$ analysis. Alleles with more than 22 repeats were rare and represented only 0.8% of spouses with PD and 0.8% in healthy octogenarians. The longest allele seen on normal chromosomes contained 25 repeats. We therefore combined both control groups for all subsequent statistical analyses.

We tested 2 related but distinct hypotheses. We first examined whether allele distribution for the entire spectrum of allele lengths differed between patients with ataxia and controls by the Wilcoxon signed rank test. This hypothesis does not require the presence of the specific threshold mentioned earlier, in which a disease association is observed. Two-tailed Wilcoxon signed rank tests determined that allele sizes differed in patients with ataxia ($P < .001$).

Subsequently, we tested the hypothesis that alleles above a threshold level of 21 repeats were associated with ataxia. Compared with our 2 healthy control groups, alleles with 22 or more CAG repeats were almost 5 times as common and were seen in 4.9% of patients with ataxia. This difference was statistically significant by $\chi^2$ analysis ($P < .001$). Setting the threshold at 21 or more CAG repeats also yielded significant differences ($P < .05$), as well as at a threshold of 23 or more CAG repeats ($P < .05$).

To examine whether the association of alleles with 22 or more repeats in patients with ataxia was specific to this form of neurodegeneration, we examined 172 chromosomes of patients with sporadic PD ranging in age from 30 to 83 years. In patients with PD, alleles with 22 or more CAG repeats had a frequency of 1.7% (Figure 1). This frequency was not different from that of controls ($P = .26$).

**GENOTYPIC ASSOCIATION**

We also analyzed whether an association with genotype existed in addition to allelic association. For this analysis we added the CAG repeat numbers in both alleles and compared the number of genotypes with 40 or more CAG repeats (Figure 2). By the 2-tailed Wilcoxon signed rank test, genotypes were different between patients with ataxia and controls ($P < .001$). In patients with ataxia, genotypes with 40 or more summed CAGs were significantly more common than genotypes in controls ($P < .001$). Genotypes for patients with PD were not different than those of controls.

**COMMENT**

The CAG repeat in the hSKCa3 channel represented a good candidate for pathologic expansion in patients with ataxia or other neurodegenerative disorders. Expansion of polyglutamine repeats in several proteins is known to cause neurodegenerative disorders. Expansion of polyglutamine repeats in several proteins is known to cause neurodegenerative disorders.23 The CAG repeat expansion in another channel protein, the CACNL4A gene, causes SCA6. In addition, a northern blot analysis revealed hKCa3-specific expression to be largely limited to the brain, striated muscle, and lymphoid tissues.20

Because of conflicting reports regarding chromosomal location, we mapped hSKCa3 using radiation hybrid mapping. Our map position in chromosome 1q21 does not agree with the initial reports by Chandy et al,21 who had mapped hSKCa3 to chromosome 22. Our results, however, confirm the report by Austin et al,15 who mapped hSKCa3 to the identical region by radiation hybrid mapping. This region has been linked to familial hemiplegic migraine, but not to schizophrenia or other neuropsychiatric disorders.26

In diseases caused by CAG repeat expansion, a threshold effect can be detected. Below the threshold, no pathological abnormality is observed, but above the threshold, expansions invariably cause disease. Length
of the abnormal poly Q tract is strongly associated with age of onset. In some diseases, such as SCA3 or dentatorubral pallidolysian atrophy, normal and abnormal repeat lengths are separated by many repeats, whereas in SCA6 the separation is only 1 repeat. In Huntington disease and SCA2, intermediate alleles are seen that predispose individuals to disease, and are associated with reduced penetrance. 27-29

Encouraged by the observation of an hSKCa3 allele with 28 repeats in a patient with sporadic ataxia we sought to identify other patients that had allele sizes not previously reported in healthy controls. No patients, however, were identified, except for a patient with SCA6 who had an hSKCa3 allele with 27 repeats. Though to our knowledge, alleles of this size have not been reported in control populations analyzed worldwide, an allele with 30 CAG repeats was identified in a Chinese patient of unspecified age with schizophrenia. 9 Results of a neurologic examination of that patient were not reported. Therefore, it is not likely that long CAG repeats in the hSKCa3 gene are a common cause of ataxia.

Although we could not prove a causative role for large hSKCa3 alleles, inspection of allele distribution in patients with ataxia suggested that larger alleles were more common in this group than reported in previous studies examining healthy controls or patients with schizophrenia. Whereas alleles with 22 or more CAG repeats were seen in less than 1% of controls or patients with schizophrenia worldwide, 4.9% of patients with ataxia had alleles with 22 or more CAG repeats, and 1.3% had alleles with 23 or more CAG repeats. To confirm this observation, we determined allele sizes in 2 control populations. Although ascertained by different methods, both of our control groups had almost identical allele distributions. Alleles with 22 or more repeats constituted 0.8% of all alleles, whereas alleles with 23 or more repeats were seen in only 0.2% of normal chromosomes. The allele distribution in our control group was in perfect agreement with previously published studies on various ethnic groups (ie, French/Alsatian, Chinese, Anglo-Saxon, Irish, and Israeli Jewish). 16

Because alleles with 22 or more CAG repeats are rare in healthy controls, we set this length as a threshold for statistical comparison by χ² analysis. The frequency of alleles with 22 or more CAG repeats was significantly increased in patients with ataxia. Even when a threshold was set at an allele size of 21 or more or 23 or more CAG repeats, distribution of alleles above this threshold was significantly different in patients with ataxia.

To determine whether large repeats were associated with neurodegenerative diseases in general, we tested a population of patients with PD, with an age-at-onset range from 31 to 83 years. The frequency of large alleles in patients with PD was not different from that seen in our 2 control groups. Further studies of patients with other neurodegenerative diseases are needed to determine whether the hSKCa3 CAG polymorphism is indeed specifically associated with ataxia.

Predisposing alleles may show additive effects when present in the homozygous state. For example, the presence of an ApoE4 allele modifies the age at onset of Alzheimer disease, and this effect is more pronounced in ApoE4 homozygotes. 30 An additive effect of pathologically expanded alleles is also seen in mouse models of SCA1 and SCA2, although this is less obvious in rare human cases with alleles homozygous for repeat expansion. In Huntington disease and other CAG-repeat diseases, the correlation of age at onset with repeat length of the pathologically expanded allele is well documented. 27 Repeat length variation of the normal allele seems to contribute relatively little to age at onset variation in HD or SCA2. 31,33 In SCA2 patients, CAG repeat length in the RAI1 gene seemed to influence age at onset. This effect was seen for contribution of the longest allele or for the CAG sum determined by adding the CAG repeats in both RAI1 alleles. 34 To determine whether the genotype at the hSKCa3 locus was associated with ataxia, we added the number of repeats of both alleles, assuming a simple additive effect. An association of genotypes with 40 or more CAG repeats with ataxia was detected, but was not seen in patients with PD. The number of patients studied, however, was too small to determine...
whether this effect was due solely to the presence of a single allele with a very long CAG repeat.

Association studies can have significant limitations owing to the presence of population stratification. Allele distribution may vary in different ethnic or geographic groups. Their differential admixture to study and control populations may mimic allelic association in the study group. Because of the high interest in the hSKCa3 polymorphism for psychiatric diseases, the CAG repeat allele distribution in the hSKCa3 gene has been extensively studied worldwide. No significant differences in allele distribution in different ethnic groups have been identified. This makes it unlikely that our observations are simply caused by population stratification. Despite this, the results of our study need to be repeated in other groups of patients with ataxia. If confirmed, it will be important to analyze whether allelic variation in the hSKCa3 channel is a disease modifier only in patients carrying other highly penetrant disease alleles at other loci, or whether it modifies an environmental risk factor. Most importantly, it will be critical to define the effects of long polyglutamine tracts on channel function and the involvement of this channel in neurodegeneration. In addition, the possibility remains that the polyglutamine repeat in hKCa3 is merely in linkage disequilibrium with an as yet unidentified functional polymorphism or a causative mutation within the gene or in its vicinity.

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