

Clinical and neuroimaging features of autosomal recessive spastic paraplegia 35 (SPG35): case reports, new mutations, and brief literature review

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Abstract

Spastic paraplegia 35 (SPG35) is a recessive condition characterized by childhood onset, progressive course, complicated by dystonia, dysarthria, cognitive impairment, and epilepsy. Mutations in the *FA2H* gene have been described in several families, leading to the proposal of a single entity, named fatty acid hydrolase-associated neurodegeneration (FAHN). Several reports have described a polymorphic radiological picture with white matter lesions of various degrees and a distinct form of neurodegeneration with brain iron accumulation. While we reviewed the pertinent literature, we also report three new patients with SPG35, highlighting the possible absence of white matter lesions even after a long neuroimaging follow-up. Three-dimensional modeling of the mutated proteins was helpful to elucidate the role of the site of mutations and the correlation with the residual enzyme activity as determined in cultured skin fibroblasts.

Keywords SPG35 · *FA2H* · Complicated hereditary spastic paraplegia

The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors.

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Introduction

Hereditary spastic paraplegias (HSPs) are a group of clinically and genetically heterogeneous disorders characterized by distal retrograde axonal degeneration of the long corticospinal tracts and posterior columns, with progressive lower extremity weakness and spasticity [1]. Up to date, at least 76 clinical forms of HSPs and 59 corresponding genes have been described with autosomal dominant (AD), autosomal recessive (AR), X-linked (XL), and maternal inheritance [2, 3]. Clinically, HSPs are classified as pure and “complicated” [4]. The main clinical feature of pure forms is the progressive spasticity, but mild sensitive disturbances and autonomic impairment may also occur [4]. In complicated forms, other clinical and neuroimaging features may be present, including cognitive and behavioral impairment, cerebellar atrophy, epilepsy, polyneuropathy, optic atrophy, and cerebral white matter involvement of variable severity [4]. Spastic paraplegia 35 (SPG35, MIM 612319) is an AR-complicated HSP caused by mutations in the gene encoding fatty acid 2-hydroxylase (*FA2H*, MIM 611026), a 372-aminoacid-long membrane-bound protein involved in the

alpha-hydroxylation of the N-acyl ceramide moiety of sphingolipid fatty acids, essential components of myelin [5, 6]. This enzyme uses free fatty acids as a substrate, and the product, free 2-hydroxy fatty acids, is then incorporated into ceramide, the precursor of galactolipids, as galactosylceramides (GalC). SPG35 is characterized by childhood onset and progressive course of spastic paraparesis with dystonia, dysarthria, cognitive impairment, and epilepsy [7]. Mutations in *FA2H* have also been reported in partially related phenotypes such as spastic paraparesis with dystonia, dysarthria, and mild cognitive decline associated with leukoencephalopathy on brain imaging [8] and in a distinct form of neurodegeneration with brain iron accumulation (NBIA) [9]. These overlapping phenotypes led to proposing a distinct entity, named fatty acid hydroxylase-associated neurodegeneration (FAHN) [9, 10] that has been reported in less than 30 patients and is characterized by a heterogeneous neuroimaging pattern, including different degrees of white matter lesions, cortical and cerebellar atrophy, thin corpus callosum, and hypointensity of globi pallidi [7–9, 11–13]. Herein, we report the clinical, neuroimaging, biochemical, and molecular findings in three novel unrelated patients with new mutations in *FA2H*, comparing their features with those previously reported in SPG35.

Methods

This study was approved by the Tuscany Regional Pediatric Ethics committee. All the procedures complied with the Helsinki Declaration of 1975. Genetic studies were performed after parental written informed consent. The three patients reported here were part of a larger group of 150 subjects with spastic paraparesis of unknown etiology studied in our laboratories. Genomic blood DNA from patients was purified by standard methods and analyzed using *SpastoPlex2.0* (methodologies outlined elsewhere [14]). Candidate pathogenic variants in *FA2H* were confirmed by Sanger sequencing and shown to segregate in the families. The three-dimensional model of the human *FA2H* was generated using a combination of threading and homology methods (see supplementary information SI). The effect of mutations on protein stability was predicted by FoldX [15], the Poisson-Boltzmann electrostatic potential surface was calculated with APBS [16], and the effect of mutations on *FA2H*-heme group affinity was quantified by mCSM-lig [17].

Determination of *FA2H* biochemical activity was performed in cultured skin fibroblasts as described in supplementary information SI.

Case reports

Patient 1

A 6-year-old girl with an unremarkable family history and childbirth exhibited a progressive spastic gait since the age of 4. Her non-consanguineous parents are both Italian and she has a 3-year-old healthy brother. Neurological examination at admission disclosed spastic gait with mild diffuse muscular hypotonia and a supinated clubfoot. Diffuse hyporeflexia and bilateral clonus were observed. Deep and superficial sensibilities were unaffected. Neuroimaging (brain and spinal 3 Tesla MRI) disclosed only minimal and non-progressive atrophy of cerebellar cortical folia (Fig. 1a–c); neurophysiological investigations (somatosensory evoked potential, SSEPs, and nerve conduction velocities) were unrevealing. Electroencephalography (EEG) during wakefulness and sleep revealed frequent left frontal focal spikes (no epileptic seizures were ever reported). Routine metabolic investigations resulted normal. Cerebrospinal fluid (CSF) glucose level, the CSF to blood glucose ratio, and liquor neurotransmitter studies were not conclusive.

Array-CGH disclosed no pathological genomic rearrangements. Partial deficiency of beta-galactocerebrosidase activity was observed on a blood spot in MS/MS and subsequently confirmed by enzyme testing in leukocytes, though sequence analysis of coding exons in the Galactosylceramidase (*GALC*) and Prosaposin (*PSAP*) genes were negative.

Cognitive evaluation (Wechsler Preschool and Primary Scale of Intelligence scale), performed while the girl was aged 5 years, reported normal scores. Nystagmus was noticed since the age of 5. Walking aids were required since the age of 6. Follow-up cognitive testing, performed at age 7 years, resulted stationary. Serial brain and spinal MRI controls ruled out novel pathological findings in gray and white matter, basal ganglia, and cerebellar structures (Fig. 1a–c).

Therapy with omega-3 fatty acids (1 g/day) was started as soon as the molecular diagnosis was obtained with no relevant effects. Her latest Spastic Paraplegia Rating Scale (SPRS) score at the latest evaluation was 27/52.

Patient 2

A 13-year-old girl presented a history of gait difficulties and frequent falls since the age of 6 years. She was the second child born to healthy, unrelated parents. Two sibs (a boy and a girl) are healthy. Family history was unremarkable and pregnancy and delivery were uneventful. The patient acquired motor and language milestones on time. Neurological examination when she was 13 showed spastic gait with hip adductor, tibialis anterior muscle weakness, and moderate leg stiffness. Diffusely brisk tendon reflexes in the lower limbs and bilateral

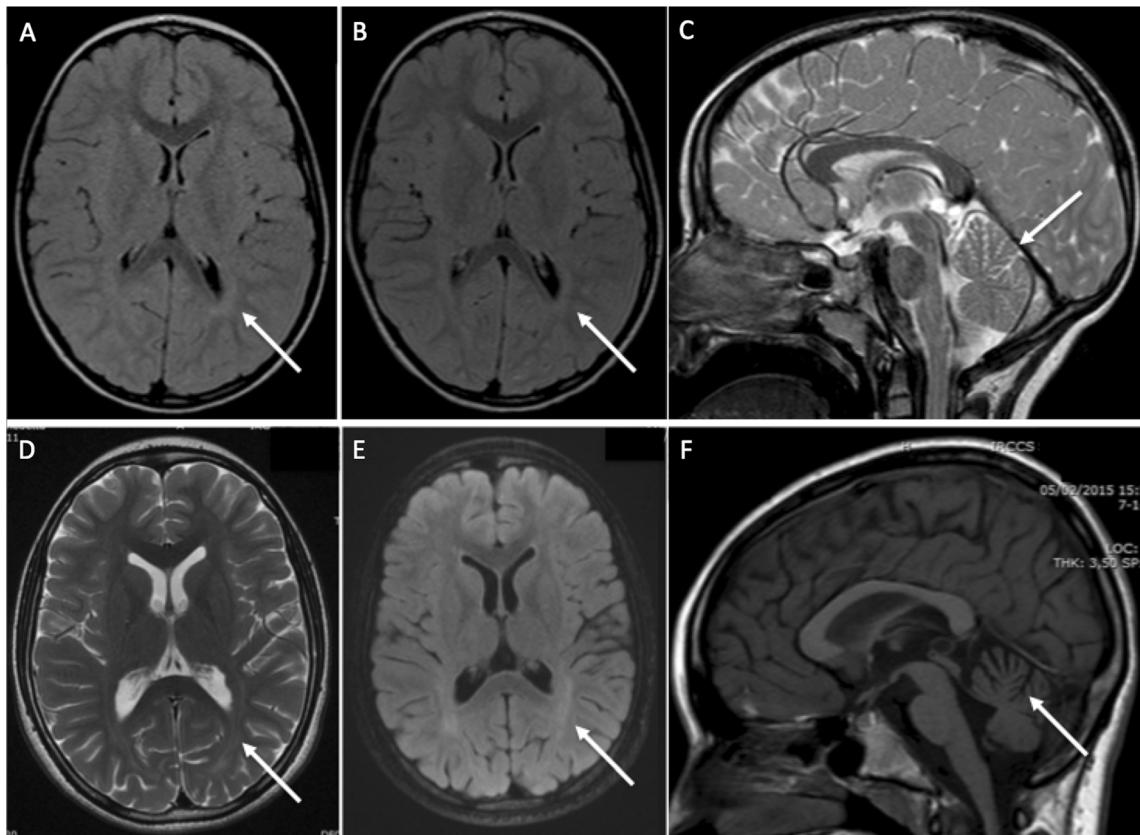


Fig. 1 MRI studies in patients 1 and 2 disclosing similar findings (solid arrows). Patient 1, axial T2 and FLAIR images at age 6 years (**a**) and 7 years (**b**), exhibiting non-progressive posterior, peritrigonal white matter alterations, and sagittal T2 image at age 7 years (**c**) with mild atrophy of

cerebellar cortical folia. Patient 2, axial T2 and FLAIR images (**d**, **e**) and sagittal T1 image (**f**) at age 12 years with posterior, peritrigonal white matter alterations, and cerebellar cortical atrophy

Babinski sign were observed. We also observed lateral nystagmus, but cerebellar function examination of gait and trunk was normal. Deep and superficial sensibility was unaffected. Her latest SPRS score was 12/52. Neuropsychological assessments were scored as within normal limits. Her EEG, during wakefulness and sleep, revealed spikes on the fronto-centro-temporal regions. EMG and nerve conduction velocities resulted normal. Beta-galactocerebrosidase activity in peripheral blood was within normal range.

Motor-evoked potentials in the lower limbs showed increased center motor conduction times with normal SSEPs. Brain MRI showed minimal bilateral white matter hyperintensity in peritrigonal and supratrigonal areas and centrum semiovale and mild cerebellar cortical atrophy (Fig. 1d–f).

Therapy with omega-3 fatty acids (1 g/day), started at age 13 years, had no relevant effects on clinical manifestations.

Patient 3

A 29-year-old Brazilian woman complained since the age of 9 of frequent falls and clumsiness. Since childhood, she exhibited slowly progressive leg stiffness; learning difficulties were

also evident when she started to attend school. Prenatal, perinatal, and family histories were unremarkable. She underwent surgery for strabismus at age 20. At age 23, she had progressed to severe motor impairment, and neurological examination showed severe lower limbs spasticity, brisk lower limbs reflexes, and bilateral Babinski sign. Cognitive evaluation (Wechsler Adult Intelligence Scale) disclosed mild intellectual disability (Full Scale Intelligence Quotient, 70). EMG showed chronic denervation in the distal muscles of the lower limbs. Brain MRI, performed at 23 years, showed periventricular white matter changes with cerebellar atrophy, mild brain atrophy, and thin corpus callosum; follow-up neuroimaging showed a stationary picture. The patient got pregnant at the age of 29 years and delivered a healthy boy.

Molecular and biochemical analyses

Using *SpastoPlex* [14], we identified, in patient 1, the c.805C>T (p.R269C) and c.1501A>G (p.S351G) in *FA2H* (MIM 611026). Both mutations were novel and segregated in healthy parents. In patient 2, we detected biallelic variants in *FA2H*, the previously described c.103G>T (p.D35Y) [8] in

compound heterozygosity with the new c.193C>T (p.P65S). The mutations segregated in healthy parents. In patient 3, we detected the novel c.1055C>T (p.T352I) and [c.340_363del24][c.363+1_8del8]. The electropherograms depicting the mutations identified in this work are presented in supplementary Fig. S1. No additional relatives were tested. Novel mutations were not found in gnomAD and ExAC browsers and were scored predictably deleterious in silico using *Polyphen 2* (<http://genetics.bwh.harvard.edu/pph2/>).

Determination of FA2H activity in cultured skin fibroblasts showed reduced enzyme activity in patients 1 and 2 (33 and 11% of values in five normal, age-matched controls, respectively) whereas enzyme testing was not performed in patient 3 who did not undergo a diagnostic skin biopsy.

Structural modeling

The effect of missense mutations on function/activity of FA2H was evaluated using computational methods based on three-dimensional protein structure. FA2H is an integral membrane enzyme of smooth endoplasmic reticulum catalyzing hydroxylation of galactosylceramide and sulfatide in myelin sheath. The three-dimensional structure of human FA2H consists of two distinct domains: the N-terminal cytochrome b5-like domain containing the heme group (residues 15–85) and the C-terminal sphingolipid fatty acid hydroxylase domain (residues 124–366) containing the catalytic di-iron cluster and the four transmembrane domains anchoring the protein to the ER membrane (Fig. 2a).

Mutations identified in our patients are distributed in both domains but invariably located on the cytoplasmic side of the protein (Fig. 2a). All missense mutations are predicted to affect the normal structure/function of FA2H protein. In particular, (i) mutation D35Y alters the cytochrome b5-like domain fold and destabilizes the protein ($\Delta\Delta G: 3.42 \pm 0.19$ kcal/mol; Fig. 2b); (ii) mutation P65S affects the hydrophobic interactions that stabilize the heme group in the cytochrome b5 domain (Fig. 2c); (iii) change of the positively charged arginine to cysteine in position 269 (R269C) decreases the density of positive charge on the hydroxylase domain's surface (Fig. 2d); and (iv) mutations S351G and T352I affect the structure/sequence of the putative phosphorylation site located in the C-terminal domain of FA2H protein (Fig. 2e).

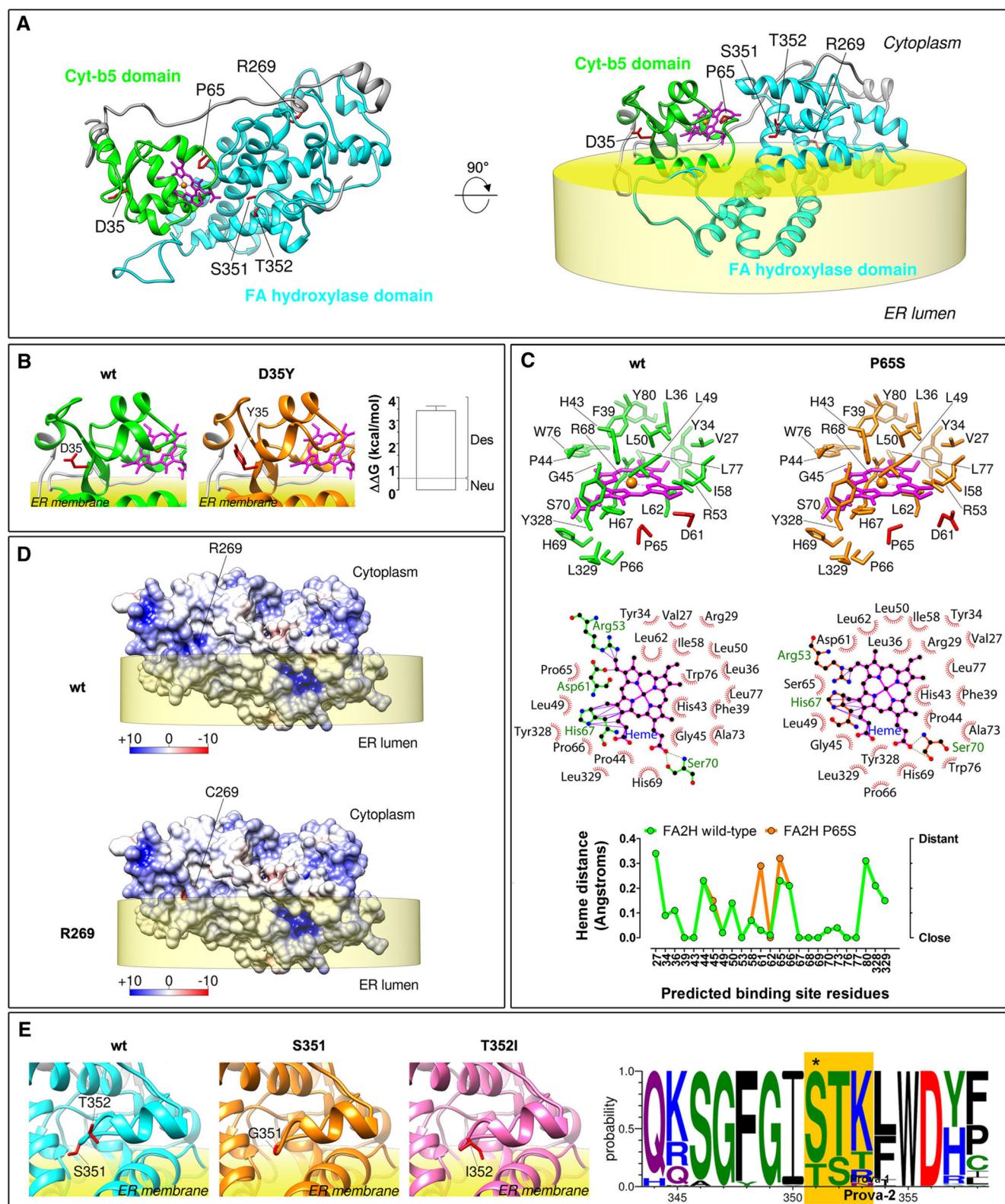
Genotype-phenotype correlation analysis

The three-dimensional model of human FA2H was used to investigate the possible relationships of FA2H missense

Fig. 2 Effects of missense mutations on FA2H structure. **a** Three-dimensional model of human FA2H (Qmean global score: 0.571). The view is from the cytoplasm (left) and membrane (right) plane. FA2H consists of two distinct domains, the cytochrome b5-like domain (residues 15–85; green) and the fatty acid hydroxylase domain (residues 124–366; cyan). The heme group in the cytochrome b5-like domain is shown as stick (magenta). All mutated residues (highlighted in red) are located on the cytoplasmic side of FA2H structure. **b** Close view of the structure around residue 35 in wild-type and mutated FA2H. D35Y mutation severely decreases the global stability of the domain ($\Delta\Delta G: 3.42$ kcal/mol; right panel). **c** Stereo view (up) and LIGPLOT diagrams (middle) of the heme binding site of wild-type (left) and P65S (right) cytochrome b5-like domain of FA2H. P65S mutation alters the network of interactions between heme group and FA2H. In particular, severely affects the binding distance between the heme and aspartic acid at residue 61 (down panel). **d** Electrostatic surface potential of FA2H wild-type and R269C mutant. The replacement of arginine for cysteine at residue 269 alters the electrostatic surface of the fatty acid hydroxylase domain of FA2H. Electrostatic surface potentials were colored according to charge with blue denoting positive charge (+10 kT/e-) and red, negative charge (-10 kT/e-). **e** Structure/sequence analysis of potential phosphorylation site in the C-terminal region of FA2H. S351G and T352I mutations determine conformation (left) and sequence (logo analysis; right) changes in the cytoplasmic loop containing the target phosphorylation site

mutations and the clinical phenotypes of SPG35 patients. All clinical reports (Supplementary Table S1) and missense variants reported as pathogenic in *FA2H* (Supplementary Table S2) were collected (last update December 2017) and carefully annotated in the protein structure. The final dataset contains 27 missense from 51 patients. These were broadly classified into three major phenotypic categories: (1) spastic paraparesis, (2) leukoencephalopathy, and (3) cases with cerebellar and basal ganglia (BG) involvement. All the pathogenic missense mutations are located within the two distinct protein domains, cytochrome b5-like domain and fatty acid hydroxylase domain (Fig. 3a). Notably, only two mutations (namely, R154C and T207M) are shared by patients classified in different phenotypic categories (Supplementary Table S2).

To understand how gene variants contribute to FA2H-disease, the full dataset of missense was analyzed for their effects on protein stability and heme binding affinity (Fig. 3b, c; see *Supplementary Material* for details). Overall, the disease-causing mutations affect thermodynamic stability ($\Delta\Delta G$ values span from -1.6 to 12.2 kcal/mol) and affinity for heme group (affinity fold change span from -1.06 to 0.04) regardless of the protein domains where mutations are located (Supplementary Fig. S2A, B). Interestingly, we observed differences in protein stability comparing mutations described in patients with white matter changes and cases associated with spastic paraparesis and cerebellar/BG involvement (Fig. 3b). A larger decrease in affinity for heme group was seen for mutations associated with cerebellar/BG involvement (Fig. 3c).



Combined, structural data suggest that the different phenotypes might be linked to specific gene effects on

FA2H function (at least considering protein stability vs heme affinity; Fig. 3d).

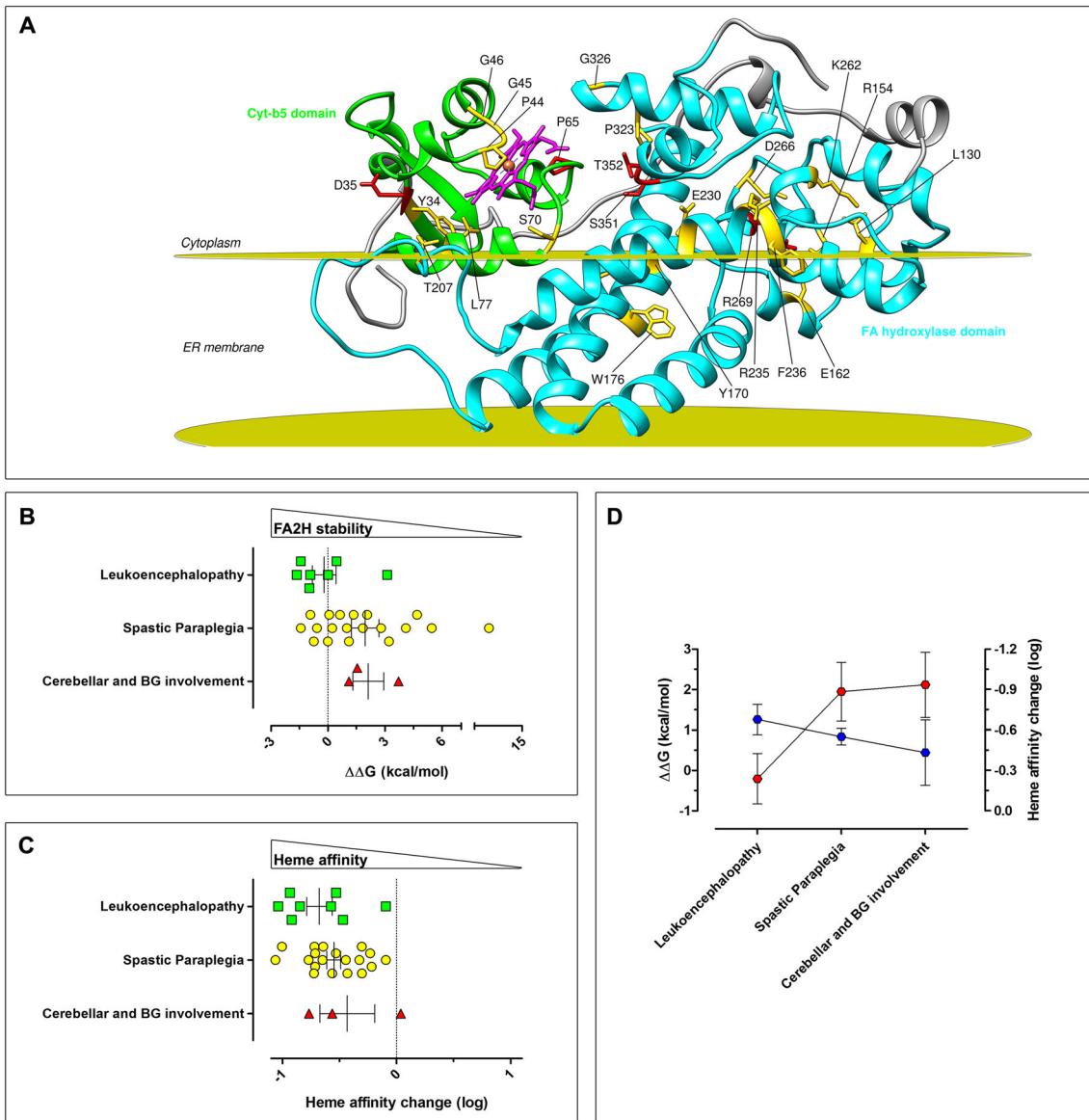


Fig. 3 Genotype-phenotype analyses of missense mutations in *FA2H*. **a** Distribution of all missense described as pathogenic in human *FA2H* (last update December 2017) across the protein structure. Mutated residues identified in this and previous studies are highlighted in red and yellow, respectively. **b** Scatter dot plot showing the effects on protein stability of the missense detected in the three different patients' groups. **c** Scatter dot plot showing the effects on heme affinity of the missense detected in the three different patients' groups. **d** Relationship between average values of global stability of the domain ($\Delta\Delta G$) (red dots) or heme affinity (blue dots) and phenotypic categories

(leukoencephalopathy, spastic paraplegia, and cerebellar/basal ganglia (BG) involvement; for details, see Supplementary Table S2). **c** Scatter dot plot showing the effects on heme affinity of the missense detected in the three different patients' groups. **d** Relationship between average values of global stability of the domain ($\Delta\Delta G$) (red dots) or heme affinity (blue dots) and phenotypic categories

Discussion

SPG35 (MIM 612319) is an infantile-onset AR complicated form of HSP linked to mutations in *FA2H* [2, 4, 7, 9, 13, 18]. The first clinical report of SPG35, describing a single and consanguineous Omani family [18], helped to define 16q21-q23 as the disease locus [14]. In 2008, homozygosity mapping documented different inactivating mutations in *FA2H* in nine patients from three consanguineous Arabi-Muslim families [8] and proposed a novel entity characterized by complicated HSP, intellectual disability, dystonia, seizures, and

neuroimaging evidence of prominent white matter lesions (leukodystrophy dysmyelinating and spastic paraparesis with or without dystonia, MIM 612443). Subsequent reports, describing different mutations in *FA2H* in other recessive families [19], could not reveal a clear genotype-phenotype correlation, though putative null mutations or deletions appeared to result in a more severe phenotype than missense variants [20]. Review of the published cases expanded the clinical and neuroradiological phenotype showing various combination of optic atrophy, dystonia, cognitive impairment, epilepsy, and cerebellar signs, associated with white matter lesions with thin

corpus callosum and brain iron accumulation in the globus pallidus [2, 7, 9, 10, 13, 19–26].

As regards to the age of appearance of the symptoms, an infantile onset has been reported in the vast majority of patients with few reports of late-infantile [21] and adult [13, 22, 27] onset.

The systematic review of the MRI data of described patients reveals that, as a common characteristic of SPG35, different cerebral structures are involved. White matter impairment, considered as the hallmark of the syndrome by some authors [8], spans from the absence of damage (cases in family 3 with milder phenotype reported in [8]), to multiple posterior periventricular lesions [19–21] to a clear-cut leukodystrophy [10, 12, 13]. The white matter impairment has not proven to be progressive in all cases with few exceptions [8, 11, 12]. Moreover, even if a long-term serial and prospective MRI evaluation of these patients is unavailable, the different degree of white matter involvement seems unrelated to the age of patients and the duration of the disease. In our study, all patients exhibited minimal and non-progressive involvement of white matter regions and cerebellar cortical structures (Fig. 1a–f). Cortical cerebral and cerebellar atrophy has been described as a main and severe feature of MRI in several cases. No evidence of progressive course or correlation between the age of patients and/or disease duration with the amount of degenerative cortical process has been proven to be significant. Hypointensity of globi pallidi has been proposed as a main neuroimaging feature in patients with SPG35, and the term FAHN has been indicated as a subtype of NBIA [9]. Serial MRI evaluations in our patients, however, did not confirm this peculiar feature and are in keeping with previous reports by others [13, 23–26].

The hypothesis that disease severity may be influenced by the residual enzyme activity has been suggested by other authors [7–9, 12] but not confirmed by a recent review [13]. Although technically laborious, determinations of enzyme activity in primary skin cells in the present work disagree with this statement. Protein function studies of FA2H seem even more helpful to try and elucidate the phenotypic variability of the disease. Herein, we used the three-dimensional protein structure to analyze the full spectrum of FA2H missense described in this work and in previous studies (for the full dataset see Supplementary Table S2) and examined their effects on the function of the two domains making-up FA2H. The hydroxylase domain is a catalytic unit of the protein, and mutations disrupting this region have the likelihood to affect severely enzyme activity. Enzyme activities determined in our cultured skin fibroblasts are in line with this assumption. On the other hand, mutations impairing the cytochrome b5-like domain (responsible for the intramolecular electron transport during the catalytic cycle) reduce but do not abolish enzyme activity [5]. Taken together, our three-dimensional analyses suggest that (1) FA2H disease-causing mutations exert their effects on protein function/stability or heme binding affinity

and (2) these effects are strictly linked to the protein structure. We also observed that (3) different effects of mutations on protein domains appear to correlate with the patients' phenotypes, though with an important overlap, offering new clues for genotype/phenotype relationships in FA2H disease.

Our report highlights some final learning points for diagnosing SPG35. Despite serial MRI evaluations, clear-cut evidence of severe white matter impairment was absent in our patients during follow-up (3 and 7 years). This finding suggests that white matter damage is unrelated to disease duration, given that in all reported cases, white matter lesions are described early in the disease course. In line with previous descriptions of SPG35, our data suggest that MRI with mild alterations is possible in the first years after onset, indicating that *FA2H* gene testing should be considered if an evocative clinical picture is present, even when brain MRI is unremarkable.

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Compliance with ethical standards

This study was approved by the Tuscany Regional Pediatric Ethics committee. All the procedures complied with the Helsinki Declaration of 1975. Genetic studies were performed after parental written informed consent.

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

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