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SHORT COMMUNICATION

Clinical and molecular studies in two new cases of ARSACS

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Abstract

Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) is an early-onset neurodevelopmental disorder characterized by the association of spastic ataxia and sensorimotor neuropathy. Additional features include retinal changes and cognitive impairment. Today, next-generation sequencing (NGS) techniques are allowing the rapid identification of a growing number of missense variants, even in less typical forms of the disease, but the pathogenic significance of these changes is often difficult to establish on the basis of classic bioinformatics criteria and genotype/phenotype correlations. Herein, we describe two novel cases of missense mutations in *SACS*. The two individuals were identified during the genetic screening of a large cohort of patients with inherited ataxias. We discuss how protein studies and specialized ophthalmological investigations could represent useful pointers for the interpretation of genetic data. Combination of these tools with NGS for rapid genotyping might help to identify new true ARSACS cases.

Keywords ARSACS · Sacsin · Genotype-phenotype correlation · Mitochondrial network · Retinal myelinated fibers

Introduction

Autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) is characterized by cerebellar ataxia, pyramidal tract signs in lower limbs, and axonal-demyelinating sensorimotor peripheral neuropathy [1–4]. Signs of the disease usually appear before the age of 5 years, but occasionally in adulthood [5]. ARSACS is caused by bi-allelic mutations in *SACS*, and more than 200 pathogenic single nucleotide variants (SNVs), mostly nonsense ones, have been identified [3, 6], distributed throughout the gene. Atypical presentations are also reported [4], apparently unrelated to specific genotypes.

With the increasingly frequent adoption of large gene panels and exome sequencing in clinical investigations of different forms of inherited ataxia, spastic paraplegia, or both [7], the number of reported SNVs is growing, making them hard to sort into neutral, benign, and disease-associated types. The large size of several ataxia genes, particularly *SYNE* (causing SCAR8) and *SACS* (causing ARSACS), also favors the detection of large numbers of SNVs of uncertain significance. Furthermore, given that combining clinical observation with the use of bioinformatics tools offering accurate allele frequency filters seems insufficient to resolve this problem, the number of so-called variants of uncertain significance looks likely to increase. Current American College of Medical Genetics guidelines [8] encourage multidisciplinary collaborations to accelerate the interpretation of SNVs, and robust functional studies to further the process of disease-related classification (not always straightforward in rare inherited brain disorders).

In the present study, we integrated data on sacsin [9] with the findings of pontine hypointensities on brain MRI [10] and a thickened peripapillary retinal nerve fiber layer (pRNFL) appearance [3] on spectral domain optical coherence tomography (SD-OCT) examination (both frequent features of ARSACS). We also explored mitochondrial dynamics as a "diagnostic biomarker" of sacsin [11]. It was reasoned that these elements, together, might allow us to prioritize potentially pathogenic SNVs and improve disease definition [12].

We here present the multiple steps we used to assess pathogenicity in two index cases with a clinical diagnosis of spastic

Ivana Ricca and Federica Morani contributed equally to this work.

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ataxia and bi-allelic missense mutations in *SACS*, once bioinformatics analyses had defined their SNVs as "likely pathogenic."

Materials and methods

Complete standard ophthalmological evaluations were performed in P1 and P2. In addition, (SD-OCT) was performed, using the iVue system (Optovue Inc.) to assess the pRNFL, ganglion cells, and macular region. iVue uses a scanning laser diode with a wavelength of 840 ± 10 nm to image ocular microstructures. The protocol provided pRNFL measurements and a 6-mm-long cross-line scan furnished high-resolution images of the macular region and papillomacular area.

A validated multigene panel [13] was used to identify causative mutations [Galatolo D., in preparation] in 298 patients presenting inherited ataxia, alone or associated with spastic paraplegia. Gene variants were confirmed by direct Sanger sequencing [13].

Mitochondrial networks in primary skin fibroblasts from P1, P2, and five age-/sex-matched healthy controls were assessed using mouse anti-porin antibodies (MitoSciences Inc.) [14]. Data analysis and evaluation of different mitochondrial morphology phenotypes were performed as described elsewhere [15]. Rabbit anti-sacsin (EMD Millipore), mouse anti-SDH70 (MitoSciences Inc.), and rabbit anti-β-tubulin (Cell Signaling Technology Inc.) antibodies were used for Western blotting (WB).

Results

Among the 298 patients, we identified five index cases with bi-allelic loss-of-function mutations in *SACS* (i.e., small insertion/deletion mutations in two patients, splice site mutations in two, and a nonsense variant in one). All were considered straightforward cases of ARSACS. Ten patients were deemed unsuitable for further studies because they harbored gene variants with a

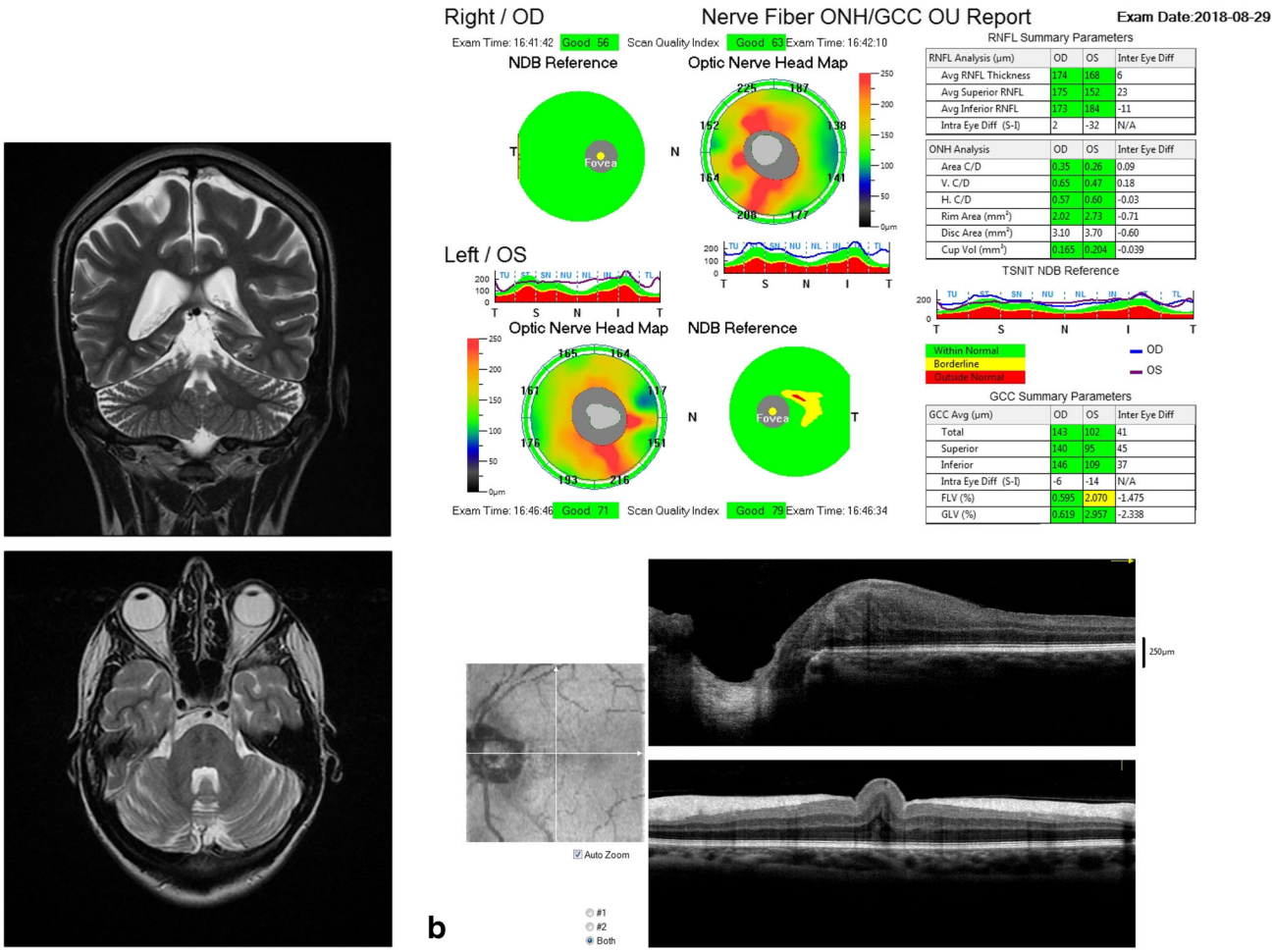

Fig. 1 **a** Brain MRI images showing mild cerebellar atrophy (above) and hypointense stripes in the pons (bottom) in patient P1. **b** In P1 and P1.2, SD-OCT scans (top images) show values indicating bilateral thickening of the peripapillary retinal nerve fiber layer (RNFL), in the upper and lower quadrants. In P1.2, spectral domain optical coherence tomography (SD-OCT) cross-line scans (6 mm long) of the papillomacular bundle (bottom images) show inner retinal thickening and the presence of a retinal fold in vertical scans

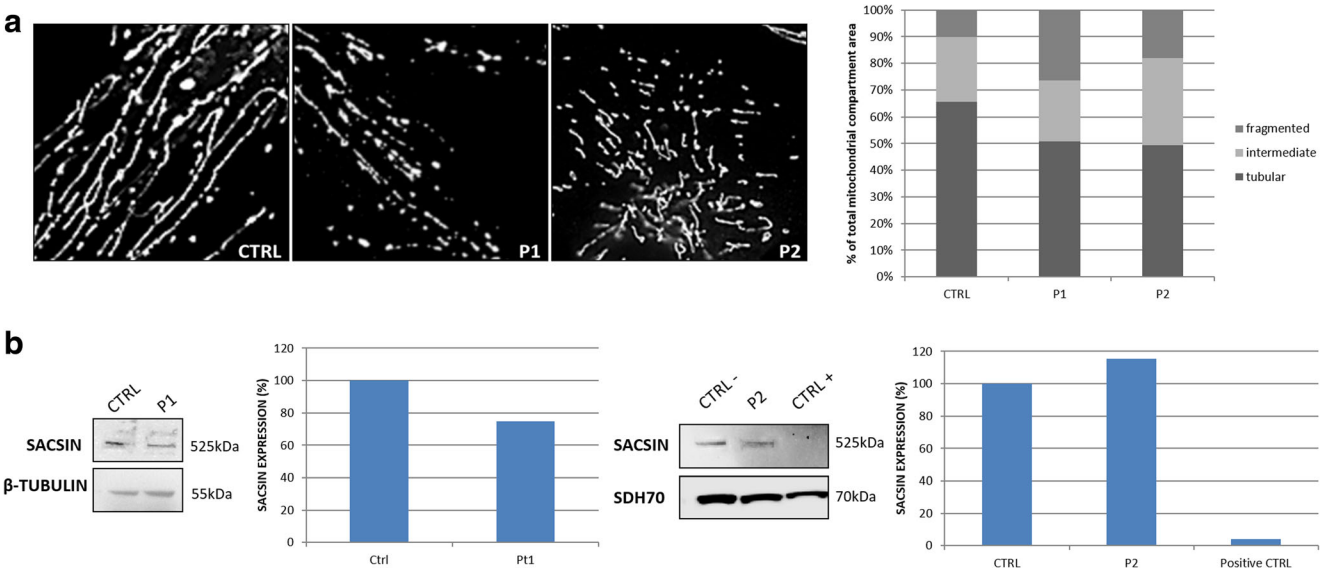

Fig. 2 **a** Analysis of the mitochondrial network morphology reveals fragmented mitochondria in P1 and intermediate mitochondria in P2 relative to a control (CTRL) (Zoom × 20, left panel). Bar graphs (right panel) show the distribution of the fibroblasts by mitochondrial morphology (tubular, intermediate, and fragmented). For each experiment, at least 20 cells derived from the patients and from five controls were used to calculate the relative percentages of the different mitochondrial groups. Data are presented as mean values ± SD of three independent experiments and were analyzed using Student's *t* test (significance $p < 0.05$). **b** Representative immunoblot analyses of fibroblast homogenates from controls (CTRL) and patients (P1 and P2) using a specific antibody against sacsin show reduction of sacsin expression in P1 but not in P2. β-Tubulin or SDH70 was used to verify equal loading. The histograms show the residual levels in patients versus CTRL whose values were arbitrarily set at 100. Data are representative of three measurements in each patient

minor allele frequency > 2% in a public database (gnomAD, http://gnomad.broadinstitute.org) [13]. Just two unrelated patients showed missense mutations of likely pathogenic significance and were therefore further investigated.

P1 is a 39-year-old woman affected by impaired gait and coordination since the age of 15 years. At the latest clinical examination, she showed moderate spastic-ataxic gait, mild dysarthria, mild diffuse leg weakness and dysmetria bilaterally, bilateral Babinski sign, *pes cavus*, and hammertoes. She also showed hypermetric saccades and lateral gaze nystagmus. On the Spastic Paraplegia Rating Scale and the Scale for the Assessment and Rating of Ataxia (SARA), she scored 16/52 and 7/40, respectively. She showed severe sensorimotor axonal and demyelinating polyneuropathy. Brain MRI showed mild cerebellar atrophy, prominent in the cerebellar vermis, and hypointense stripes in the pons (Fig. 1a). Spine MRI was normal. Her 38-year-old brother (case P1.2) has presented a similar disorder since the age of 16.

P2 is a 6-year-old girl affected by congenital ataxia, diagnosed at the age of 18 months. Her latest examination revealed severe ataxia, very slow ocular saccades, severe dysmetria, dysdiadochokinesia and rebound phenomenon, stiff legs, and bilateral *pes cavus*. Cognitive assessment with WISC-V was normal. MRI showed slight atrophy of the superior cerebellar vermis, corpus callosum thinning, and cervical spinal cord atrophy. The neurological deficits were stable over time. Although her movements appeared clumsy, she could walk unsupported, but not run. Neurophysiological examination was consistent with sensorimotor axonal polyneuropathy. SARA score was 7/30.

Ophthalmological, visual function and funduscopic examinations were normal in P1, her brother, and P2. Nystagmus was observed only in lateral gaze-holding positions. While SD-OCT was normal in P2, the test revealed pRNFL thickening, particularly at the level of the papillomacular bundle, with the presence of a retinal fold in this area, in P1, P1.2, and her brother. The macular area showed mild foveal distortion, but not foveal hypoplasia (Fig. 1b).

In P1, we detected the [c.563G>A (p.Gly188Glu) + c.7394C>T (p.Ser2465Leu)] mutations in compound heterozygosity with c.11747T>G (p.Leu3916Trp). P2 harbored the c.962G>A (p.Arg321Gln) and c.8330G>A (p.Arg2777Lys) mutations (Supplementary Figure S1). All variants were absent in gnomAD and predicted to be "likely pathogenic" in silico [13].

Evaluation of mitochondrial networks and sacsin levels in skin fibroblasts revealed a significant relative increase (P1) or a mild increase (P2) in the number of fragmented mitochondria as opposed to the tubular shape seen in controls (Fig. 2a); WB showed a significant reduction of sacsin expression only in P1 (Fig. 2b).

Discussion

Our work showed that a "multi-biomarker approach," combining MRI, retinal, mitochondrial network, and WB investigations, could help to define the disease role of bi-allelic missense mutations in *SACS*. Such approaches are becoming

critical now that rapid access to multigene analyses and exome sequencing is expanding the array of potential SNVs and making their interpretation challenging.

Our two index cases harbored "likely pathogenic" SNVs and had compatible imaging and mitochondrial network phenotypes. However, it is underlined that classic biomarkers, such as hypointense pontine stripes and mitochondrial fragmentation, often considered disease specific, also occur in other clinical conditions mimicking ARSACS [12, 16]. Thus, WB of sacsin remains the single robust marker of a disease-related *SACS* genotype. Alternatively, highly specialized retinal studies might be used to support neurological investigations [17]. In our P1, but not in P2, the classic OCT finding of pRNFL thickening [18, 19] was identified using SD-OCT, a tool offering better resolution of retinal layers and segmentation. Interestingly, we observed a retinal fold in papillomacular bundle associated with mild foveal distortion [20], suggesting a disorder of retinal development in ARSACS.

Our findings prompt a final consideration. Since most pathogenic *SACS* mutations are associated with a predictably truncated protein, the usefulness of commercial antibodies (raised against the C-terminus of sacsin) is limited as they do not allow exhaustive assaying of residual sacsin levels. There is therefore a need for new antibodies to refine the results of cellular studies and increase the chances of establishing genotype-phenotype correlations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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