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Two Novel Homozygous SACS Mutations in Unrelated Patients Including the First Reported Case of Paternal UPD as an Etiologic Cause of ARSACS

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Abstract Autosomal recessive spastic ataxia of Charlevoix–Saguenay, more commonly known as ARSACS, is an early-onset cerebellar ataxia with spasticity, amyotrophy, nystagmus, dysarthria, and peripheral neuropathy. SACS is the only gene known to be associated with the ARSACS phenotype. To date, 55 mutations have been reported; of these, only five in Italian patients. We *found* two novel homozygous nonsense mutations in the giant exon of SACS gene in two unrelated patients with classical *ARSACS* phenotype. Characterization of the homozygous nature of the mutations through genotyping of the parents, quantitative DNA analysis and indirect STS studies permitted us to confirm in one of the cases that uniparental isodisomy of the paternal chromosome 13 carrying the mutated SACS gene played an etiologic role in the disease.

Keywords ARSACS · SACS gene · Mutations · Chromosome 13 · UPD · Isodisomy

Abbreviations

STS	Sequence-tagged sites
UPD	Uniparental disomy
qPCR	Real-time quantitative PCR

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Introduction

Autosomal recessive spastic ataxia of Charlevoix–Saguenay (ARSACS; MIM 270550) phenotype is due to the alteration of the activity of sacsin (MIM 604490), a protein which function is not yet fully known. The existence of a carboxy terminus domain which harbors a "DnaJ" (aa 4322–4370) motif suggests a potential interaction with members of the HSP70 family of heat shock proteins stimulating their ATPase activity (Parfitt et al. 2009; Engert et al. 2000). At the C terminus of the protein, directly after the DnaJ domain (aa 4451–4567), is located a HEPN domain which is supposed to be involved in nucleotide binding (Parfitt et al. 2009; Engert et al. 2000). A UbL domain (aa 1–124) that can interact with the proteasome was also recently identified at the N terminus of sacsin. Analogous regions are present in proteins involved with protein folding, suggesting a similar function for sacsin (Parfitt et al. 2009). Sacsin is encoded by the SACS gene which is located on chromosome 13q12.12 and comprises nine coding exons consisting of 13,737 base pairs; the last coding exon is a "giant" and consists of 12,794 bp (this exon is the largest identified in vertebrate organisms) (Engert et al. 2000).

SACS is to date the only gene known to be associated with the ARSACS phenotype: causal mutations in the SACS gene include two common mutations responsible for 95% of the cases in individuals from north-eastern Quebec (Mercier et al. 2001), but this founder effect is absent in other regions, and prevalence of SACS mutations associated with ARSACS phenotype outside of Canada are still uncertain.

To date, a total of 55 different disease-causing SACS mutations were described worldwide; of these, only five were reported in Italian patients (Criscuolo et al. 2004;

Grieco et al. 2004; Terracciano et al. 2009). Almost all mutations outside the Charlevoix–Saguenay region are limited to the single families. Very frequently, the mutations are present as homozygous (43%) and, excluding consanguinity, these observations arise some questions concerning the real frequency of ARSACS mutations among the populations (Human Gene Mutation database, http://www.hgmd.cf.ac.uk).

Patients and Methods

Patients

The first patient (patient 1) is a 26-year-old male presenting moderate ataxia and mild dysarthria. The disease became evident at the age of 2 years with deambulation difficulties. Up to 25 years of age, the difficulties were minor, the patient reports he played soccer, but in the last 2 years the ataxia worsened to the point when he can no longer walk without support. The father of the patient is from the USA while the mother is of Italian origin.

The second patient (patient 2) is a 35-year-old female presenting severe ataxia and moderate dysarthria. The disease was first noticed in infancy with bipodal deambulation at the age of 26 months. The disease remained stable roughly to 20 years of age; afterwards, there was a marked worsening of the ataxia. Actually the patient walks with two supports and is wheelchair bound for most of the daily activities. Parents are not consanguineous and are both of Italian origin but from different regions.

Methods

Genomic DNA for the genetic analysis was extracted from peripheral venous blood samples by conventional methods (Quick-gDNA MiniPrep, ZymoResearch, USA). We performed molecular analysis of the SACS gene through polymerase chain reaction (PCR) followed by direct sequencing and qPCR. We analyzed also the DNA of available parents to study the segregation of the mutations.

Polymerase Chain Reaction In total, 43 original primer pairs were designed to cover the complete coding sequence as well as flanking intronic sequences of the SACS gene [NM_014363.4]. The regions close to the found mutations were sequenced with overlapping primer pairs to exclude the presence of possible polymorphisms which could impair correct primer annealing. Primers were designed by NCBI Primer Design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) avoiding low complexity regions and nucleotides involved in known polymorphisms. Primer sequences and reaction conditions are available on request.

Direct Sequencing Sequencing reactions were performed using BigDye Terminator v.3.1 Cycle Sequencing kit (Applied Biosystems, USA). Sequence products were purified through fine columns (Sephadex G50, GE Healthcare Life Science, UK) and resolved in an ABI PRISM® Sequencer 3130 (Applied Biosystems, USA).

Real-time quantitative PCR Real-time experiments were performed on four ARSACS gene fragments using SyBR Green chemistry on ABI PRISM® 7000 (Applied Biosystems, USA). For each assay, four normal DNA controls and three standard no-template controls were analyzed. Reactions were performed in a final volume of 25 µL containing 2× SYBR Green PCR Master Mix (Applied Biosystems, USA), 300 nM of each primer, and 5 to 40 ng genomic DNA, according to serial dilution protocol. FANCB [NM_152633.2] localized on chromosome X in region Xp22.31 was employed as reference gene. Each reaction was performed in triplicate, and each experiment was repeated two times.

Results were analyzed with $\Delta\Delta$Ct method. FANCB was employed as the reference gene; only values with standard deviation within the range of ±10% were considered valid. A standard curve for human FANCB was constructed using serial dilutions of this calibrator.

Microsatellites Analysis Haplotypes were constructed using ten microsatellite markers flanking the SACS gene on chromosome 13 spanning a region of 50 cM (D13S175, D13S1236, D13S232, D13S292, D13S787, D13S1243, D13S283, D13S1285, D13S221, and ATXN8OS/ATXN8). These markers were selected from the NCBI-UniSTS website (http://www.ncbi.nlm.nih.gov/unists). Following amplification by PCR, fluorescently labeled products were analyzed on an ABI Prism 3130 (Applied Biosystems, USA) by capillary electrophoresis. Peak analysis was performed by Genemapper Analysis Software (Applied Biosystems, USA).

Results

We performed the molecular genetic analysis by direct sequencing of the SACS gene in two Italian patients with a clinical suspect of ARSACS based on their symptoms and clinical history.

In patient 1, direct sequencing allowed the identification of a homozygous nonsense mutation in exon 10 of the SACS gene [c.13132C>T]. The mutation leads to the formation of a premature stop at codon 4378 [p.Arg4378X].

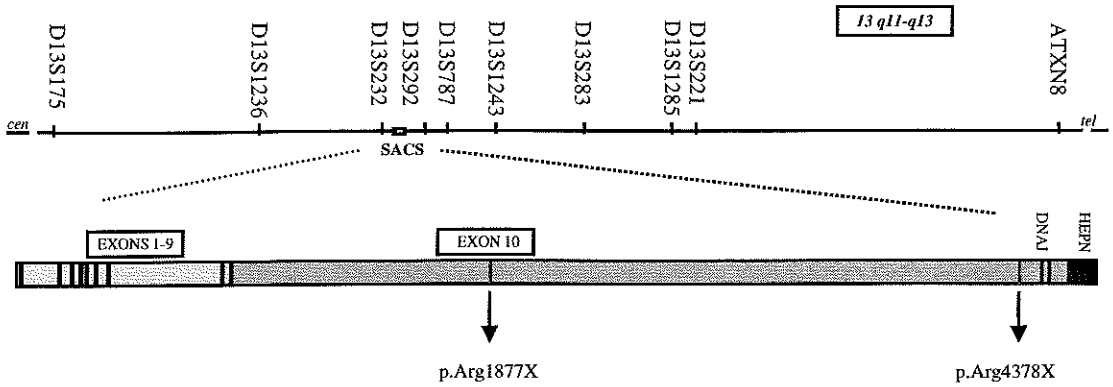

Fig. 1 Localization of the STS used to perform the haplotype analysis on chromosome 13 q11–q13 and schematic representation of SACS gene exons and known domains are represented. *Arrows* indicate the location of both mutations found in our patients

This mutation was present in heterozygosis in the healthy father, but was absent in the mother. Primer annealing sites did not contain known polymorphisms and no variations were identified by the sequencing of these locations with overlapping primers. qPCR performed in the regions −1,186 bp upstream and +66 bp downstream of the mutation excluded the presence of a deletion in the patient and a long range PCR between the qPCR-analyzed regions confirmed the homozygous nature of the mutation.

In patient 2, direct sequencing allowed the identification of a different homozygous nonsense mutation in exon 10 of the SACS gene [c.5629C>T]. The mutation leads to the formation of a premature stop at codon 1877 [p.Arg1877X]. The mutation was present in heterozygosis in the proband's healthy father and brother. The deceased mother's DNA was not available for the analysis. Also in this case primer annealing sites did not contain known polymorphisms and no variations were identified by the sequencing of these locations with overlapping primers. qPCR performed in the regions −137 bp upstream and +88 bp downstream of the mutation excluded the presence of a deletion in the patient and a long range PCR between the qPCR analyzed regions confirmed the homozygous nature of the mutation also in this case.

Having excluded the presence of gross or small deletions in SACS gene in both patients, we performed haplotype analysis in both families to identify the mutation containing alleles and to shed light on the mechanism underlying the observed homozygosity. Ten microsatellites spanning a region of 50 cM located on chromosome 13 were studied (Fig. 1).

In the case of patient 1, STS analysis was performed considering both his parents (Fig. 2a). All the tested STS in the patient resulted homozygous for one of the paternal alleles; more precisely, three microsatellites were not informative (D13S175, D13S292, D13S787), while seven microsatellites (D13S1236, D13S232, D13S1243, D13S283, D13S1285, 13S221 ATXN8OS/ATXN8) were consistent with paternal uniparental isodisomy. The inherited paternal allele was the one carrying the SACS mutation.

In the case of patient 2, the STS analysis was performed considering only the patient's father. Since the mother of patient 2 died several years ago, we extended the STS test also to the proband's brother, carrier of the mutation

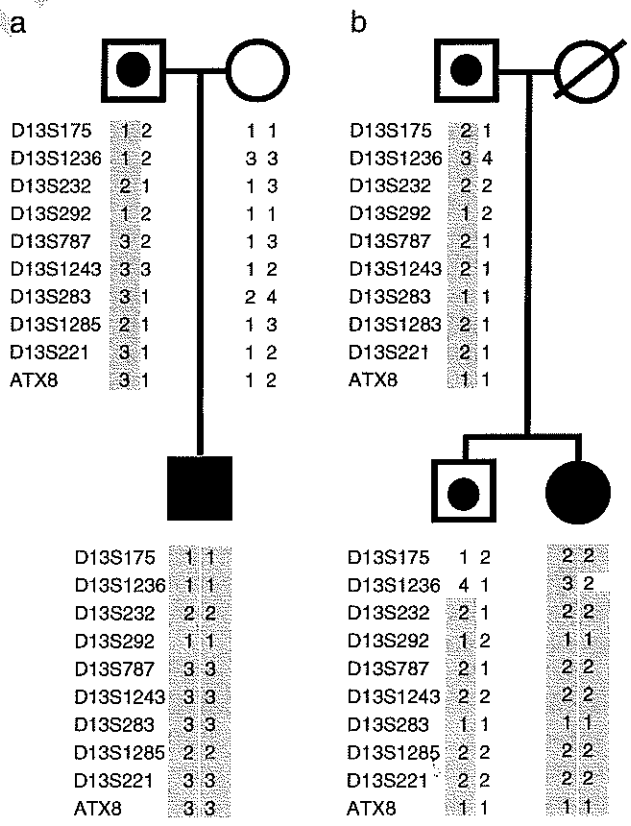

Fig. 2 Pedigree with haplotype analysis results are shown for patient 1 (**a**) and for patient 2 (**b**). The putative paternal alleles which segregate with the pathogenetic mutation are painted *grey*

(Fig. 2b). Nine of the tested STS were homozygous and compatible with a paternal allele while D13S1236 showed a second allele shrunken by a single repeat in comparison to the one present in the father.

Discussion

In this study, we identified two yet undescribed homozygous nonsense mutations in the *SACS* gene in two ARSACS patients. Although never previously reported, the nonsense mutations here presented, p.Arg1877X and p.Arg4378X, truncate prematurely the protein severely impacting its structure. In both patients the HEPN domain and the DnaJ motif together with the C terminus are lost. Likely these truncated proteins are unable to interact normally with other proteins belonging to their functional pathway and their functions appear to be impaired.

We attempted to study the segregation of these mutations in the parents of the probands: in both cases the fathers resulted to be heterozygous carriers of the mutation, the mother of the patient 1 resulted non carrier while no information was available for the mother of patient 2.

qPCR allowed us to exclude the presence of deletions in the region of the respective mutations; in fact, dosage determination confirmed the presence of two copies of the SACS gene.

To ensure the absence of little deletions or single-nucleotide variations that could impair the correct attachment of the original primers used to detect the mutation, we performed a long-range PCR with a primer pair located on the sides of the region of interest that were confirmed not to be deleted by qPCR. The regions encompassing PCR amplicons containing the mutation sites and both primers were sequenced with long range PCR. In both cases, the long-range PCR confirmed the homozygous status of the mutation.

STS analyses in the families were consistent with paternal uniparental isodisomy as the genetic base of the disease in the case of the patient carrying the homozygous p.Arg4378X mutation. In the patient with the p.Arg1877X mutation, UPD is one of the possible explanations of the disease considering a single-repeat shrink of the heterozygous allele. The other explanation would be the presence of an ancestral allele shared by the parents.

To the best of our knowledge, only few reports of paternal UPD 13 have been documented (Slater et al. 1995; Berend et al. 1999; Tsai et al. 2004). Furthermore, chromosome 13 is not subject to paternal or maternal imprinting (Slater et al. 1995) thus the pathogenic role of UPD could only be associated with recessive traits.

Both our homozygous mutations differ to classical Canadian mutations and were never previously reported. This fact raises questions regarding the real frequency of SACS mutations in the Italian population.

The presence of UPD as the etiologic cause of the disease alters the recurrence risk of ARSACS for the parents of the patient, reducing the risk way below the 25%, usually associated with autosomal recessive diseases.

In summary, this is the first time UPD has been reported as an etiologic base of ARSACS and a further example of UPD of chromosome 13. Moreover, doing a more general consideration, our report contributes to the idea that the study of DNA of both parents in cases of homozygosity of an autosomal recessive inherited mutation might help improve genetic counseling, resulting, in the case of UPD, in a decreased recurrence risk. This study underlines also the importance of a detailed analysis of the parents in case of a recessive disease where a homozygous mutation is found.

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Conflict of Interest The authors state no conflict of interest.

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