Heterozygous missense variants of *SPTBN2* are a frequent cause of congenital cerebellar ataxia

Francesco Nicita[1#], Marta Nardella[1#], Emanuele Bellacchio[2], Paolo Alfieri[3], Gaetano Terrone[4], Giorgia Piccini[3], Federica Graziola[5], Claudio Pignata[4], Alessandro Capuano[5], Enrico Bertini[1] and Ginevra Zanni[1]*

[1] Unit of Neuromuscular and Neurodegenerative diseases, Department of Neurosciences, Bambino Gesù Children's Hospital, Rome, Italy

[2] Genetics and Rare Diseases Research Division, Bambino Gesù Children's Hospital, Rome, Italy

[3] Unit of Child Neuropsychiatry, Department of Neurosciences, Bambino Gesù Children's Hospital, Rome, Italy

[4] Department of Translational Medical Sciences, Section of Pediatrics, University of Naples Federico II, Naples, Italy

[5] Unit of Neurology, Department of Neurosciences, Bambino Gesù Children's Hospital, Rome, Italy

[#] These authors have contributed equally to this work

*** Corresponding author:**
Ginevra Zanni MD, PhD
Bambino Gesù Children's Hospital, Rome, Italy.
Email: ginevra.zanni@opbg.net

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Running title: *SPTBN2*-related congenital ataxia

ABSTRACT

Heterozygous missense variants in the *SPTBN2* gene, encoding the non-erythrocytic beta spectrin 2 subunit (beta-III spectrin), have been identified in autosomal dominant spinocerebellar ataxia type 5 (SCA5), a rare adult-onset neurodegenerative disorder characterized by progressive cerebellar ataxia, whereas homozygous loss of function variants in *SPTBN2* have been associated with early onset cerebellar ataxia and global developmental delay (SCAR14). Recently, heterozygous *SPTBN2* missense variants have been identified in a few patients with an early-onset ataxic phenotype. We report five patients with non-progressive congenital ataxia and psychomotor delay, 4/5 harboring novel heterozygous missense variants in *SPTBN2* and one patient with compound heterozygous *SPTBN2* variants. With an overall prevalence of 5% in our cohort of unrelated patients screened by targeted next generation sequencing (NGS) for congenital or early-onset cerebellar ataxia, this study indicates that both dominant and recessive mutations of *SPTBN2* together with *CACNA1A* and *ITPR1,* are a frequent cause of early-onset/congenital non-progressive ataxia and that their screening should be implemented in this subgroup of disorders.

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Key words: congenital ataxia , spinocerebellar ataxia , SCA5, SCAR14, SPTBN2, beta-III spectrin

INTRODUCTION

Congenital ataxias account for about 10% of non-progressive infantile encephalopathies and are characterized by severe neonatal hypotonia and developmental delay, followed by cerebellar ataxia in the first years of life. They are generally classified into pure cerebellar and syndromic forms and their inheritance can be autosomal recessive autosomal dominant or X-linked recessive (reviewed by [1,2]). More than 20 genes are known to date (see supplemental Table S1). Homozygous loss of function mutations of *SPTBN2* have been identified in four families with early-onset cerebellar ataxia associated with cognitive impairment, eye movements abnormalities and variable additional neurological signs, defined as autosomal recessive spinocerebellar ataxia 14 (SCAR14; MIM#615386) [3-6]. Heterozygous missense variants of *SPTBN2* have been initially associated to autosomal dominant spinocerebellar ataxia 5 (SCA5; MIM#600224) characterized by a slowly progressive cerebellar syndrome with downbeat nystagmus and tremor, beginning in the third decade with a tendency toward anticipation in later generations [7-11]. The identification of 3

unrelated cases of early-onset cerebellar ataxia and developmental delay carrying the same de novo pathogenic variant (p.R480W) in *SPTBN2* has challenged the previous SCA5/SCAR14 genotype-phenotype correlation [12-15]. We used targeted next generation sequencing in 100 unrelated patients with non-progressive congenital or early onset ataxia associated with cerebellar atrophy. .

MATERIAL and METHODS

Clinical reports

The clinical and neuroradiological features of the five probands (Figure 1, Table 1 and Supplemental files): congenital onset (within the first months of life), nonprogressive cerebellar symptoms (nystagmus, ataxia, hypotonia) and developmental delay, are almost constant findings. For detailed neuropsychological profiling of patients 1 and 5 (see supplemental files and Table S2).

Next-Generation Sequencing and Bioinformatic Analysis

All samples (from 100 index patients: 87 sporadic cases, 8 probands with autosomal dominant, 4 with autosomal recessive and one with X-linked inheritance) were included in a next-generation sequencing (NGS) panel of genes whose mutations are causative of various forms of cerebellar ataxias (Table S3), including all known SCA genes. Genomic DNA was extracted from peripheral blood of the patients and their parents. Informed consent was obtained from all participating subjects according to the Declaration of Helsinki. The panel was designed using Nextera technology

on a MiSeq platform (Illumina, San Diego, CA, USA), following the manufacturer's protocol, with expected coverage of 99% of the targeted genomic regions. Mapping of sequences against the hg19 reference genome was performed by Bowtie2. Bioinformatic tools HaplotypeCaller (GATK ver. 4.3) and ANNOVAR were used to call and annotate the variants, respectively. Subsequent filtering allowed to exclude intronic variants, synonymous variants not affecting splicing, and variants with frequency >1% in human variation databases. Segregation was verified by Sanger sequencing in the families. Accession numbers are as follows: human *SPTBN2* mRNA: NM_006946; human SPTBN2 protein: NP_008877. The pathogenicity of the identified missense variant was investigated using PolyPhen-2, SIFT, Mutation Assessor, and CADD, while conservation of the affected residue was assessed by ClustalW2 (http:// www.ebi.ac.uk/Tools/msa/clustalw2/). We filtered rare *SPTBN2* variants in 15/100 ataxic patients (a list of all *SPTBN2* variants found in our screening is available as supplemental Table S4); only one third (5/15) of the variants were confirmed to be pathogenic (highly conserved, predicted to be damaging by in silico tools and molecular modeling), after segregation analysis and careful clinical (and in some case MRI) evaluation by two experienced pediatric neurologists, of carrier parents.

Modelling

The p.T62I and p.F160C mutations were mapped on the PDB structure 6ANU (cryo-electron microscopy structure of F-actin complexed with spectrin beta chain). The p.Y272H mutation was mapped on the conformer 4 of the PDB structure 1WYQ (NMR structure of the second CH domain of human spectrin beta chain). To model the p.R437W and p.R437Q mutations, the homology model of SPTBN2 spectrin repeats 1-3 was built employing the following procedure. The amino acid residues 305-638 of SPTBN2 and the residues of the ROD domain of alpha-actinin from the

PDB entry 1HCI (used as the template structure), were aligned (the two sequences share 33% amino acid identity; Supplementary file 1). All side chain atoms in the template structure were deleted, the amino acids were renamed and renumbered to the corresponding human SPTBN2 residues according to the above pairwise sequence alignment, and the modified PDB structure file was parsed to SIDEpro [16] for side chain reconstruction.

RESULTS

Genetic data

Six novel mutations in *SPTBN2* were detected. In particular, four patients harbored heterozygous missense variants (NM_006946): c.185C>T;p.T62I, c.479T>G;p.F160C, c.1309C>T;p.R437W, and c.1310G>A;p.R437Q and one patient harbored compound heterozygous variants (a nonsense c.6267G>A; p.W2065* and a missense c.888T>C; p.Y272H) (Figure 2, Table 1) . Segregation analysis demonstrated a *de novo* origin of the mutations in three cases (patient 1, 2 and 3) whereas in one case (patient 4) segregation analysis could not be completed because the father was not alive. All mutations are predicted deleterious or probably damaging by in silico prediction tools. None of the six mutations have been reported in available databases (i.e., dbSNP146, 1000 Genomes, ExAC and GnomAD).

Molecular Modeling

The p.T62I and p.F160C pathogenic variants fall in the first Calponin-homology (CH) domain of SPTBN2 (NP_008877) affecting a highly conserved residue. The replacement of Thr62 with an isoleucine residue introduces a novel hydrophobic interaction with Trp154, which modify the

interface of the CH-1 domain and actin (Fig. 3B). The replacement of Phe160 (highly conserved) with a cysteine causes the loss of the hydrophobic interactions with Trp66 and Leu98 (Fig. 3C), modifying also in this case the interface of the CH-1 domain with actin. The p.Y272H mutation affects a highly conserved residue and disrupts the hydrophobic interactions with nearby residues in the second CH domain of SPTBN2 (Fig. 3D). Superposition of the SPTBN2 CH-2 domain with the CH2 domain of human alpha-actinin, which, like SPTBN2 also presents two CH domains arranged in tandem, suggests that the Y272H amino acid substitution likely perturbs the interface between the two CH domains and possibly their mutual three-dimensional arrangements. The p.R437W or p.R437Q mutation implies the replacement of the cationic and highly conserved arginine 437 with the aromatic and large tryptophan (W) or the polar glutamine (Q) residue in a region that has structural importance for the second spectrin repeat (Fig. 3E). Both the tryptophan and glutamine residues should cause important reorganizations in the structure of the spectrin repeat 2 also affecting its arrangement with the flanking repeats (spectrin repeats 1 and 3). The p.W2065* mutation causes the loss of a functional domain of SPTBN2 (Fig. 3A). Based on conservation and structural analysis, all the mutations presented in this work are expected to have important impacts on functional regions of the SPTBN2 protein, providing a basis for rationalizing their pathogenic effects.

DISCUSSION

Spectrins are heterotetrameric cytoskeletal membrane proteins composed of two main alpha subunits (SPTA1, SPTAN1) and five beta subunits (SPTB, SPTBN1, SPTBN2, SPTBN4, SPTBN5) (reviewed by [17]) . The non-erythrocytic beta spectrin 2 subunit (beta-III spectrin) is a 2,391-

amino acid protein encoded by *SPTBN2* (11q13.2) and expressed throughout the soma and dendritic tree of cerebellar Purkinje cells. SPTBN2 is required for the maintenance of dendritic architecture, and for the trafficking and stabilization of several membrane proteins: Ankyrin-R, cell adhesion proteins, voltage-gated sodium channels, glutamate receptors (mGluR1) and transporters (EAAT4) [18]. It contains the actin/ARP1 binding site at the N-terminal (calponin-homology domains), 17 spectrin repeats involved in the formation of the heterotetrameric ±-² -spectrin complex and a pleckstrin homology (PH) domain which binds phosphatidylinositol lipids, at the C-terminal [19]. Loss of SPTBN2 in the mouse mutant results in altered dendritic morphology and density, changes in Purkinje cells intrinsic excitability: reduced sodium currents and deficits in glutamatergic neurotransmission. [20-22].

To date, three cases of dominant *SPTBN2* (p.R480W) mutations and four cases of homozygous recessive mutations have been reported in early-onset ataxia (Figure S1). In a recent targeted exome analysis of patients with ataxic phenotypes, de novo or dominant *SPTBN2* mutations were identified in 4/88 (4,5%) two of them presenting with infantile onset ataxia and developmental delay [23]. Here we show novel pathogenic *SPTBN2* variants in 5% of our cohort of patients with congenital or early-onset non-progressive ataxia; of note that only one third (5/15) of the rare *SPTBN2* variants identified in our study were confirmed to be pathogenic. The five novel pathogenic missense variants localized in the actin binding domain or in the spectrin repeats domains of SPTBN2 and one stop variant localized in the PH domain of the protein. Thermodynamic changes caused by different mutations may affect the overall dimerization capability of the protein. The hotspot R480W mutation, located in the second spectrin repeat domain (as the R437Q and R437W variants identified in the present study) has been predicted to increase protein stability, whereas SCA5-

related T472M found in the same domain or L253P found in the actin binding domain were either neutral or destabilizing the protein [14]. Yet the molecular mechanisms for differences in timing of onset, progression, mode of inheritance and presence of cognitive deficits in ataxic patients, remain unknown. Interestingly, the two brothers carrying a homozygous missense mutation reported by [6] and patient 5, carrying a nonsense and a missense variant, appear to have a milder phenotype with normal cognitive development (Supplemental file 2 and Table S2) if compared with previously reported SCAR14 patients with homozygous loss of function mutations [3-5]. Similar frequencies have been observed for mutations in other SCA-associated loci such as *ITPR1* [24, 25, 26], *CACNA1A* [27], whereas both autosomal recessive and dominant modes of inheritance associated with neurodevelopmental and/or neurodegenerative features have been reported in *KCNC3* [28] or *GRM1* [29, 30] mutated families. The challenge will be to decipher subtle changes underpinning Purkinje cells dysfunction both in early and later stages of these various forms of ataxia. This study further delineates the clinical, mutational and epidemiological spectrum of congenital and early onset *SPTBN2*-related ataxias and suggest that screening of *SPTBN2* should be implemented in this subgroup of disorders.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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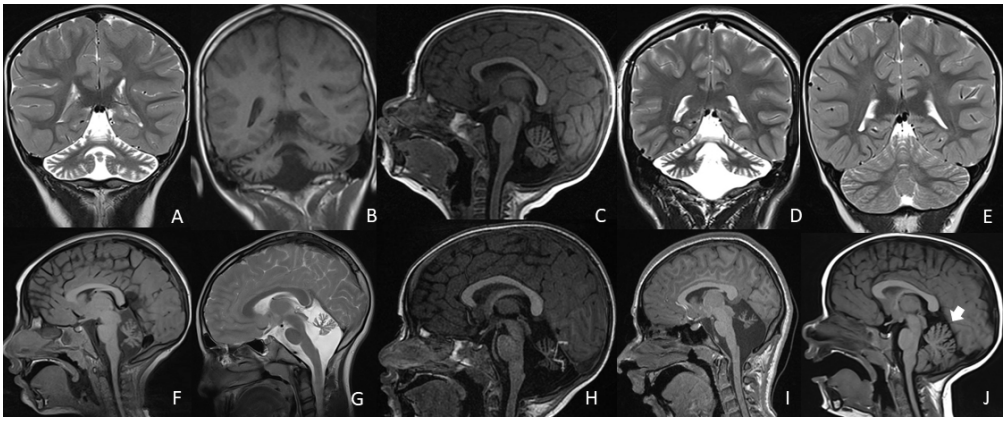
LEGEND FOR FIGURES

Figure 1A-J: Brain MRI of patient 1 at the age of 2y8mo (A,F), patient 2 at the age of 10y (B,G), patient 3 at the age of 11mo (C) and 2y10mo (H; with artefacts), patient 4 at the age of 16y5mo (D,I), and patient 5 at the age of 2y8mo (E,J). Severe atrophy of cerebellar hemispheres and vermis is evident in patients 1-4; a milder atrophy in the upper cerebellar vermis is present in patient 5 with compound heterozygous *SPTBN2* variants (white arrow in J). No abnormalities of the pons, corpus callosum, cerebral white or grey matter are observed in all patients.

Figure 2: Pedigree structure and sequencing chromatograms of *SPTBN2* mutations identified by targeted resequencing in the affected individuals and corresponding wild-type sequences in unaffected parents. Vertical arrows indicate the site of nucleotide exchange.

Figure 3A-E: Structural analysis and sequence conservation around the sites affected by mutations in SPTBN2. (A) Scheme of SPTBN2 protein indicating the mutations presented in this work. (B) Structure of SPTBN2 in complex with actin (PDB 6ANU) showing the site of the p.T62I mutation. (C) Crystal structure of SPTBN2 in complex with actin (PDB 6ANU) highlighting the site of the p.F160C mutation. (D) NMR structure of the CH-2 domain of SPTBN2 (PDB 1WYQ , blue ribbons) superposed on the structure of the CH1-CH2 domains of human alpha-actinin (PDB 2EYI, white ribbons) indicating the site of the p.Y272H mutation. (E) Homology model of SPTBN2 spectrin repeats 1-3, showing the site of the p.R437W mutation. In the protein structures, residues

interacting with the amino acids affected by mutations are shown (for clarity not all residues are displayed). In the sequence alignments, the grayed columns represent invariant residues.



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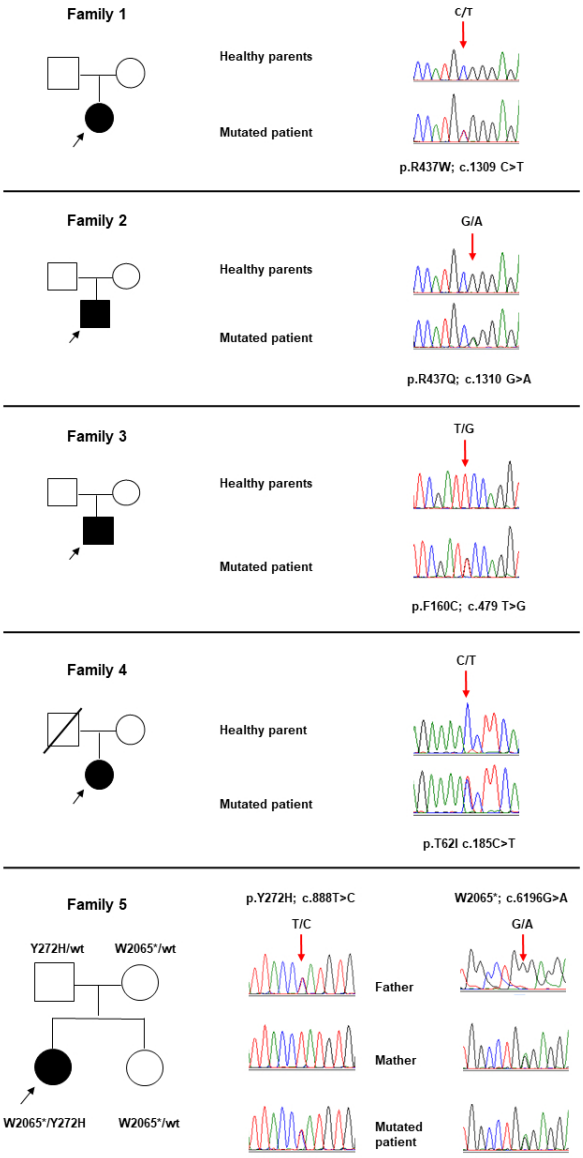

Figure 2: Pedigree structure and sequencing chromatograms of *SPTBN2* mutations identified by targeted resequencing in the affected individuals and corresponding wild-type sequences in unaffected parents. Vertical arrows indicate the site of nucleotide exchange.

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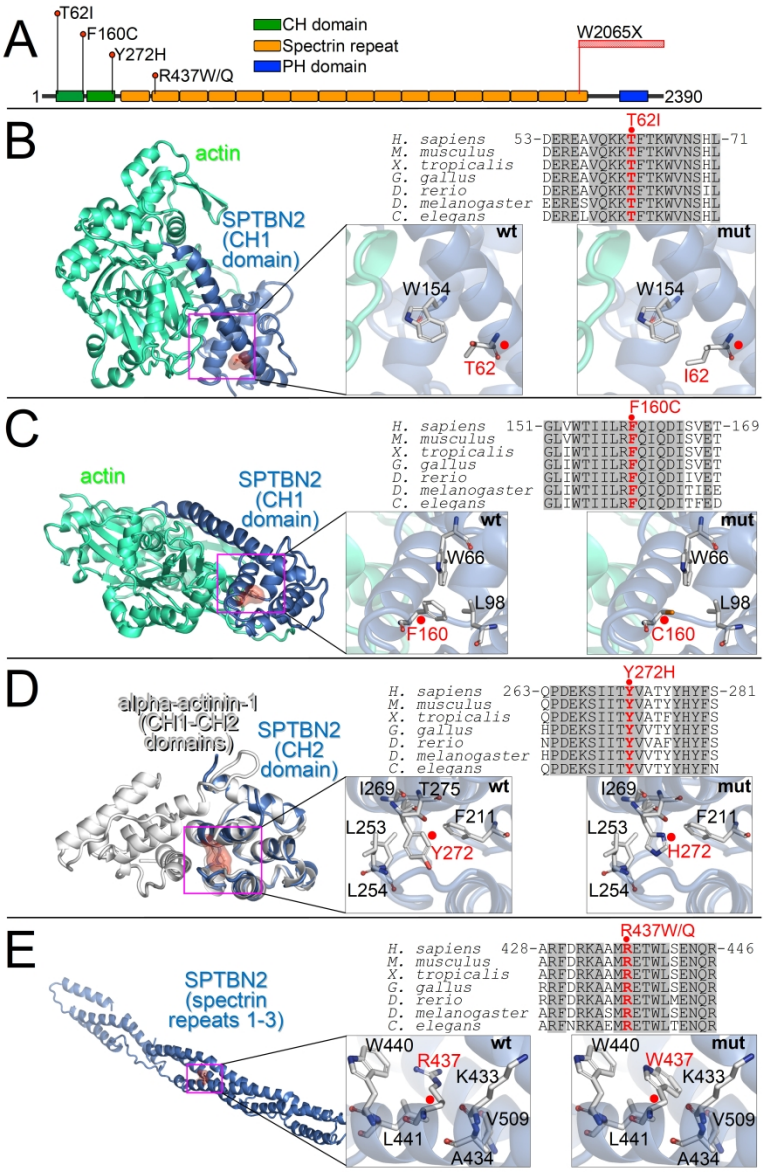

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316x475mm (300 x 300 DPI)

Table 1: Summary of the clinical, genetic and neuroradiological features of the reported patients

Patient	**1**	**2**	**3**	**4**	**5**
Sex, Age	F, 8 years	M, 18 years	M, 5 years	F, 18 years	F, 9 years
***SPTBN2* variant**	**c.1309C>T**	**c.1310G>A**	**c.479T>G**	**c.185C>T**	**c.888T>C; c.6267G>A;**
Protein	**p.R437W**	**p.R437Q**	**p.F160C**	**p.T62I**	**p.Y272H; p.W2065***
Domain	**2nd SPEC**	**2nd SPEC**	**ABD**	**ABD**	**17th SPEC, PH**
Age of onset	10 mo	5 mo	5 mo	8 mo	12 mo
First sign/symptom at onset	Psychomotor delay	Hypotonia	Psychomotor delay and strabismus	Psychomotor delay Microcephaly	Motor delay
Sitting unsupported	1 y	10 mo	13 mo	1 y	8 mo
Walk (ataxic) unassisted	5 y	2 y	Not acquired	10 y	2 y 4 mo
First words	2 y 6 mo	1 y	Not acquired	3 y	1 y 6 mo
Ocular anomalies	-	Nystagmus (horizontal)	Strabismus	Nystagmus (horizontal and vertical)	-
Congenital hypotonia	+	+	+	+	+ (mild)
Pyramidal signs	-	+ (increased OTR)	-	-	-
Tremor	-	+ (intention tremor)	-	-	-
Dystonia	-	-	-	-	-
Facial myokymia	-	-	-	-	-
Bulbar dysfunction	-	-	-	-	-
Cognitive delay	+	+	+	+	-
Behavioral problems	-	-	-	-	-
Additional findings	-	-	-	Mild bradykinesia	-
Cerebellar atrophy	+	+	+	+	+ (mild)
Progression of atrophy	NA	+	+	NA	NA
Additional MRI findings	-	-	-	-	-
Course	Non progressive	Non progressive	Non progressive	Non progressive	Non progressive

SPEC= Spectrin repeats; ABD= Actin binding domain; n.d = not determined in the father, absent in the mother; OTR= osteotendineous reflexes; PH= Pleckstrin homology domain