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Mutations of the *puratrophin-1* (*PLEKHG4*) gene on chromosome 16q22.1 are not a common genetic cause of cerebellar ataxia in a European population

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Abstract Autosomal dominant cerebellar ataxia (ADCA) is a genetically heterogeneous group of neurodegenerative disorders with overlapping clinical presentation. Recently, a single nucleotide substitution in the 5'-untranslated region (UTR) of the *puratrophin-1* (*PLEKHG4*) gene on chromosome 16q22.1 has been shown to be associated with ADCA in 52 unrelated Japanese families. As this mutation has so far not been investigated in other populations, we have screened 537 European patients with a clinical diagnosis of cerebellar ataxia for this specific nucleotide substitution. The mutation was not identified in our cohort. In addition, we screened the complete 5'-UTR as well as the entire coding region of this gene in 120 patients for variations that might account for their clinical symptoms. Several new rare variations were found. For none of the variations could an obvious pathogenetic relevance be postulated at this point, albeit some findings should be followed up in additional populations and by functional assays. We conclude that mutations of the *puratrophin-1* gene are not a common cause of hereditary ataxia in our Caucasian population.

Keywords Ataxia · ADCA · Puratrophin-1 · Mutation · SCA4

Introduction

Hereditary cerebellar ataxias represent a heterogeneous group of disorders linked to many genes or genomic loci. Numerous genetic loci have been mapped for the subgroup of autosomal dominant cerebellar ataxias (ADCA), with SCA28 being the most recently published

subtype (Cagnoli et al. 2006). The gene responsible has been identified for only some of these loci: SCA-1, -2, -3 (Machado Joseph disease, MJD), -6, -7, -8, -10, -12, -14, -17, -27, and DRPLA. Many of the other SCA types have been described in only one or a few families. A considerable number of ADCA loci may still remain to be discovered (reviewed by Schols et al. 2004).

In several SCA subtypes (SCA-1, -2, -3, -6, -7, -17, DRPLA) a pathological expansion of a polymorphic coding CAG microsatellite leading to an expanded polyglutamine stretch within the respective gene product has been identified as the common mutation mechanism (reviewed by Koeppen 2005). Similarly, in other SCA types (SCA-8, -10, -12) expanded microsatellites within non-coding gene regions with potential impact on gene expression have been associated with the corresponding disease. So far, SCA14 and SCA27 are the only SCA subtypes for which single-nucleotide substitutions have been exclusively identified as the underlying mutation mechanism.

Although many hereditary forms of ataxia occur in both Caucasian and Japanese populations, the frequency of each respective subtype is often quite different. For example, DRPLA is quite a common cause of ataxia-related phenotypes in Japan, while it is extremely rare in Europe, where only a few families have been described (reviewed by Schols et al. 2004). Recently, a single-nucleotide substitution (C.−16C>T; all nucleotide and amino acid positions numbered from the beginning of the ATG start codon) in the 5'-untranslated region (UTR) of the *puratrophin-1* (*PLEKHG4*, NCBI gene ID 25894, OMIM*609526) gene on chromosome 16q22.1 has been shown to be associated with ADCA in 52 unrelated Japanese families (Ishikawa et al. 2005). All patients shared the same substitution and no other mutation was found in this gene or another gene at that specific genomic region. The mutation leads to decreased *puratrophin-1* mRNA expression and is also specifically associated with the occurrence of puratrophin-1 protein aggregates in the cytoplasm of Purkinje cells, the pathological impact of which remains elusive.

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Although the exact mechanisms by which *puratrophin-1*-associated pathology is mediated are still unknown, it is essential to clarify whether the c.-16C>T change or other mutations within this gene are associated with ADCA cases in other ethnic populations. We have therefore screened a large cohort of Caucasian patients suffering from cerebellar ataxia for variations within the *puratrophin-1* gene, including the c.-16C>T mutation.

Methods

A total of 537 unrelated patients were included in this study. All patients were referred by their neurologist with a clinical diagnosis of unexplained cerebellar ataxia suggestive of ADCA. Of these 537 patients, 129 had a family history consistent with a dominant form of hereditary ataxia, 269 were sporadic cases, and for the remaining 139, no sufficient family history was available. Nearly all patients presented with adult onset phenotypes. The vast majority of patients were of German origin and only a few ($n < 20$) originated from other European countries. None was from Japan or other non-European countries. The major European SCA mutations [SCA-1, -2, -3 (MJD), -6 and -17] were excluded by molecular genetic methods in each patient. In cases with ambiguous family history and a clinical picture potentially resembling an atypical presentation of Friedreich ataxia—the most common recessive form of ataxia in Europe—the latter was also been ruled out by molecular genetics ($n = 57$). Family-based linkage studies had not been performed in any of the patients previously. All patients gave informed consent for molecular genetic diagnostics.

In order to screen for the c.-16C>T mutation in the *puratrophin-1* gene, we established a PCR protocol modified from that used by Ishikawa et al. (2005). Primers 4F and 4R (Table 1) were used to generate a 253 bp PCR product from genomic DNA. All PCR reactions were run with 1× PCR buffer (Qiagen, Hilden, Germany), 3.0 mmol MgCl₂, 0.2 mmol of each deoxyribonucleoside triphosphate, 4 pmol of F and R primer, 0.25 U of HotStar *Taq* polymerase (Qiagen) and 50 ng DNA. PCR products were digested enzymatically with 4 U *XagI* (Fermentas, St. Leon-Rot, Germany), an isoschizomer of *EcoNI*, following the manufacturer's instructions. Restriction resulted in two fragments (158+95 bp) in the case of the wildtype c.-16C allele and in one undigested fragment (253 bp) in cases harbouring the mutated c.-16T allele. Fragments were separated on 2.5% agarose gels stained with ethidium bromide. Results were confirmed in four patients by direct sequencing using the primers mentioned above. Sequencing reactions were carried out using a DYE-namic ET Terminator kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's instructions. Analyses were run on a capillary sequencer (MegaBACE 1000, Amersham Biosciences).

Mutation screening of the entire *puratrophin-1* gene and its 5' UTR was performed by PCR and subsequent polyacrylamide gel electrophoresis under single strand conformation polymorphism (SSCP) conditions. DNA from 120 patients with a positive family history (consistent with an autosomal dominant mode of inheritance) for cerebellar ataxia was included in these analyses. PCR was performed as described above and products were radioactively labelled with [α -³²P]dCTP. In order to increase SSCP sensitivity, two different gel conditions with different proportions of (bis-) acrylamide, glycerol and different buffer concentrations were run for each PCR system. For the same reason large PCR products (>300 bp) were digested with an appropriate restriction enzyme (Table 1) before electrophoresis. Samples showing band shifts in SSCP analyses were analysed by direct sequencing using the same primers as for SSCP and under the conditions described above.

Statistical analyses of allele and genotype frequencies of selected single nucleotide polymorphisms (SNPs) were performed using contingency tables and Chi-square statistics and Fisher's exact test when appropriate. A well established cohort of healthy German blood donors was used as controls (Godde et al. 2005).

Results

c.-16C>T mutation screening

All 537 patients were typed homozygous for the wildtype c.-16C allele. The mutated c.-16T allele was not observed at all in our cohort.

SSCP mutation screening

All SNPs that are located within our PCR systems and that are listed as validated in the NCBI database (<http://www.ncbi.nlm.nih.gov/>) were identified in our analyses: rs11860295, rs8044843, rs3868142, rs17680862 (all coding, non-synonymous), rs785029, rs11556908 (coding, synonymous) and rs7200919 (intronic). All but the intronic SNP (rs7200919) were rare (frequency of the minor allele < 0.1). For two of the SNPs [rs3868142 and rs7200919, which have also been analysed for the HapMap project (<http://www.hapmap.org/>)], allele and genotype frequencies were compared between our patients ($n = 120$) and a healthy control group ($n = 180$). No statistically significant differences in allele and genotype distribution were observed, and frequencies were virtually identical with those from the HapMap database (data not shown).

In addition to the known polymorphisms, some novel nucleotide exchanges were found. One patient carried an intronic single nucleotide substitution in heterozygous state 20 bp upstream of exon 2 [c.-219-20A>G; all exons are numbered according to the data from the

Table 1 Primer sequences and restriction enzymes used for polymerase chain reaction (PCR) and single strand conformation polymorphism (SSCP) analyses

PCR system	Exon ^a	Forward (F) and reverse (R) primer	Restriction enzyme
1	1 (5'UTR)	1F:5'-TAGGGATGGAGCAGGACAA-3' 1R:5'-GGGATTACAACAGGCACAGT-3'	None
2	2 (5'UTR)	2F:5'-CCCAGTCCTCACGGCAAC-3' 2R:5'-GCAGGAGGCTGGTGTCTGA-3'	None
3	3 (5'UTR)	3F:5'-CGTGAGGACGCTGAGGGT-3' 3R:5'-CGCGCTGAAGACAGTCGA	None
4	3	4F:5'-GCCTGAATTGCGTTCCTG-3' 4R:5'-GAAACTGCACACAGCAAATCT-3'	<i>XapI</i> (<i>EcoNI</i>) ^b
5	3	5F:5'-ATTCCCTTCCCAGACTTCCCTA-3' 5R:5'-AAAGGCCCCCTGGAGAAATGG-3'	<i>PstI</i>
6	4 + 5	6F:5'-TCTCTGGATAGGTGGTCCA-3' 6R:5'-CCTGACTCCCAGTCCTCA-3'	<i>AvaI</i>
7	6	7F:5'-CTGAGCCAGCTGTGAGGACT-3' 7R:5'-TGAGGGGAGCTACAATCCTG-3'	None
8	7 + 8	8F:5'-AGAGTTGGGGATAGTGCTGA-3' 8R:5'-CTATATGACTGCCCCATGC-3'	<i>PstI</i>
9	9 + 10	9F:5'-GAAGCCCAGGACCAACACT-3' 9R:5'-CCAGATGAGTCCTGGCATAGA-3'	<i>PvuII</i>
10	11 + 12	10F:5'-AAGGCAGGGGGTCTAAGATG-3' 10R:5'-CCTAGTTCCTGGCATTGCAT-3'	<i>CfrI</i>
11	13	11F:5'-TATCTGAGTCTGGGGGCTAGA-3' 11R:5'-TCATGGATCACTTGGAGCTG-3'	None
12	14	12F:5'-GGCAGGATCTTCTCTAATGC-3' 12R:5'-GGACCCAAGACATCTCCT-3'	<i>CfrI</i>
13	15	13F:5'-GGGAGGGCAGTAAAGAGAG-3' 13R:5'-TGTGGCAGAACGAGCTGATA-3'	<i>XmnI</i>
14	16 + 17	14F:5'-TAGCCTGTGCCAGCCTAG-3' 14R:5'-CCTTCTTCTCACACCCACTG-3'	<i>XcmI</i>
15	18 + 19	15F:5'-GCAGTGGGTGTGAAGAAGAAG-3' 15R:5'-AGCTCTCCAGTGAGGGGAGT-3'	<i>EcoO109I</i>
16	20	16F:5'-GGGTGGTAGAGGCCCAAG-3' 16R:5'-CCCAGATCCCAAGGAAAGTG-3'	None
17	21	17F:5'-CGCACTTTCCTTGGGACTCT-3' 17R:5'-GACTGGGTGAACTGTCAGCA-3'	<i>RsaI</i>
18	22 + 23	18F:5'-GCCAGCCCTCTTTGAGTATG-3' 18R:5'-TGGTCACAGCAGATCCAGAG-3'	<i>HinfI</i>

^aExons are numbered according to the data from the UCSC database (comprising also untranslated exons 1 and 2 which were not included by Ishikawa et al. (2005))

^bRestriction enzymes used only for specific c.-16C>T mutation screening, not for single strand conformation polymorphism (SSCP) analyses

UCSC database (<http://www.genome.cse.ucsc.edu/>). This numbering additionally comprises untranslated exons 1 and 2, which were not included by Ishikawa et al. (2005)].

An interesting finding concerns a heterozygous substitution in exon 5 (c.601C>T; p.201R>W) in one single patient, which resulted in the loss of an *AvaI* recognition site (C/YCGRG) as demonstrated by SSCP and direct sequencing. We searched for this change in a control cohort ($n=180$) by PCR and *AvaI* (New England Biolabs, Frankfurt, Germany) restriction and detected it in 1 out of the 360 chromosomes (female, aged 43), as confirmed by direct sequencing of the respective sample.

Two patients carried a nucleotide substitution in exon 6 (c.764G>C) in heterozygous state, which leads to a change of the respective amino acid (p.255R>P). Again, 180 healthy controls were screened for this variation by PCR and *XapI* (Fermentas) restriction, but the c.764G>C nucleotide substitution was not found. Subsequently, we screened two further independent control cohorts from northern ($n=90$) and western ($n=90$) Germany. By this means the substitution was

found in two control chromosomes (samples from a male aged 45, and from a female aged 65).

Samples from five patients showed a heterozygous change (c.1707T>G) in exon 14 that does not lead to an amino acid change (p.569A>A). Two patients carried a heterozygous G>A change in intron 4 (c.595+37G>A). One patient showed a heterozygous change in intron 5 (c.719+36C>T) that was also seen in a control sample.

Discussion

Autosomal dominant cerebellar ataxias are a group of disorders characterised by overlapping phenotypes that are often hard to distinguish by exclusively clinical parameters. Therapies for most ataxias are still purely symptomatic, and prophylactic strategies for persons at risk for hereditary ataxia are completely lacking to date. The precise molecular genetic determination of ADCA subtypes is essential in the light of upcoming therapeutic approaches emerging from the comparatively exorbitant amount of research performed in this area.

Although the c.-16C>T mutation in the *puratrophin-1* gene is very likely the result of an ancient founder effect and may possibly be restricted only to families of Japanese origin, it is essential to know whether this mutation is also the cause of certain ADCA subgroups in other ethnic populations. We therefore screened a large European ataxia population for the c.-16C>T mutation in the *puratrophin-1* gene. Since we were unable to detect the c.-16C>T mutation in our cohort, we conclude that this specific change is not a common cause of hereditary ataxia in Caucasian populations.

To date, single-nucleotide mutations are a rare cause of ADCA. Yet, different mutations within one gene can, by all means, be responsible for ADCA in various ethnic populations, including Japanese and Caucasians. This has recently been shown for SCA14, which is also caused (among other mutations, e.g. small deletions) by single-nucleotide substitutions (Yabe et al. 2003; van de Warrenburg et al. 2003). Therefore we screened the rest of the 5'-UTR and the entire coding sequence of the *puratrophin-1* gene for variations that might be pathogenic for ADCA. The fact that all the validated SNPs listed in the NCBI database were detected by our SSCP screening indicates that the sensitivity of our approach is probably close to 100%. This is in agreement with data from studies that have thoroughly investigated the efficiency of the SSCP method (Jaekel et al. 1998).

Beside these known SNPs, several new, mostly infrequent, nucleotide exchanges were found. For those changes which are intronic (C.-219-20A>G; c.595+37G>A; c.719+36C>T) or do not lead to a change in the amino acid sequence (c.1707T>G, p.569A>A), functional relevance is unlikely. On the other hand, as the pathogenesis of the c.-16C>T mutation is mediated via downregulation of mRNA expression, a pathogenetic relevance of these changes cannot be ruled out completely. Further studies in different populations and on mRNA levels will be useful to evaluate this issue. Unfortunately, no RNA or tissue samples were available from these patients.

Even more difficult is the evaluation of the two new non-synonymous exchanges we detected. For both the c.601C>T (p.201R>W) and the c.764G>C (p.255R>P) substitutions the pathogenetic relevance cannot be clarified definitively at this point. According to the InterPro database of the European Bioinformatics Institute (EBI; [http://www.ebi.ac.uk/]) amino acids p.201R and p.255R are both located within the cellular retinaldehyde-binding/triple function, c-terminal domain (*CRAL_TRIO_C*, amino acids 183-341) of the *puratrophin-1* protein. As this domain is found in proteins that may be functional components of the visual cycle, any impact of such variations on *puratrophin-1* related ataxia is not obvious. Neither are the nucleotide substitutions in the immediate vicinity of any known polymorphism. Therefore, one cannot deduce any potential functional relevance from their location. The fact that both the c.764G>C and the

c.601C>T exchanges were also seen in one (or two, respectively) control individuals in each case may indicate that they are both rare physiological variants. On the other hand, the respective control individuals are younger (43, 45 and 65 years, respectively) than the highest age of onset mentioned in the literature (72 years; Nagaoka et al. 2000). Thus, in theory they might still develop symptoms of ataxia in later life (no family history was available for control individuals). However, altogether we found no change on the DNA level for which an obvious pathogenetic relevance can be postulated at this time.

A specific subtype of ADCA, SCA4, has also been mapped to chromosome 16q22.1 in European families (Flanigan et al. 1996; Hellenbroich et al. 2003). It is, however, still unclear whether the Japanese form of 16q22.1-linked ataxia and European SCA4 have the same genetic background (i.e. whether they are allelic or not), as the 'Caucasian' *SCA4* gene has still not been identified. Hellenbroich et al. (2005) have screened the coding sequences of 34 genes within the critical genomic region, including the *puratrophin-1* gene, in a German SCA4 family. They also found variations within the *puratrophin-1* gene that were, however, not further specified and did not segregate with the disease. Thus, our study supports their findings that coding *puratrophin-1* variations are not a common hereditary cause for ataxia in Europe. As neither study excludes the possibility that mutations in other regulatory regions of the *puratrophin-1* gene might be causal for ADCA in Caucasian populations, further studies with regard to altered *puratrophin-1* gene expression are required. Such investigations may constitute a challenging undertaking as extensive regions of non-coding, regulatory sequences in *cis* and in *trans* will have to be included.

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