

SHORT REPORT

Identification of a novel in-frame *de novo* mutation in *SPTAN1* in intellectual disability and pontocerebellar atrophy

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Heterozygous in-frame mutations (p.E2207del and p.R2308_M2309dup) in the α -II subunit of spectrin (*SPTAN1*) were recently identified in two patients with intellectual disability (ID), infantile spasms (IS), hypomyelination, and brain atrophy. These mutations affected the C-terminal domain of the protein, which contains the nucleation site of the α/β spectrin heterodimer. By screening *SPTAN1* in 95 patients with idiopathic ID, we found a *de novo* in-frame mutation (p.Q2202del) in the same C-terminal domain in a patient with mild generalized epilepsy and pontocerebellar atrophy, but without IS, hypomyelination, or other brain structural defects, allowing us to define the core phenotype associated with these C-terminal *SPTAN1* mutations. We also found a *de novo* missense variant (p.R566P) of unclear clinical significance in a patient with non-syndromic ID. These two mutations induced different patterns of aggregation between spectrin subunits in transfected neuronal cell lines, providing a paradigm for the classification of candidate variants.

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INTRODUCTION

The targeting and maintenance of groups of proteins at specific membrane domains of neurons, such as the synapse and the axon, are critical for the brain function and development. Not surprisingly, mutations in proteins that participate in the assembling of these domains, such as the spectrins, have been shown to cause neurological disorders. Spectrins consist of α - and β -subunits that form antiparallel heterodimers, which are assembled to form heterotetramers in a head-to-head configuration.^{1,2} Heterozygous mutations in the β -III subunit (*SPTBN2*) were shown to cause spinocerebellar ataxia type-5.³ Recently, *de novo* in-frame mutations in the α -II spectrin subunit (*SPTAN1*) were identified in patients with severe intellectual disability (ID), infantile spasms (IS) with hypsarrhythmia, hypomyelination, and atrophy of various regions of the brain, including the cerebellum and brainstem.⁴ These mutations are located in a C-terminal domain that includes the nucleation site involved in α/β spectrin heterodimer formation.⁵ Biochemical studies indicated that these mutations destabilized this interaction, leading to aggregation of α -II(mut)/ β -II and α -II(mut)/ β -III spectrin heterodimers in mouse cortical neurons and lymphoblastoid cells from the patients.⁴

Recent studies suggest that *de novo* point mutations in the germ line, which in humans are estimated to occur at a rate of $\sim 1.1 \times 10^{-8}$ per base, may explain a large fraction of ID cases.^{6–8} In the course of a project aimed to identify such mutations in candidate synaptic genes,

we sequenced *SPTAN1* in patients with idiopathic ID. We identified *de novo* mutations in *SPTAN1* in a patient with non-syndromic ID and in a patient with ID, epilepsy and pontocerebellar atrophy, further expanding the phenotypical spectrum associated with mutations in *SPTAN1*. Moreover, we found that these mutations induce different patterns of aggregations of spectrin subunits in cultured neuronal cells, providing a paradigm to validate candidate variants and to establish correlations between genotypes and phenotypes.

METHODS

Subjects and DNA sequencing

Ninety-five cases of idiopathic ID (46 males and 49 females), mostly of French–Canadian ethnicity, without growth abnormalities or specific dysmorphic features, as well as 190 French–Canadians controls were studied. A subset of the ID cases (25/95) also displayed epilepsy. Genomic DNA was extracted from blood samples (Qiagen/Gentra, Toronto, ON, Canada) and paternity/maternity was confirmed using six informative unlinked microsatellite markers (Supplementary Table S1). Polymerase chain reaction amplification of all *SPTAN1*-coding exons and their splice junctions (Supplementary Table S2 for primers), DNA sequencing, and mutation analyses are detailed in the Supplementary Information.

Expression vectors

SPTAN1 mutants (c.1697G>C/p.R566P, c.6605_6607del/p.Q2202del, c.6619_6621del/p.E2207del; positions based on Refseq NM_001130438.2) were generated by site-directed mutagenesis using KOD-Plus-Mutagenesis kit

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(Toyobo, Osaka, Japan), and verified by sequencing. A full-length human *SPTAN1* cDNA (α -II spectrin) with a C-terminal Flag-tag was cloned into a CAG-promoter vector to express C'-Flag-tagged α -II spectrin.

Cell culture, transfection, and immunofluorescence

Mouse neuroblastoma 2A (N2A) cells and primary cultures of mouse cortical neurons were grown, transfected, and tested by immunofluorescence for the expression of wild type and mutant spectrin subunits, as previously described,^{4,9} and detailed in the Supplementary Information.

RESULTS

We identified three heterozygous variants in *SPTAN1* in different patients, including two missense (c.1679A>G/p.E560G and c.1697G>C/p.R566P) and one in-frame amino-acid deletion (c.6605_6607del/p.Q2202del). p.E560G was transmitted from a healthy mother and is predicted not to affect protein function by various algorithms (Supplementary Table S3). In contrast, p.R566P (patient-1) and p.Q2202del (patient-2) were absent from the blood DNA of the corresponding healthy parents, indicating that they were

de novo, and were not detected in 190 French-Canadian controls, nor reported in dbSNP134. p.R566P affects a well-conserved residue that lies at the N-terminus of SPTAN1 and is predicted to damage protein function by SIFT¹⁰ and polyphen.¹¹ The deleted p.Q2202 residue is part of a C-terminal domain required for α/β spectrin subunit heterodimer formation.⁵ Interestingly, p.Q2202del is in close proximity to the recently reported *SPTAN1* in-frame mutations, p.E2207del and p.R2308_M2309dup, identified in patients with ID, IS, and structural brain defects (Figure 1).⁴

The phenotypes of the patients with p.R566P and p.E2207del were different (Supplementary Information). Patient-1 (p.R566P), a 9-year-old boy, shows mild non-syndromic ID without epilepsy. The brain CT-scan did not reveal any abnormality. He has a sister who also shows non-syndromic ID but who does not carry the mutation. Patient-2 (p.Q2202del), an 11-year-old boy, shows severe ID. He did not present with IS but developed mild generalized epilepsy. The brain MRI showed severe atrophy of the cerebellum and mild atrophy of the brainstem, without any hypomyelination or other structural defects (Figure 2).

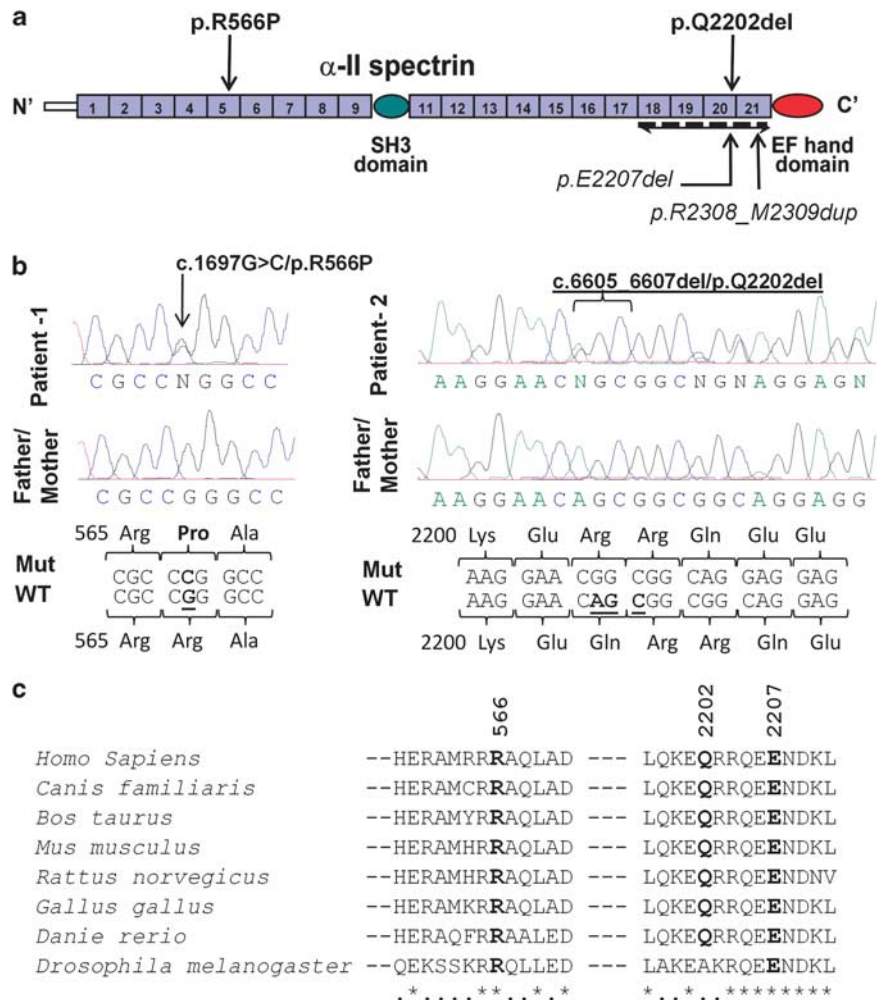


Figure 1 *De novo* mutations identified in *SPTAN1*. (a) Schematic representation of the SPTAN1 protein consisting of 22 domains, including 20-spectrin repeats, an SH3 domain, and an EF hand domains. The last four spectrin repeats required for α/β spectrin heterodimer associations are indicated (bidirectional arrow). Mutations identified in this study (p.R566P and p.Q2202del) and those by Saitsu *et al*⁴ (p.E2207del and p.R2308_M2309dup) are shown. (b) Chromatograms of the *de novo* p.R566P and p.Q2202del mutations (Mut) and wildtype (WT) *SPTAN1* sequences. (c) Amino-acid conservation of the SPTAN1 residues affected with *de novo* mutations (p.R566P, p.Q2202del and p.E2207del). Amino-acid alignments were generated by homogene (NCBI) and the amino-acid sequences flanking the mutations are shown.

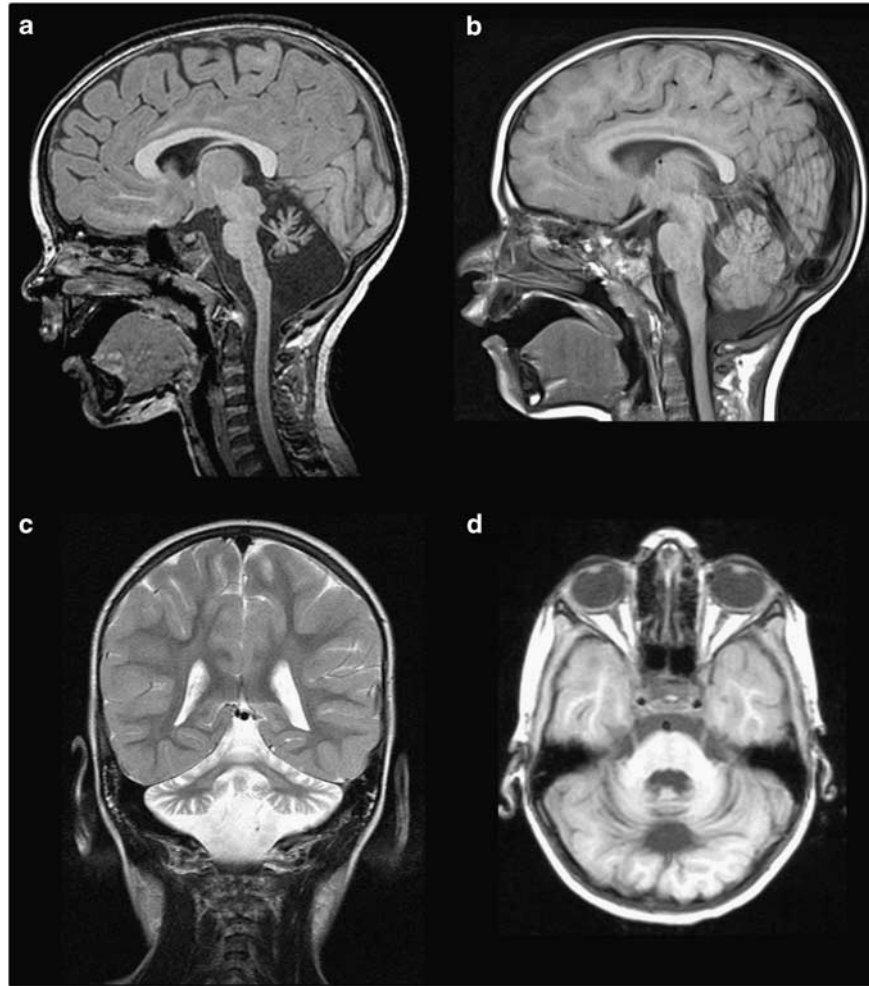


Figure 2 The brain MRI of the patient with p.Q2202del showing pontocerebellar atrophy. (a) Sagittal T1 image at midline showing severe atrophy of vermis with hypoplastic brainstem. The corpus callosum is larger than normal for the age of the patient. (b) Sagittal T1 image of an age and gender matched control showing normal pons and normal cerebellum (c) Coronal T2 image of the patient at the level of the posterior fossa, showing severe atrophy of both cerebellar hemispheres with T2 hypersignal of both middle cerebellar peduncle. (d) Axial T1 image of the patient at the level of the cerebellum showing diffuse cerebellar atrophy.

Expression of α -II spectrin containing p.E2207del or p.R2308_M2309dup induced subunit aggregates in primary neuronal cultures.⁴ We took advantage of this assay to compare the impact of p.R566P, p.Q2202del, and p.E2207del in mouse N2A cells and mouse cortical neurons (Figure 3). Expression of wild-type α -II spectrin in N2A cells resulted in its localization at cell periphery, without the formation of aggregates (Figures 3a and b). The p.Q2202del mutant showed a similar pattern of expression whereas the p.E2207del induced small aggregates in 6% of transfected cells. In contrast, the p.R566P mutant induced large aggregates in 86% of transfected N2A cells. These mutant α -II spectrin aggregates colocalized with endogenous β -II spectrin (Supplementary Figure S1). No endogenous β -III-spectrin subunits were detectable in N2A cells by immunofluorescence. In primary neuronal cultures, p.E2207del induced aggregates in most cells; these aggregates were composed of both β -II and β -III spectrin subunits (Figures 3b-d). The p.Q2202del mutant showed a similar aggregation profile, but in a lower proportion of cells (15–20%). In contrast, p.R566P induced aggregates in a much smaller number of cells (<5%). Moreover, double immunostaining revealed that these aggregates were composed of the β -II but not of the β -III spectrin subunit.

DISCUSSION

Saitsu *et al*⁴ recently showed that in-frame deletion (p.E2207del) or duplication (p.R2308_M2309dup) in the C-terminal region of SPTAN1 causes a specific syndrome characterized by severe ID, IS, hypomyelination, and brain atrophy.⁴ We describe here a novel in-frame deletion in the same domain (p.Q2202del). The phenotype of the patient with this mutation (patient-2) overlaps with that of the previously described patients with in-frame C-terminal SPTAN1 mutations, although being milder. For instance, all three patients showed severe ID, but patient-2 was less impaired than the others. Moreover, the previously described patients presented early in life with IS, and their course was characterized by intractable seizures. In contrast, patient-2 did not show IS and his seizures were well controlled with a single antiepileptic drug. Finally, MRI studies revealed pontocerebellar atrophy in all three patients with the in-frame mutations but patient-2 did not show atrophy of other structures or hypomyelination, like the others.

All three in-frame mutations induced the aggregation of the α -II with the β -II and β -III spectrin subunits in primary neuronal cells, suggesting that they have a dominant-negative effect. Although the spectrin aggregation profile associated with p.Q2202del was similar to

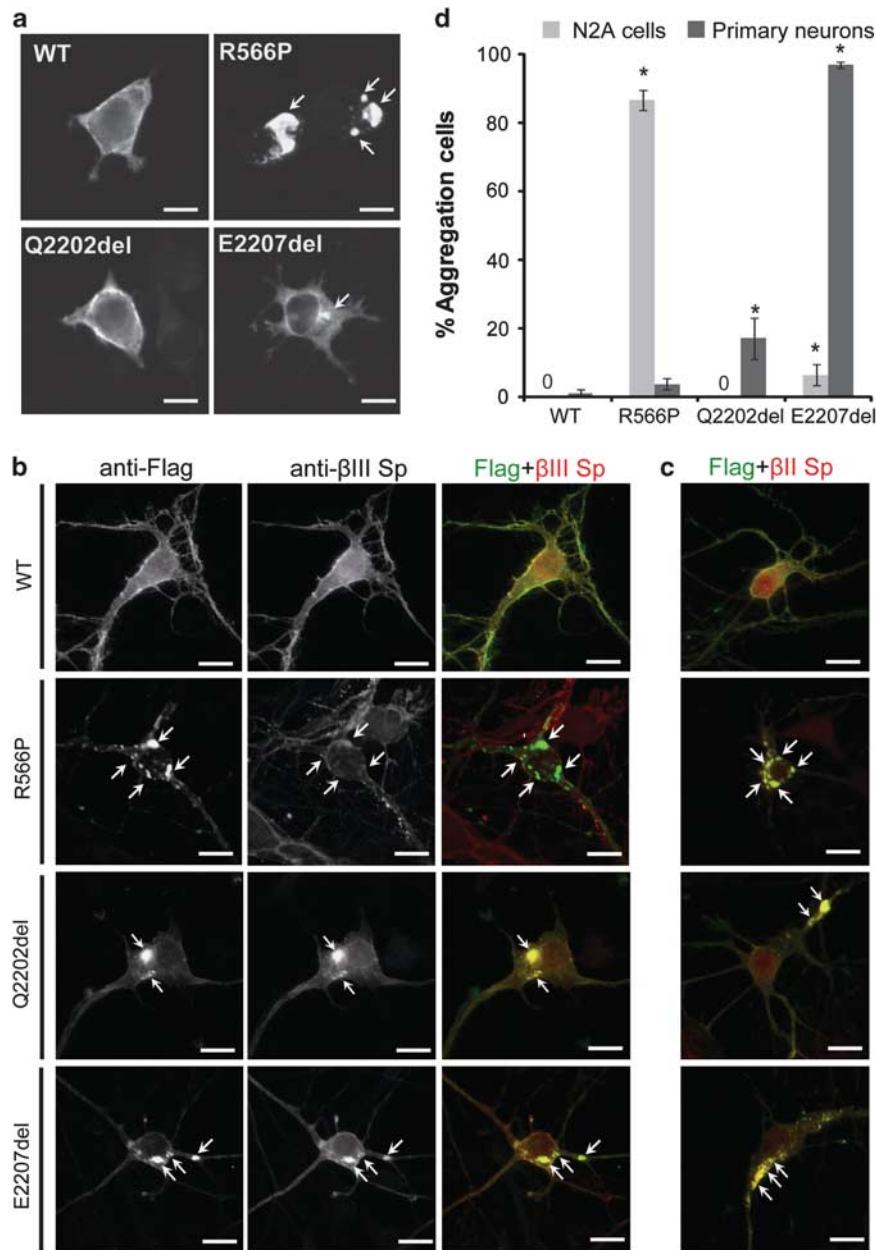


Figure 3 Mutant α -II spectrin cause aggregation of α/β spectrin heterodimers. **(a)** The wildtype (WT) and the three mutant α -II spectrins were detected by immunofluorescence in transfected N2A cells. The WT α -II spectrin and p.Q2202del mutant were similarly expressed at cell periphery. However, the p.R566P and p.E2207del α -II spectrin mutants showed large and small aggregations (arrows), respectively. **(b, c)** Expression of the WT and the three mutant α -II spectrins at 7 days *in vitro* in primary cortical neurons. Flag tagged WT α -II spectrin was expressed at cell extensions and periphery, overlapping with the expression of β -II and β -III spectrins. Three mutant α -II spectrins (R566P, Q2202del, and E2207del) showed aggregation in cell bodies and neurites (arrows). Aggregations caused by the Q2202del and E2207del mutants were colocalized with both β -II and β -III spectrins (lower two panels). Aggregations caused by the R566P mutant were colocalized with β -II spectrin, but their colocalization with β -III spectrin was not evident. The scale bar represents 10 μ m. **(d)** N2A cells and primary cortical neurons showing α -II spectrin aggregation were counted: Numbers of aggregated/total numbers of counted cells (expressing transfected α -II spectrin) in three experiments: N2A, WT: 0/194, R566P: 212/244, Q2202del: 0/241, E2207del: 9/180; primary neurons, WT: 3/300, R566P: 11/300, Q2202del: 51/300, E2207del: 291/300. Asterisks indicate that a significant difference ($P < 0.01$) was observed compared with WT by Bonferroni's posttest analysis. The scale bar represents 10 μ m.

that of p.E2207del, it was observed at a lower frequency correlating with the less severe phenotype observed in patient-2. Heterozygous mutations in β -III spectrin (*SPTBN2*) were previously shown to cause spinocerebellar ataxia type-5.³ It is tempting to speculate that cerebellar atrophy in patients with mutations in *SPTAN1* is caused at least in part by the aggregation of β -III spectrin. This is consistent with the

fact that β -III, unlike β -II spectrin, is abundantly expressed in the human cerebellum (Allen Brain Atlas; <http://human.brain-map.org/>). All together, these observations strengthen the causal relationship between in-frame mutations at the C-terminus of *SPTAN1*, severe ID, and pontocerebellar atrophy, while expanding the phenotypical spectrum associated with these mutations.

We also identified a *de novo* missense (p.R566P) in *SPTAN1* in a patient with mild non-syndromic ID without epilepsy or any gross brain abnormalities. Interestingly, the aggregation profiles induced by p.R566P and by the in-frame mutations are different. First, p.R566P induced aggregates in a great proportion of N2A cells but only in a negligible proportion of primary neuronal cells. The reverse pattern was found with the in-frame mutations. Second, p.R566P only aggregated with β -II spectrin whereas the in-frame mutations showed aggregations with both β -II and β -III spectrin subunits in cortical neurons. The phenotypic differences between the patient with the missense and the patients with the in-frame mutations may thus be explained by the preferential aggregation of p.R566P α -II with β -II spectrin. It is important to underline that the sister of patient-1 was also diagnosed with non-syndromic ID but lacked p.R566P in her blood DNA. Although it is possible that she is a phenocopy, it appears more likely that p.R566P is not responsible for her brother's condition. The identification of other patients with deleterious mutations in the N-terminal region of *SPTAN1* would help to distinguish these possibilities.

In summary, our work indicates that in-frame mutations in the C-terminus of *SPTAN1* cause a core set of manifestations that include severe ID, generalized epilepsy, and pontocerebellar atrophy. Although it is unclear whether p.R566P is pathogenic, we found that this mutation induces a distinct pattern of spectrin aggregation. Additional studies are needed to determine whether this pattern of aggregation is associated with a specific phenotype. Our study thus provides a paradigm to validate candidate variants in *SPTAN1* and to establish correlations between genotypes and phenotypes.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (<http://www.nature.com/ejhg>)