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KCNC3: Phenotype, mutations, channel biophysics – a study of 260 familial ataxia patients

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Abstract

We recently identified *KCNC3*, encoding the Kv3.3 voltage-gated potassium channel, as the gene mutated in SCA13. One g.10684G>A (p.Arg420His) mutation caused late-onset ataxia resulting in a non-functional channel subunit with dominant-negative properties. A French early-onset pedigree with mild mental retardation segregated a g.10767T>C (p.Phe448Leu) mutation. This mutation changed the relative stability of the channel's open conformation. Coding exons were amplified and sequenced in 260 autosomal-dominant ataxia index cases of European descent. Functional analyses were performed using expression in Xenopus oocytes. The previously identified p.Arg420His mutation occurred in three families with late-onset ataxia. A novel mutation g.10693G>A (p.Arg423His) was identified in two families with early-onset. In one pedigree, a novel g.10522G>A (p.Arg366His) sequence variant was seen in one index case but did not segregate with affected status in the respective family. In a heterologous expression system, the p.Arg423His mutation exhibited dominant negative properties. The p.Arg420His mutation, results in a non-functional channel subunit was recurrent and associated with late-onset progressive ataxia. In two families the p.Arg423His mutation was associated with early-onset slow progressive ataxia. Despite a phenotype reminiscent of the p.Phe448Leu mutation, segregating in a large early-onset French pedigree, the p.Arg423His mutation resulted in a nonfunctional subunit with a strong dominant-negative effect.

Keywords

Ion Channel gene defects; Spinocerebellar Ataxia; SCA13; KCNC3

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INTRODUCTION

Spinocerebellar ataxias (SCAs) represent a diverse group of neurodegenerative diseases which affect the cerebellum and its connections and result in motor incoordination. SCAs show an autosomal dominant inheritance and are caused by mutations in a number of genes. Most commonly, the type of mutation is a DNA CAG repeat expansion resulting in an enlarged poly-glutamine domain in the respective protein. In addition to translated CAG-repeat mutations, a number of non-CAG ataxia genes (SBTBN2,TTBK2, PRKCG, ITPR1, AFLG3L2; MIM#s 600224, 604432, 605361, 606658, 610246) have been identified with functions as diverse as protein kinases, cytoskeletal and mitochondrial proteins, and proteins regulating intracellular calcium stores (Houlden, et al., 2007; Ikeda, et al., 2006; Mariotti, et al., 2008; van de Leemput, et al., 2007; Yabe, et al., 2003). We described KCNC3 (Kv3.3; MIM# 605259) as the gene mutated in SCA13 (Waters, et al., 2005; Waters, et al., 2006a). Two different mutations each segregating in large families were identified. A g.10767T>C (p.Phe448Leu) mutation (nucleotide is based on KCNC3 transcript variant 1, RefSeq NG_008134.1, NP_004968.2 and NM_004977.2 in GenBank)(den Dunnen JT, 2000; Pruitt KD, 2007) segregated with affected individuals in a French pedigree with early-onset ataxia (Herman-Bert, et al., 2000; Waters, et al., 2006a). This mutation is located at the cytoplasmic end of the pore and affects channel gating by increasing the relative stability of the open conformation.

A second pedigree segregated a p.Arg420His mutation. This Filipino family had a late onset with slow progression of ataxic symptoms. The p.Arg420His mutation is located in the S4 transmembrane segment, an essential element of the voltage sensor domain, and results in a non-functional subunit with dominant negative effects specific to the Kv3 channel family (Waters, et al., 2006a).

These two families were identified based on their individual linkage to human chromosome 19q. We now describe the analysis of a large number of index patients from European pedigrees with autosomal dominant ataxia of early or late onset. We show that the p.Arg420His mutation, originally described in Filipinos, is recurrent in Europeans. Another conserved arginine in S4 was mutated to histidine (p.Arg423His) in two families in whom the clinical phenotype in affected individuals was characterized by an early-onset ataxia. This novel mutation had strong dominant-negative characteristics similar to p.Arg420His.

MATERIALS AND METHODS

Identification of Index cases

The study group was composed of 260 index cases from European autosomal dominant ataxia families. All patients had undergone clinical examinations and genetic tests to exclude ATNX1, ATXN2, ATXN3, CACNA1A, ATNX7, and TBP (MIM#s 164400, 183090, 109150, 183086, 164500, 607136) CAG expansion mutations.

The control group included 284 alleles contributed by 77 normal individuals living in France, 10 index individuals from the French families in the CEPH pedigree set and 55 US individuals that represented spouses of patients with Parkinson disease.

All subjects were consented at the institution of origin and all work was approved by the IRB. DNA samples were collected and stored using standard procedures.

Mutation detection

For mutation screening, intronic and exonic PCR primer pairs (Table 1) were designed from genomic sequence to amplify each exon of the *KCNC3* gene, including the flanking splice

sites with a set of 5 PCR primer pairs. Due to the complexity in the amplification of exon 1, three different primer pairs overlapping at least 100 base pairs were designed to amplify the entire exon. Exon 2 was amplified utilizing one primer pair composed of a total of 1330 base pairs and Exon 3 and 4 were amplified as one amplicon with primer pairs flanking each exon respectively, thus encompassing the entire intron 3. PCR products were checked on agarose gels, and then sequenced as detailed below. Each fragment containing mutations was PCR amplified and sequenced a second time to confirm that the identified mutations were not due to PCR artifacts.

The PCR reactions were done under the following conditions: 50ng (5ul) of genomic DNA, 1× (2ul) of 10× Buffer (Qiagen), 1unit (0.2ul) of Hot Taq (Qiagen), 2ul of Q solution (Qiagen) and dd H_20 up to 20ul. The PCR cocktails were amplified as follows: Exon 1 (all 3 amplicons) and exon 3–4 used an initial denaturation of 94°C for 15min, followed by 10 cycles at 95°C 1min10sec, 67°C 30sec, 72°C 1min30sec followed by 30 cycles of 95°C 1min10sec, 65°C 30 sec, 72°C 1min30sec followed by an extension of 72°C for 5min. Exon 2 was amplified at 95°C for 15min, 35 cycles of 95°C 1min, 60°C 30 sec, 72°C 2min with a final extension of 72'C 7min.

Sequence analysis

DNA sequencing was performed using the ABI BigDye Terminator v3.1 cycle sequencing kit and the following protocol: 10ng (2ul) of purified PCR amplicon, 3ul sequencing reaction premix, 2ul 5× sequencing buffer, 80ng (2ul) of primer and 11ul of DD H20. The reaction mix was run in a PCR thermocycler (Bio-Rad MyCycler v 1.065) and cycled as follows: 96'C for 3 min followed by 25 cycles consisting of 96'C for 10s, 50 for 5s and 60'C for 4 min. Sequencing products were purified using ABI Centri-Sep spin columns. Resuspended samples were electrophoresed on an ABI 377 DNA sequencer. All sequences were analyzed using BioEdit biological sequence alignment editor (v5.0.9.1, Tom Hall, Isis Pharmaceuticals) All samples were sequenced bidirectionally (sense and antisense) and with multiple primers.

Analysis of channel function

Mutations were introduced into the human wildtype Kv3.3 clone (kind gift of Dr. James L Rae, Mayo Foundation) using the QuikChange (Stratagene)(Rae and Shepard, 2000b).

Sequence variants were verified by sequencing. Run off transcripts were prepared in vitro using the mMessage mMachine kit (Ambion). Wild type and mutant subunits were expressed alone or in carefully controlled ratios by injecting RNA into Xenopus oocytes. Channel activity was recorded 2–3 days later using a two electrode voltage clamp (Papazian, et al., 1991). During electrophysiological experiments, oocytes were bathed in a solution containing 4 mM KCL, 85 mM NaCl, 1.8 mM $CaCl_2$, and 10mM HEPES, pH 7.2. Electrode resistances ranged from 0.3 to 0.8 MΩ.

RESULTS

Genetics

KCNC3 is composed of 5 exons encompassing a 13,870 kb region of chromosome 19; the mRNA contains 3176bp, of which 2274bp are coding. We screened the entire coding region for DNA variants in 260 index cases of European descent with autosomal dominant ataxia. The index cases were from families with late as well as early disease onset.

Table 2 and 3 show the DNA sequence changes found in 260 index cases and 142 controls. Table 2 shows variants that meet established criteria for disease-causing mutations including

absence in controls, change of highly conserved amino acids, and significant effects on channel function. Table 3 summarizes variants found in controls or cases that likely represent rare normal alleles. All variants were present in the heterozygous state. Exon 5 was not analyzed as it did not contain any coding sequences. We identified a total of 15 allelic variants. These consisted of 14 single nucleotide changes, and one 9bp duplication. Eight variants were located in the coding region, and five resulted in amino acid changes. The 9bp g.5932_5940dup (GCCGCCAAC) resulted in the addition of 3 amino acids (p.Asn215_Ala217dup) in exon 1 and was observed in 1 control. There were 3 single nucleotide changes that resulted in amino acid changes, which changed an arginine to a histidine (p.Arg366His, p.Arg420His, and Arg423His). These coding variants were observed in cases, but not in controls.

A number of variants that did not change the predicted amino acid sequence were identified either in the 5-UTR, or as synonymous changes in exons 1 or 2. These variants each occurred at a frequency of <1% in either cases or controls and likely represent rare normal alleles. Subtle effects on channel abundance, however, cannot be excluded at this time. No variants were detected in exons 3 and 4 or within 10bp of exon/intron junctions.

Other than exon-intron boundaries, we did not attempt to sequence introns with the exception of intron 3 owing to its small size. Not surprisingly, DNA variants were most common in intron 3. Four sequence variants were detected. One single nucleotide change, located at position g.13858G>A in intron 3, was relatively common and found in 7.31% of cases (3.65% of alleles), but only in 0.70% (0.35% of alleles) of controls. The significance of this finding is unknown as our study was not designed to examine differences in frequencies of variants found in cases and controls.

We found a total of 6 index cases with variants in the coding region that resulted in changes of the amino acid sequence. These variants were not detected in normal control samples. As one variant (p.Arg366His) did not co-segregate with the disease phenotype in the respective family, 5 index cases remained that carried disease-causing mutations. The two mutations (p.Arg420His and p.Arg423His) were recurrent. In order to confirm that these mutations had arisen independently we analyzed haplotypes of affected individuals with markers in the *KCNC3* region. Markers included MF1 located within the gene itself and others flanking *KCNC3* with a distance of less than 1Mb to 4Mb (D19S412, D19S553, D19S606, D19S879 and D19S902). We did not detect shared haplotypes for p.Arg420His or pArg423His mutation carriers, respectively, indicating independent mutational events. Thus, mutations in KCNC3 in our series are recurrent, but are a rare cause of autosomal dominant SCA.

Clinical phenotypes of five index cases and three first-degree relatives are shown in Table 2. We identified 3 European index cases with the p.Arg420His mutation, which we had previously identified in a Filipino pedigree (Waters, et al., 2006b). Two of the index cases lived in Germany and one in France.

All individuals with the p.Arg420His mutation shared adult onset, predominantly cerebellar signs, and slowly progressive course. Age of onset ranged from 25 to 51. Imaging showed cerebellar atrophy in all cases (Fig. 1). One individual (MR 3-3), daughter of a male index case, had a disease onset at age 28 with an ataxic gait, but also developed seizures. Interestingly, these seizures emanated from a temporal lobe focus, and the patient is currently being evaluated for resective epilepsy surgery. In contrast to other individuals with the p.Arg420His mutation, this father-daughter pair also exhibited lower leg spasticity.

We also identified a novel p.Arg423His mutation in two index cases. Similar to the p.Phe448Leu mutation previously described in a large French family, these individuals had an early onset, but in contrast did not exhibit mental retardation or seizures. Age of onset

was described as being in infancy and detected as delay in acquisition of motor milestones. One individual had a computerized tomography scan at age 3 with normal results. Three individuals were examined at ages 17, 33, and 47 and had had little progression of disease with regard to gait ataxia and dysarthria. Seizures were not reported in these individuals, but one individual had lower leg spasticity.

One male index case with an age of ataxia onset at 65 carried a p.Arg366His variant in the heterozygous state. His affected nephew with an age of onset at 45 did not carry this change. The nephew's father was unaffected. This family was lost to follow-up and no imaging findings were available. A brother of the index case had a rapidly progressive disease with ataxia and speech problems just prior to death. This patient was negative for the p.Arg366His mutation as well as for a *PRN* mutation, but tested positive for the 14-3-3 protein in CSF by western blot analysis. Paternity testing was consistent with the stated relatedness of the three individuals. Thus, despite functional alterations caused by this variant (see below), the p.Arg366His change does not meet stringent genetic criteria for a disease causing variant.

Functional Analysis

To characterize the effects of the newly-identified amino acid substitutions on channel function, the p.Arg423His mutation and the p.Arg366His variant were generated in wild type human Kv3.3 (Rae and Shepard, 2000a). Wild type and mutant subunits were expressed in Xenopus oocytes and channel activity was recorded with a two electrode voltage clamp (Papazian, et al., 1991).

The p.Arg423His mutant was non-functional when expressed alone (Fig. 2Ai). Upon co-expression with wild type, the p.Arg423His subunit suppressed activity consistent with a strong dominant negative effect (Fig. 2Aii). At a 1:1 expression ratio of wild type and p.Arg423His, which is the expected ratio found in SCA13 patients, current amplitudes measured at +60 mV were significantly smaller than wild type expressed alone. Current amplitudes were further suppressed at ratios of 1:2 and 1:4 (wild type: p.Arg423His) (Fig. 2Aiii). The currents generated by expression of wild type alone or by co-expression of wild type and p.Arg423His at a 1:1 ratio had similar functional properties (Fig. 2Aiv). Although there was a small difference in inactivation kinetics in the example shown in Fig. 2Aiv, the significance of this finding is difficult to assess. We have observed variability in the rate of Kv3.3 inactivation in oocytes, perhaps due to differences in the basal level of protein kinase C (PKC) activity. Phosphorylation of Kv3.3 by PKC slows inactivation (Desai, et al., 2008).

The p.Arg366His variant was non-functional when expressed alone (Fig. 2Bi). Subunits with the p.Arg366His alteration had a dominant negative effect on expression of wild type (Fig. 2Bii). At a 1:1 expression ratio of wildtype and p.Arg366His, current amplitudes were significantly less than wild type expressed alone. Increasing the amount of the mutant subunit led to further decreases in current amplitude (Fig. 2Biii). The functional properties of currents generated by expression of wild type alone or by co-expression of wild type and p.Arg366His at a 1:1 ratio were similar (Fig. 2Biv).

DISCUSSION

This study answered several questions relating to frequency, nature, recurrence, and phenotype/genotype correlations in mutations of the KCNC3 (Kv3.3) ion channel. We found that *KCNC3* mutations were relatively rare, but that two mutations were recurring and involved the S4 domain. Specific disease causing mutations were predictive of the clinical phenotype with little variability within and between families.

Overall, the number of variants discovered after complete sequencing of the four coding exons and adjacent exon-intron junctions was small. Although we sequenced close to 700 alleles, many DNA variants were only observed a single time, either in controls or in affected index cases. These were located in the 5'UTR, in exons as synonymous or non-synonymous variants, and in the 3'UTR. The exception was one SNP in intron 3 g. 13858G>A that reached a frequency of 7.3% in ataxia patients, but had a frequency of 0.70% in controls. As our study was not designed to examine the significance of allele frequency differences between cases and control, the biologic consequences of variants seen in cases and controls remain unknown at this time. Even though functional effects cannot be immediately gleaned from the sequence changes, it is possible that these variants could contribute to development or progression of disease.

In addition to rare non-coding variants, we identified three amino acid changing DNA variants. Of 260 index cases with a family history consistent with autosomal dominant inheritance, only five carried a mutation in the *KCNC3* gene. Thus, it is highly unlikely that *KCNC3* mutations explain a large proportion of the 30–40% of SCA families that have no identified mutations (Pulst, 2001).

The two recurring mutations and the variant of unknown significance seen in a single sample occurred in highly conserved arginines. The arginines at positions 366, 420 and 423 are virtually invariant in all voltage-gated potassium channels. Arg423 is one of several arginine residues in the S4 transmembrane segment that sense and respond to changes in voltage (Aggarwal and MacKinnon, 1996; Seoh, et al., 1996). The positively charged residues in S4, including Arg420 and Arg423, are responsible for voltage-dependent conformational changes that result in channel opening or closing.

The p.Arg420His mutation, originally described in a Filipino pedigree (Waters, et al., 2006a), was identified in three additional pedigrees from different European countries. These mutations had arisen independently as shown by haplotype analysis. The p.Arg420His mutation results in a non-functional subunit with dominant-negative properties on channel expression. In all four pedigrees, the p.Arg420His mutation was associated with late-onset progressive ataxia and cerebellar atrophy. Age of onset was highly variable within and between pedigrees and ranged from 25 to 51 in this study (Table 2) and from 22 to more than 60 years of age in the previously reported Filipino pedigree.

The variant p.Arg366His is located at the intracellular end of the S2 transmembrane segment in the voltage sensor domain. Recently reported X-ray structures of voltage-gated potassium channels indicate that this positively-charged residue is involved in a network of electrostatic interactions that likely stabilize the voltage sensor domain (Long, et al., 2005a; Long, et al., 2005b). The p.Arg366His variant, which was seen in one individual with late onset, also resulted in a non-functional subunit with dominant-negative effects. When wild type and mutant subunits were expressed in Xenopus oocytes at a 1:1 expression ratio, current amplitudes were reduced to 50% (Fig. 2). Despite the likely importance of Arg366 for channel function, we cannot designate the p.Arg366His variant as disease-causing due to its lack of recurrence and absence of segregation in the family. The possibility remains, however, that the affected nephew without the p.Arg366His change represents a phenocopy.

A novel p.Arg423His mutation was observed in two pedigrees. The p.Arg423His mutation was associated with delayed development of motor milestones and lifelong, relatively stable ataxia, and thus resembled the phenotype observed in the large French pedigree segregating the p.Phe448Leu mutation. In contrast to presence of mild mental retardation in all carriers of the p.Phe448Leu mutation and seizures in some, none of the four individuals with the p.Arg423His mutation showed mental impairment or seizures.

Despite the shared early-onset phenotype, the effects of the two mutations in a heterologous expression system were distinct. The p.Arg423His mutation generated non-functional subunits with a strong dominant negative effect on channel expression, whereas the p.Phe448Leu mutation increased the stability of the open state of the channel. Therefore, the initial notion that gain-of-function mutations in KCNC3 would be associated with early-onset and dominant-negative mutations with late-onset and progression is not supported. It is currently unknown why the two dominant-negative mutations, p.Arg420His and p.Arg423His, have such different phenotypes. It remains possible that p.Arg423His has gain of function effects that were not detected in our experiments. For instance, mutations in conserved S4 arginine residues of the Nav1.4 voltage-gated Na^+ channel associated with hypokalemic periodic paralysis generate aberrant leak currents through putative 'gating pores' in the voltage sensor domain that may contribute to the disease phenotype (Sokolov, et al., 2007; Struyk, et al., 2008).

This study extends the mutational and phenotypic spectrum of SCA13 and confirms the importance of voltage-gated potassium channels in cerebellar development and function. The p.Arg420His, p.Arg423His, and the previously identified p.Phe448Leu changes meet accepted criteria for mutations by segregation in pedigrees, recurrence, change of evolutionarily conserved amino acids, lack in control samples, and abnormal function *in vitro*. The p.Arg366His variant meets some of these criteria, but has not been observed a second time. Therefore, despite the significant effects on channel function, its status as a disease-causing mutation should be interpreted with caution. The previously reported p.Phe448Leu mutation (Herman-Bert, et al., 2000;) remains the only KCNC3 mutations with a cellular gain-of-function phenotype. Our study suggests that SCA13 will likely be a relatively rare cause of autosomal dominant ataxia.

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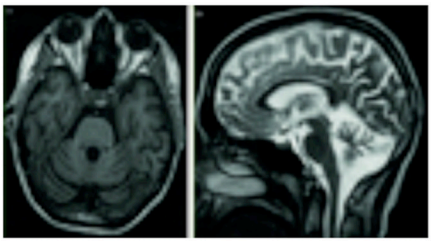

Figure 1.
Cerebellar atrophy in individual MR 3-3 with an p.Arg420His mutation. A) T1-weighted axial image of posterior fossa. B) T2-weighted midsaggital image, note lack of brainstem atrophy.

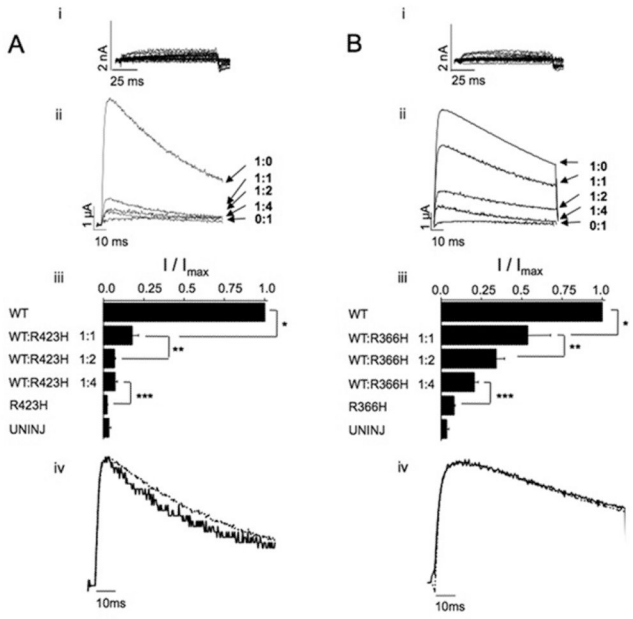

Figure 2.

Dominant negative effects of *KCNC3* mutations. Ai and Bi: p.Arg423His (A) or p.Arg366His (B) was expressed in Xenopus oocytes for functional analysis. Currents were evoked by stepping from −90 mV to voltages ranging from −80 to +70 mV in 10 mV increments. Aii and Bii: Shown are representative current traces evoked by stepping from −90 mV to +60 mV for wild-type Kv3.3 expressed alone or in the presence of p.Arg423His or p.Arg366His at the indicated ratios. Aiii and Biii: Normalized peak current amplitudes measured at +60 mV are shown for wild-type expressed alone or with p.Arg423His or p.Arg366His at the indicated ratios. For comparison, current amplitude values for p.Arg423His or p.Arg366His expressed alone or for uninjected oocytes (Uninj) are also provided. Values are provided as SEM ± mean, n=4–10. Statistical significance was tested by one-way analysis of variance (ANOVA). P< 0.05: *, significantly different from 1:0; **, significantly different from 1:1, ***, significantly different from 1:4. Aiv and Biv: Representative current traces recorded at +60 mV of wild type expressed alone (dotted traces) and co-expression of p.Arg423His (Aiv) or p.Arg366His (Biv) at a 1:1 ratio with wild type (solid traces) have been scaled and overlaid for comparison.

Table 1

Primer Table

Exon	Primer Sequence 5' to 3'	Primer name	Amplicon size	Annealing Temp
1	TAG GTG AGG GCG TGC GAT CTG TT GCC CGC GAA AGG ACG AGA C	1A-F 1A-R	584	65°C
1	CTC CCA CCC AAT CCC GTC GGT C GCG ATG CTG CCG GTA GGT CAT CC	1B-F 1B-R	658	64°C
1	TTC GCG TAC GTG CTC AAC TAC TA TGG GGA AGA GGC TTC TAG GAG	1C-F 1C-R	583	64°C
2	CTG GAA GGG TCT TCT GGA TGT TA TTT TCT CCC TCA CCT CTT CGA C	2A-F 2A-R	1330	60°C
2	CGC CAC CAT GAT TTA CTA CGC TTT TCT CCC TCA CCT CTT CGA	2B-F* 2B-R*	NA	NA
3/4	ATC TTG CCC CAC CGC GTG TTC A CGG TCA GTG GGG GCT GCA TGT TC	3-4-F 3-4-R	779	64°C

* Nested (Exon 2) sequencing primers

Table 2

Demographic, clinical and mutation findings of selected index patients and their relatives

Family ID	Index Patient	Age at Onset	Age at Exam	Sign at Onset	Cerebellar Signs	LL Pyramidal Signs	Mental Retardation	Imaging	Seizures	Progression	Coding Position	Amino acid Change
TUB 164-3	Yes	>25	43	Ataxic gait	Yes	No	No	CCA	No	Yes	c.1259G>A	p.Arg420His
TUB 3-1	Yes	42	66	Ataxic gait and seizures	Yes	Yes	No	CCA	No	Yes	c.1259G>A	p.Arg420His
TUB 3-3	No	28	31	Ataxic gait	Yes	Yes	No	CCA	Yes	Yes	c.1259G>A	p.Arg420His
BOR 542-3	Yes	51	57	Ataxic gait	Yes	Yes	No	Vermian atrophy WM changes	No	NA	c.1259G>A	p.Arg420His
SAL 289-6	Yes	Infancy	47	Motor delay with ataxic gait, falls, dysarthria, writing difficulties	Yes	No	No	NA	No	Stable	c.1268G>A	p.Arg423His
AAD-SAL 289-12	No	2	17	Motor delay with ataxic gait and falls	Yes	No	No	Normal (age 3)	No	Stable	c.1268G>A	p.Arg423His
STR 198-14	Yes	Infancy	3	Ataxia	Yes	No	No	NA	No	NA	c.1268G>A	p.Arg423His
STR 198-10	No	3	33	Motor delay with ataxic gait, dysarthria	Yes	Yes	No	NA	No	Stable	c.1268G>A	p.Arg423His

Nucleotide numbering for cDNA –based nomenclature uses 1+ as to the A of the ATG translation initiation codon in GenBank RefSeq NM_004977.2 according to journal guidelines. (www.hgvs.org/mutnomen). The initiation codon is codon 1. RefSeq NG_008134.1, NP_004968.2.

Table 3

Other Sequence Variants Found in Index Cases And Controls

Location	Type	Flanking Sequence	Genomic Position	Coding Position	Amino Acid Change	% Cases	% Controls
5' UTR	Non-coding	GGTCTC**T**/**C**CTCTAT	g.4938T>C			0.19	0.00
5' UTR	Non-coding	TCCCCT**C**/**T**CTCCCT	g.4893C>T			0.19	0.00
5' UTR	Non-coding	CGTCTT**T**/**G**AAATAG	g.4870T>G			0.38	0.00
Exon 1	Synonymous	AGTCTG**C**/**T**GTCTCG	g.5313C>T	c.18C>T	p.=	0.19	0.00
Exon 1	Coding	AAC/**GCCGCCAAC**/GCC	g.5932_5940dup	c.637_645dup	p.Asn215_Ala217dup	0.00	0.35
Exon 2	Synonymous	CACCAA**C**/**T**GAGTTC	g.10763C>T	c.1338C>T	p.=	0.19	0.00
Exon 2	Coding	TCATGC**G**/**A**CATCAC	g.10522G>A	c.1097G>A	p.Arg366His	0.19	0.00
Exon 2	Synonymous	CTACGT**G**/**A**GAGGGG	g.10478G>A	c.1268G>A	p.=	0.19	0.00
Intron 3	Non-coding	GCCCCC**C**/**A**CTACTG	g.13799C>A	c.2170+14C>A		0.58	0.70
Intron 3	Non-coding	AGGAGA**G**/**A**GGGGAT	g.13855G>A	c.2170+70G>A		0.19	0.00
Intron 3	Non-coding	AGAGGG**G**/**A**GATGGG	g.13858G>A	c.2170+73G>A		3.65	0.35
Intron 3	Non-coding	CCCAAC**T**/**C**CTCTGG*	g.14003T>C	c.2171-26T>C		0.19	0.35

RefSeq NG_008134.1, NM_004977.2, NP_004968.2, % reflects allele frequency

*The starting and end position of the mutation on the gene (KCNC3_v001) lie 26 positions upstream of the 5' splice site of exon 4.