

## ARTICLE

# Mutations in phospholipase DDHD2 cause autosomal recessive hereditary spastic paraplegia (SPG54)

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Hereditary spastic paraplegias (HSP) are a genetically heterogeneous group of disorders characterized by a distal axonopathy of the corticospinal tract motor neurons leading to progressive lower limb spasticity and weakness. Intracellular membrane trafficking, mitochondrial dysfunction and myelin formation are key functions involved in HSP pathogenesis. Only recently defects in metabolism of complex lipids have been implicated in a number of HSP subtypes. Mutations in the 23 known autosomal recessive HSP genes explain less than half of autosomal recessive HSP cases. To identify novel autosomal recessive HSP disease genes, exome sequencing was performed in 79 index cases with autosomal recessive forms of HSP. Resulting variants were filtered and intersected between families to allow identification of new disease genes. We identified two deleterious mutations in the phospholipase *DDHD2* gene in two families with complicated HSP. The phenotype is characterized by early onset of spastic paraplegia, mental retardation, short stature and dysgenesis of the corpus callosum. Phospholipase *DDHD2* is involved in intracellular membrane trafficking at the golgi/ endoplasmic reticulum interface and has been shown to possess phospholipase A1 activity *in vitro*. Discovery of *DDHD2* mutations in HSP might therefore provide a link between two key pathogenic themes in HSP: membrane trafficking and lipid metabolism.

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## INTRODUCTION

The genetically diverse group of hereditary spastic paraplegias (HSP) is clinically defined by a progressive spasticity and weakness of the lower limbs, caused by distal axonopathy of the long motor axons of the corticospinal tract. Identification of more than 39 HSP<sup>1</sup> genes highlights intracellular membrane trafficking, mitochondrial metabolism and myelin formation as key functions involved in HSP pathogenesis.<sup>2</sup> Only recently lipid metabolism has emerged as another main theme in HSP pathophysiology, backed by the discovery of mutations in a number of genes involved in lipid metabolism and signaling including *CYP2U1*,<sup>3</sup> *CYP7B1*,<sup>4</sup> *DDHD1*,<sup>3</sup> *FA2H*,<sup>5</sup> *GBA2*<sup>6</sup> and *PNPLA6*.<sup>7</sup>

In this study, we have identified deleterious mutations in phospholipase *DDHD2*, the sister enzyme of *PA-PLA1/DDHD1* in two families with complicated HSP.

## MATERIALS AND METHODS

Exome sequencing was performed in 79 index patients with familial forms of autosomal recessive HSP. The SureSelect Human All Exon 50 Mb kit (Agilent, Santa Clara, CA, USA) was used for in-solution enrichment; exome sequencing was performed using the HiSeq2000 instrument (Illumina, San Diego, CA, USA). Paired-end reads of 100 bp length were produced. BWA and GATK software packages<sup>8,9</sup> were used to align sequence reads to the reference and call variant positions. The data were then imported into Gem.app, a web-based

database and analysis tool for next generation sequencing data developed by the group of S Züchner (<https://secureforms.med.miami.edu/hihg/gem-app/>)<sup>10</sup> for further analysis. An average of 82 613 347 sequence reads was produced per sample, 98.7% of which could be aligned to the targeted sequence. Mean coverage was 75.9-fold; 85.5% of the targeted sequence was covered by at least 10 reads. Variants were filtered for impact on the coding sequence, presence of either one homozygous or two heterozygous variants in one gene, frequency in public databases (minor allele frequency in dbSNP135 and NHLBI ESP6500 < 0.5%), conservation (GERP score > 2 or PhastCons score > 0.6) and genotyping quality (GATK quality index > 30 and genotype quality GQ > 30). Additionally, variants segregating in more than two families in Gem.app were removed. In addition to 79 families with complex HSP, Gem.app contained ~130 families with pure HSP and ~450 families with non-HSP phenotypes at the time of analysis.

Informed consent was obtained from all individuals and the Institutional Review Boards at the participating medical centers approved the study.

## RESULTS

### Gene identification

In family TH126003, seven homozygous SNVs and one homozygous Indel were present; two of the SNVs as well as the Indel didn't segregate with the disease. Segregating homozygous missense variants were found in *C14orf166* (NM\_016039.2: c.311C>T, p.Pro104Leu; rs149288575), *FUT10* (NM\_032664.3: c.473T>C, p.Leu158Pro), *IMPA1* (NM\_001144879.1: c.542C>T, p.Thr181Ile) and *OTOGL*

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(NM\_173591.3: c.3461A>G, p.Asp1154Gly); a homozygous nonsense variant in *DDHD2* (NM\_001164234: c.859C>T, p.Arg287\*) was the only truncating change present. Details on all the five segregating variants in family THI26003 are given in Supplementary Table 1. As it was not possible to further narrow down the list of candidate genes in family THI26003 alone based on the available in silico parameters, the resulting list of five candidate genes was used as a seed and intersected with the resulting candidate gene lists obtained by exome sequencing in the remaining 78 autosomal recessive HSP families. Another deleterious *DDHD2* variant was identified in family IHG25194 (NM\_001164232.1: c.1982\_1983delAT, p.Tyr661Cysfs\*8) that segregates with the disease (Figure 1). Parents of the two affected siblings of this family were not available for genetic analysis, therefore a genomic deletion of one *DDHD2* allele cannot be ruled out with certainty. Independent analysis of the exome data of family IHG25194 did not yield any nonsense or truncating variants other than the above described *DDHD2* mutation.

In none of the other four candidate genes additional variants were found in the remaining autosomal recessive HSP families.

Both *DDHD2* mutations will destroy the integrity of the DDHD domain and thereby affect catalytic function, membrane localization, phosphoinositide binding and homo-oligomerization (Figure 1). Both mutations therefore likely lead to a complete loss of phospholipase *DDHD2* function.

### Clinical description

The two siblings of family THI26003 originating from Azerbaijan (Iran) from a consanguineous family background (first degree cousins) developed a progressive spastic gait disorder since early childhood. At the time of examination (disease duration 22–13 years) they were still able to walk unsupported. Due to mental retardation both had attended a special school for mentally disabled children and were working in a sheltered workshop. Spastic paraplegia was further complicated by short stature, high arched palate and dysgenesis of the corpus callosum especially in the dorsal parts (Figures 2a and b).

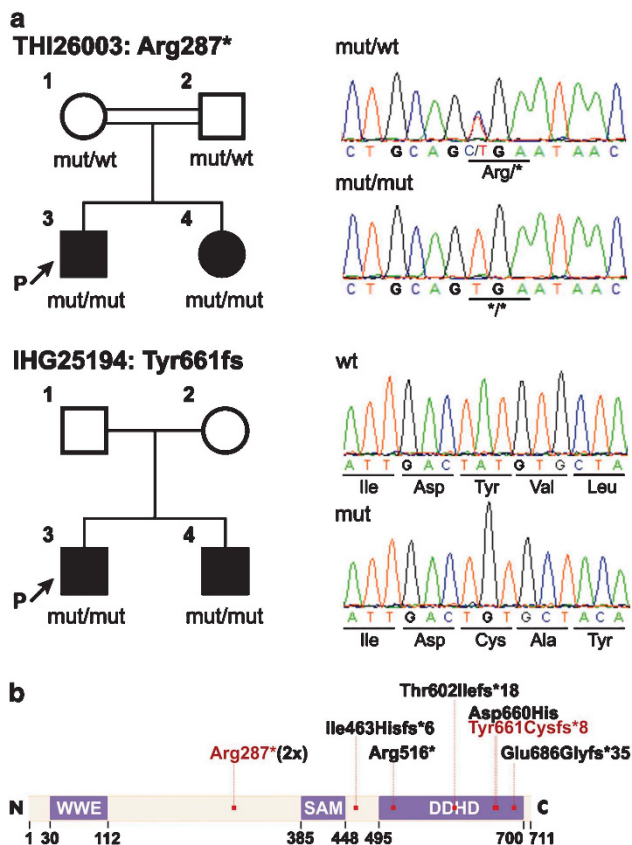
The two brothers of family IHG25194 were of Indian origin and had spastic paraplegia from early childhood. No consanguinity was reported, but both parents originated from the same village and were part of the Muslim community, explaining a possible founder effect. Additional clinical signs and symptoms in both the siblings included mental retardation, mild facial dysmorphism, short stature and dysgenesis of the corpus callosum (Figures 2c and d).

Considering the usually high phenotypic variability in HSP, the phenotype between the two unrelated *DDHD2* families is astonishingly similar. Key features of *DDHD2*-related HSP appear to be spastic paraplegia, mental retardation, short stature and dysgenesis of the corpus callosum (Table 1).

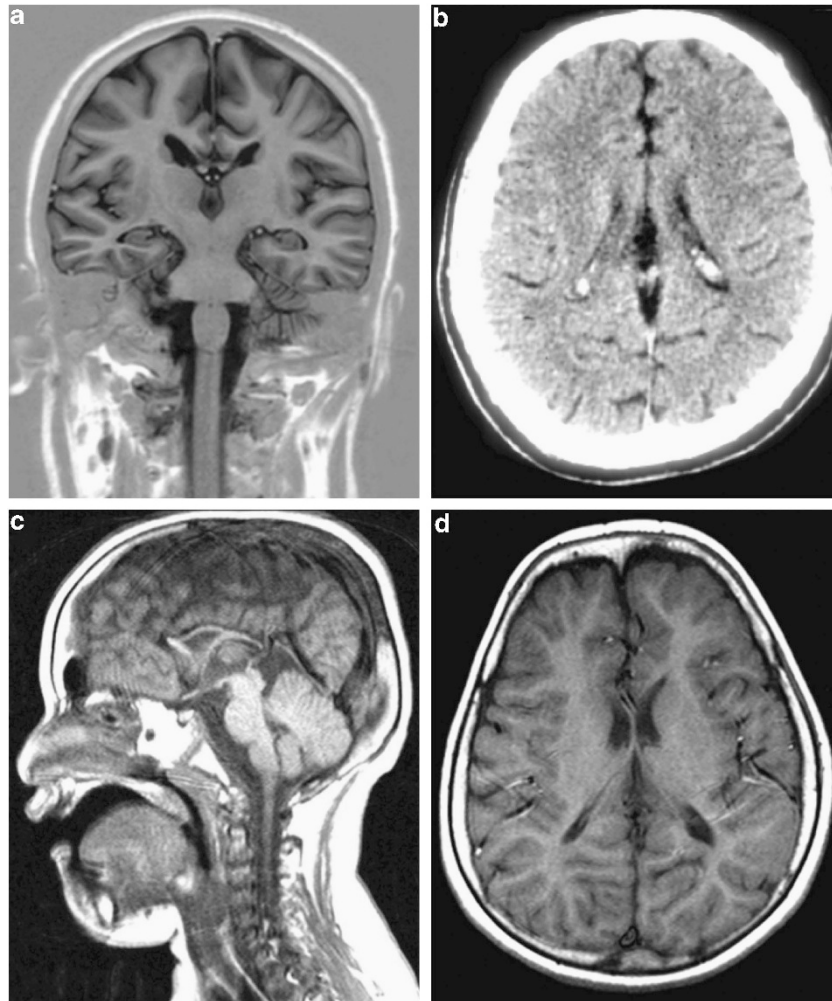
### DISCUSSION

Phospholipids are a key component of biological membranes. They are metabolized by the large family of phospholipases that can be classified according to their site of cleavage. The phospholipase A<sub>1</sub> family, consisting of extracellular and intracellular enzymes, hydrolyzes the ester bond at the sn-1 position of phospholipids, producing 2-acyl-lysophospholipids and fatty acids. In contrast to most eukaryotic organisms, mammals have three different intracellular phospholipase A<sub>1</sub>s: phosphatidic acid preferring phospholipase A<sub>1</sub> (PA-PLA1/iPLA1 $\alpha$ ; *DDHD1*), the SEC23-interacting protein p125 (iPLA1 $\beta$ ; *SEC23IP*) and phospholipase *DDHD2* (iPLA1 $\psi$ ; *DDHD2*).

The biological function of phospholipase *DDHD2* is not fully understood. *DDHD2* is ubiquitously expressed; a cytosolic and a membrane-associated pool, localizing to the cis-Golgi and the ER-Golgi intermediate compartment (ERGIC) are in a dynamic equilibrium.<sup>11–13</sup> Overexpression of *DDHD2* leads to dispersion of the Golgi and enlargement of the perinuclear ERGIC.<sup>12,14</sup> Conflicting data exists about the effect of *DDHD2* depletion. Morikawa *et al*<sup>13</sup> report a specific defect of retrograde transport from the Golgi to the ER; this defect is not confirmed by Sato *et al*<sup>11</sup> who instead postulate a anterograde transport defect from the Golgi to the plasma membrane. The authors contribute these differences to their use of different oligonucleotides for the knockdown of *DDHD2* in HeLa cells and possible off-target effects. In spite of these contradictions, the role of *DDHD2* in intracellular membrane trafficking is further supported by its homology to another member of the PLA1 family – p125 – that has been shown to be involved in vesicular transport from the ER to the Golgi by interacting with Sec23p, a component of the COP II complex.<sup>15</sup>



**Figure 1** Pedigrees and mutations. (a) Pedigrees and sequence traces of the *DDHD2* families. The c.859C>T mutation segregates in family THI26003 and leads to the formation of a preterminal stop signal at codon 287. The c.1982\_1983delAT mutation segregates in family IHG25194; it results in a frameshift at amino acid position 661. (b) Schematic of the *DDHD2* gene. The *DDHD2* gene contains three known protein domains. The WWE domain is predicted to mediate protein interactions in ubiquitin and ADP ribose conjugation systems. The tandem SAM (sterile alpha motif domain) – DDHD domain is required for phosphoinositide binding.<sup>14</sup> Integrity of the family-defining DDHD domain, present in *DDHD2* as well as its homolog *DDHD1*, is necessary for the PLA1 catalytic activity and homo-oligomerization of *DDHD2*. Catalytic function as well as a positively charged cluster in the SAM domain (Arg434-Lys435-Lys436) also required for phosphoinositide binding are necessary to promote membrane localization.<sup>11,14</sup> Mutations previously described<sup>28</sup> are indicated in black, novel mutations in red.



**Figure 2** Cranial MRI/CT scans of DDHD2 patients. MRI/CT scans of DDHD2 patients show dysgenesis of the corpus callosum as well as some paucity of the periventricular white matter (d). (a): coronal MRI (T1 inversion recovery) of THI26003-4. (b): axial CT-scan of THI26003-3. (c): sagittal MRI (T1) of IHG25194-3. (d): axial MRI (T1) of IHG25194-3.

Changes in morphology and dynamics of Golgi and ER functionally connect several subtypes of HSP: Atlastin-1 (*SPG3*), the CNS expressed member of the atlastin family of GTPases, localizes predominantly to the tubular ER and to a lesser extent to the ERGIC and the cis-Golgi. It is required for the formation of three-way junctions of the ER.<sup>16</sup> Receptor expression-enhancing proteins (REEPs) and reticulons form large oligomeric complexes in the ER membrane that are involved in curving the ER membrane, thereby forming the characteristic tubular structure of the smooth ER. Mutations in *REEP1* (*SPG31*) as well as reticulon 2 (*RTN2*, *SPG12*) genes cause autosomal dominant forms of HSP.<sup>17</sup> Point mutations in *BSC12*, causing *SPG17*, lead to upregulation of ER stress markers.<sup>18</sup> The long isoform of spastin mutated in the most common autosomal dominant subtype of HSP (*SPAST*, *SPG4*) forms a protein complex with atlastin-1 and REEP1 in the tubular ER network and is thought to coordinate microtubule regulation and membrane modeling.<sup>2,17</sup> The identification of mutations in the gene encoding DDHD2, another protein acting at the ER-to-Golgi interface therefore comes as no surprise.

In addition to its involvement in membrane trafficking, DDHD2 has been shown to possess phospholipase A1 catalytic activity towards

phosphatidic acid and other phospholipids,<sup>12,19</sup> a function shared also by DDHD1. Phosphatidic acid has diverse biological functions. It is a precursor for the biosynthesis of triacylglycerols and phosphoglycerols. In addition to its structural functions in biological membranes,<sup>20</sup> it is also involved in lipid signaling.<sup>21</sup> This phospholipase A1 catalytic activity puts DDHD2 into context with a number of recently discovered HSP genes involved in lipid metabolism. Both seipin (*SPG17*)<sup>22</sup> and spartin (*SPG20*)<sup>23</sup> are involved in formation and regulation of lipid droplets. These dynamic organelles consist of a core of neutral lipids that are covered by a monolayer of amphiphatic lipids containing cholesterol and phospholipids. Phosphatidic acid, preferred substrate for DDHD1 and DDHD2, is essential for lipid droplet assembly.<sup>24</sup> The acetyl-CoA transporter *SLC33A1* located in the ER membrane and required for the formation of O-acetylated gangliosides is mutated in *SPG42*. Defects in the metabolism of complex lipids cause at least four more HSP subtypes: in *SPG39* the deacetylation of phosphatidylcholine, the major membrane phospholipid, is defective due to mutations in phospholipase B/neuropathy target esterase (*PNPLA6*),<sup>7</sup> mutations in fatty acid-2 hydroxylase (*FA2H*) affect synthesis of 2-hydroxysphingolipids in



**Table 1 Clinical features of genetically confirmed DDHD2 patients**

Identifier	Gender	Age at onset	Age at examination	Brisk tendon		Extensor plantar response	Bladder disturbance	Sensory deficits	Other signs/symptoms	Imaging	Neurophysiology
				Spasticity, UL/LL	reflexes, UL/LL						
THI26003-3	M	~3	25	-/+	+/+	+	-	None	Mental retardation short stature high arched palate hypertelorism	TCC	NCV normal; SEP/MEP: prolonged latencies to the LL
THI26003-4	F	6	19	-/+	+/+	+	-	Vibration sense	Mental retardation saccadic eye pursuit short stature high arched palate IgA deficiency	TCC	NCV normal; SEP/MEP normal
IHG25194-3	M	0	2	-/+	+/+	+	Unknown	Unknown	Mental retardation short stature antemongoloid slant, telecanthus	TCC	Not done
IHG25194-4	M	0	9	-/+	+/+	+	Unknown	Unknown	Mental retardation short stature	TCC	NCV normal; SEP/MEP normal

Abbreviations: F, female; LL, lower limbs; M, male; MEP, motor evoked potentials; NCV, nerve conduction velocity; SEP, sensory evoked potentials; TCC, thin corpus callosum; UL, upper limbs; WMC, white matter changes.

SPG35<sup>25</sup> and *CYP2U1* mutations in SPG49 lead to disturbed  $\omega$ - and  $\omega$ -1 fatty acid hydroxylation.<sup>3</sup> In *GBA2*, mutated in autosomal recessive HSP SPG46, the conversion of glucosylceramide to free glucose and ceramide by the non-lysosomal glucosylceramidase is deficient.<sup>6</sup> In SPG5 mutations in the  $7\alpha$ -hydroxylase gene *CYP7B1* not only lead to accumulation of oxysterols in SPG5 patients<sup>26</sup> but may also influence neurosteroid metabolism.<sup>27</sup>

During preparation of this manuscript Schuurs-Hoeijmakers et al<sup>28</sup> reported mutations in *DDHD2* in four families with autosomal recessive HSP. The phenotype in these families is strikingly similar to the clinical features reported in families THI26003 and IHG25194 with early onset spasticity, mental retardation and TCC being the overlap between all affected family members. The seven DDHD2 mutations described in Schuurs-Hoeijmakers et al<sup>28</sup> and our study comprise four frameshift, two nonsense and only one missense mutation so far (Figure 1). Of note, most mutations cluster in the DDHD domain, located in the C-terminal half of the protein. Further studies will have to clarify whether a toxic gain of function of potentially expressed truncated or mutant DDHD2 protein contributes to the phenotype. The nonsense mutation Arg287\* that we identified in the Iranian family THI26003 has been described by Schuurs-Hoeijmakers et al<sup>28</sup> in another Iranian family; a founder effect is therefore possible.

The identification of mutations in *DDHD2*, which is involved in Golgi-/ER membrane trafficking and lipid metabolism further demonstrates the critical roles of these essential cellular processes in motor neuron function and helps to understand the molecular mechanisms underlying the pathogenesis of HSPs.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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