Clinical and molecular heterogeneity of *VPS13D*-related neurodevelopmental and movement disorders

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Abstract

Background: The VPS13 family of proteins has been implicated in lipid transport and trafficking between endoplasmic reticulum and organelles, to maintain homeostasis of subcellular membranes. Recently, pathogenic variants in each human *VPS13S* gene, have been linked to distinct human neurodevelopmental or neurodegenerative disorders. Within the *VPS13* family of genes, *VPS13D* is known to be implicated in mitochondria homeostasis and function.

Methods: We investigated a Pakistani sibship affected with neurodevelopmental impairment and severe hyperkinetic (choreoathetoid) movements. Whole exome sequencing (WES) and Sanger sequencing were performed to identify potential candidate variants segregating in the family. We described clinical phenotypes and natural history of the disease during a 3-year clinical follow-up and summarized literature data related to previously identified patients with *VPS13D*-related neurological disorders.

Results: We identified by WES an homozygous non-synonymous variant in *VPS13D* (c.5723 T>C; p.Ile1908Thr) as the potential underlying cause of the disease in our family. Two young siblings developed an early-onset neurological impairment characterized by global developmental delay, with impaired speech and motor milestones, associated to hyperkinetic movement disorders as well as progressive and non-progressive neurological abnormalities.

Conclusion: In this study we delineated the heterogeneity of *VPS13D*-related clinical phenotypes and described a novel *VPS13D* homozygous variant associated with severe neurological impairment. Further studies will be pivotal to understand

the exact *VPS13D* function and its impact on mitochondria homeostasis, brain development and regulation of movements, to further clarify genotype-phenotype correlations and provide crucial prognostic information and potential therapeutic implications.

Word count: 245

Keywords: *vacuolar protein sorting 13*; *VSP13D; epilepsy; neurodevelopmental disorders; movement disorders; chorea; mitochondria function*

Introduction

The vacuolar protein sorting 13 D (VPS13D) belongs to the VPS13 family of proteins, highly conserved in eukaryotes (Durand et al., 2022) and involved in mitochondrial integrity and function and in peroxisome biogenesis (Anding et al., 2018; Baldwin et al., 2021; Insolera et al., 2021) (Figure 1A). The VPS13 family of proteins is implicated in lipid transport, trafficking and sorting between organelles such as mitochondria and peroxisomes to maintain physiological homeostasis of sub-cellular membranes (Dall'Armellina et al., 2023; Insolera et al., 2021). The VPS13- proteins share high homology with proteins involved in lipidic balance such as perilipins, also involved in the interaction with sub-cellular membranes of different organelles (Dall'Armellina et al., 2023).
Neurons are cells with high energy demand and rely on mitochondrial function and integrity to ensure their homeostasis (Insolera et al., 2021). Interestingly, biallelic pathogenic variants in genes encoding different VPS13 proteins, (*i.e.*, VPS13A, VPS13B, VPS13C and VPS13D) have been implicated in neurological disorders with variable associated clinical features (Dall'Armellina et al., 2023).

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The VPS13A protein acts as intermembrane bridge between mitochondria and endoplasmic reticulum (ER) and mediates lipidic transfer. Biallelic *VPS13A* variants have been associated with an autosomal recessive neurodegenerative disorder, characterized by global developmental delay, hyperkinetic movements (chorea and dystonia), frequent (and often refractory) epilepsy, and a (peculiar) abnormal erythrocyte morphology (*i.e.*, acanthocytosis) referred as 'choreoacanthocytosis', (**MIM**: 200150) (Peikert et al., 2018; Weber et al., 2019). The VPS13C protein presents biological affinity to VPS13A, is located at ER-lipid droplet and endo-lysosome junctions and has been associated with an autosomal recessive early-onset Parkinsonism with a rapid and severe disease progression and premature cognitive decline (Dall'Armellina et al., 2023; Lesage et al., 2016) (**MIM**: 616840). Furthermore, biallelic *loss of function* (LoF) variants in *VPS13B* have been related to Cohen syndrome (MIM: 216550), an autosomal recessive disorder characterized by the variable combination of microcephaly, global developmental delay, distinctive cranio-facial features, retinal dystrophy, and intermittent neutropenia (Kolehmainen et al., 2003; Rodrigues et al., 2018). *VPS13D* has been associated with spastic paraplegia or spastic ataxia (OMIM 607317), with 33 cases described to date (Durand et al., 2022; Gauthier, Meijer, Lessel, et al., 2018; Huang & Fan, 2022; Koh et al., 2020; Öztop-Çakmak et al., 2022; Pauly et al., 2023; Petry-Schmelzer et al., 2021; Seong et al., 2019). Patients with *VPS13D*-related disorders usually develop an ataxic and/or spastic gait disorder with variable age at onset and other associated clinical features, including neurodevelopmental impairment with intellectual disability, hyperkinetic or abnormal movements such as chorea or dystonia, neuropathy, tremors, oculomotor abnormalities with frequent saccadic intrusion, chorioretinal dystrophy and abnormalities on brain imaging such as a Leigh-like leukoencephalopathy pattern (Huang & Fan, 2022; Koh et al., 2020; Pauly et al., 2023; Swartz et al., 2002).

Disease-causing variants in *VPS13A-C* are frequently biallelic truncating and result in null mutations with no detectable protein; VPS13D is the only member of VPS13 family protein to be essential in mitochondrial homeostasis and its complete loss of function appear to be lethal and probably not compatible with life in humans (Durand et al., 2022; Dziurdzik et al., 2020). Thus, compound heterozygous truncating and missense variants or homozygous missense variants are the ones most frequently observed in individuals affected with *VPS13D*-related disorders (Durand et al., 2022). Also deletions and duplications have been described, without an evidence of a clear genotype-phenotype correlation (Pauly et al., 2023).

VPS13D, the fourth member of the protein family, maps to chromosome 1p36.22 between the positions 36.22 and 36.21, and it contains 69 exons, encoding 4,388 amino acids (Figure 1B). Evidences from previous animal studies highlighted the importance of *VPS13D* in the early stages of development, and the knockout (KO) of this gene in mice and Drosophila Melanogaster models, leads to lethal outcomes in the embryonic stages (Seong et al., 2019).

Specifically, VPS13D protein resulted crucial for maintain mitochondria homeostasis, modulating mitochondrial fission, mitophagy and phagophore elongation in several cell types (Insolera et al., 2021; Pickles et al., 2018). Mitochondrial fission is mainly regulated by inter-organelles phospholipid transport, mediated by VPS13D protein, located at ER-mitochondria and ER-peroxisomes junctions. Therefore, VPS13D protein is involved in mitochondria clearance and its loss of function leads to fission impairment with mitochondria damage, that induces mitophagy. Similarly, *VPS13D* loss of function has been associated with phagophore elongation disruption with accumulation of stalled mitophagy intermediates and lack of matrix content (Burté et al., 2015; Insolera et al., 2021; Misgeld & Schwarz, 2017). In this study, we identified an homozygous missense variant on VPS13D

protein (c.5723 T>C; p.Ile1908Thr), inherited with an autosomal recessive model of inheritance from their heterozygous parents (WT/ c.5723 T>C, p.Ile1908Thr), leading to the substitution of an Isoleucine (Ile) amino acid to a Threonine (Thr) at the exon 22 of the *VPS13D* gene.

Our autosomal recessive homozygous missense *VPS13D* variant was found in two siblings affected with neurodevelopmental delay, epilepsy, choreo-athetosis, oculomotor anomalies and cardiac abnormalities. We also provide a literature survey to compare and expand both the molecular and the clinical spectrum, associated with *VPS13D* variants.

Material and methods

Patient recruitment, clinical and imaging phenotyping

We recruited as part of the SYNaPS Study Group collaboration, 2 individuals affected with neurodevelopmental impairment, seizures, cardiac abnormalities and a severe choreo-athetoid movement disorder. We compared their phenotypic, radiological and genetic data with the 33 *VPS13D*-mutated individuals reported so far (Table 1). The family involved in the study provided informed consent for being part of this study. The study was approved by the Ethical Committee at the participating centers.

Genetic studies

We collected blood samples from the two patients and their parents, and extracted DNA using standard procedures. To investigate the genetic cause of the disease, WES was performed in both the affected patients (Figure 1C). Nextera Rapid Capture Enrichment kit (Illumina) was used according to the manufacturer instructions. Libraries were sequenced in an Illumina HiSeq3000 using a 100-bp paired-end reads protocol. Sequence alignment to the human reference genome (*VPS13D*), and variant calling, and annotation were performed as described elsewhere (Mencacci et al. 2016). In total, 72,881,112 (II-1) and 70,866,620 (II-2) unique reads were generated. After removing all synonymous changes and variants not segregating between the two probands and the unaffected parents, we filtered single nucleotide variants (SNVs) and indels, only considering exonic and donor/acceptor splicing variants. In accordance with the pedigree and phenotype, priority was given to rare variants (<1%) in public databases, including 1000 Genomes project, NHLBI Exome Variant Server, Complete Genomics 69, and GnomAD database)] that were fitting a recessive model.

Results

Identification of the homozygous p.Ile1908Thr VPS13D variant

We identified an ultra-rare and potentially damaging homozygous variant in the affected siblings, c.5723 T>C that causes the change of Isoleucine (Ile) amino acid to Threonine. This emerged as the most likely explanation for the disease pathogenesis as supported by existing reports, linking *VPS13D* pathogenic variants to clinical and neurological phenotypes with frequently associated neurodevelopmental and movement anomalies (Gauthier, Meijer, Rossignol, et al., 2018; Seong et al., 2019). Segregation analysis by traditional Sanger sequencing

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[primers used: forward [(5'-CCTTGATTGCTCTGCCGTTT-3') and reverse (5'-ACACCTTACTCAGCACGTGA-3')] confirmed the autosomal recessive mode of inheritance with the probands carrying the homozygous variant and the healthy parents being carriers of the variant at the heterozygous state (Figure 1C). The identified mutation which is a missense variant leading to an amino acid change (p.Ile1908Thr) is located at exon 22.

Clinical phenotypes of patients carrying the homozygous VPS13D p.Ile1908Thr variant

The two affected individuals are siblings, part of a Pakistani family from Punjab (Lahore). These are a 5.5 years old boy and a 4-year-old girl, born from second-cousin parents. Both children presented global developmental delay with a speech disorder. They showed the first clinical features at two-three months of age, when a diplopia and a form of convergent strabismus presented by the little girl and a divergent form by the brother were noted (figure 1B). They didn't achieve neurodevelopmental milestones, and appeared unable to maintain the head, also showing a moderate axial hypotonia. The siblings quickly developed a hyperkinetic -choreoathetoid- movement disorder at 2.6 years and 10 months of age, respectively. It was characterized by slow involuntary movements of flexion-extension of the hands' fingers, flexion-extension and prono-supination of the ankles and tongue protrusion, observed at rest as well as during voluntary activity. Movements appeared continuous and fluctuating from one part of the body to the other, configuring a choreo-athetosis disorder more severe in the 2 years and 6 months old patient. Computed tomography (CT) and brain magnetic resonance imaging (MRI) studies were performed at 4 and 5.5 years of age, respectively, and documented a normal

brain conformation with adequate myelination for the age, without signal abnormalities in both affected children. Furthermore, an EEG was performed and characterized by a background activity dominantly in the range delta (2-3 Hz) and theta (6-7 Hz), with intermixed small amount of low voltage fast beta activity over both hemispheres. Sleep spikes were present but poorly performed, K-complex were seen. Globally was described a multifocal epileptiform activity with interictal epileptic discharges congruent with an epilepsy diagnosis reported in both siblings. They developed different type of seizures, starting at 1 year of age, including tonic-clonic and myoclonic seizures, controlled by one antiseizure medication (ASM) (*i.e.*, levetiracetam). In addition, a transposition of great arteries with intact inter-ventricular septum and a regressed left ventricular size were described at echocardiography exam of the young boy.

At the last neurological examination of the two siblings, at the age 5.5 and 4 years, respectively, they were seizure-free and presented with neurodevelopmental impairment, including speech delay, hypotonia and unability to walk, in addition to oculomotor abnormalities and choreoathetosis.

Literature revision of VPS13D-mutated individuals

We summarized the clinical phenotypes associated with *VPS13D* biallelic variants reported so far in the literature. Thirty-three individuals (14 males; 48%) were examined. The mean age at onset was 20 years (range: 0-63 years). The first symptom was gait disturbance in 20 patients (60%), tremor in 2 patients (6%), intellectual disability, chorioretinal dystrophy, and hypotonia in 3 cases (9%) while 3 individuals and both our siblings showed neurodevelopmental delay as first feature of the disorder. Ataxia of lower and/or upper limbs was reported in 23 patients (69 %) while

spasticity of upper and/or lower limbs with Babinski sign was detected in 9 patients (27%) and 22 patients (66%), respectively. Paresis was described in 10 probands (30%) and ambulation appeared independent only in five patients (15%). Seven probands (21%) presented a movement disorder such as dystonia and/or chorea while our siblings showed choreo-athetosis. Furthermore, hyperreflexia was present in 24 patients (72%) while tremor was detected only in 3 patients (9%). 11 patients (33%) developed dysarthria while 8 (21%) and 3 (9%) probands showed oculomotor square waves jerks and nystagmus, respectively. Rarely, an epileptic phenotype has been reported in patients with *VPS13D*-related disorders (percentage was not available). Specifically, in one study (Lee et al., 2020) were described two clinical cases of two 4-year-old patients who developed seizures, promptly controlled by administration of one ASM. All patients underwent brain MRI, which was abnormal in 15 patients (45%). Brain anomalies included: cerebellar atrophy (n=11, 33%) and leukoencephalopathy (n = 4, 12%).

Please refer to Table 1 for more details.

Discussion

In the last decade, the advent of next-generation sequencing (NGS) technologies led to an increased understanding of monogenic causes underlying many pediatric neurodevelopmental and movement disorders (Dias et al., 2019; Niestroj et al., 2020; Steel et al., 2015). These discoveries shed a new light on several emerging pathways (e.g., cAMP metabolism, synapse regulation and physiology) and their implication in pediatric hyperkinetic movement disorders (Niccolini et al., 2018; Salpietro et al., 2018). Furthermore, different genetic mechanisms related to brain developmental disorders, have been recently identified, highlighting the importance of the screening

process of genes, potentially implicated in these conditions (Baldassari et al., 2020; Donkervoort et al., 2020; Manole et al., 2020; Neuray et al., 2020).

During the last decade, VPS13 family proteins have been found to be critically involved in biogenesis and homeostasis of sub-cellular organelles such as mitochondria and peroxisomes, modulating phospholipidic transport, sorting and trafficking between ER-membranes and organelles (Bean et al., 2018; Dall'Armellina et al., 2023; Friedman et al., 2011; Kumar et al., 2018). Specifically, neurons are high energy cells, particularly sensitive to mitochondria disturbance and organelle dis-homeostasis (Insolera et al., 2021). Mutations in *VPS13*s genes have been, therefore, associated with neurodevelopmental and/or neurodegenerative disorders (Velayos-Baeza et al., 2004).

Interestingly, *VPS13D* seems to be the most important gene involved in cell viability. Specifically, among the VPS13 proteins, the knockout of only *VPS13D* appears embryonically lethal in both mice and flies (Anding et al., 2018; Seong et al., 2018; Ugur et al., 2020; Vonk et al., 2017), further highlighting the importance of this paralog and its intolerance to genetic variations. This protein has been found to be essential for mitochondria fission, mitophagy and phagophore elongation in some cell types (Insolera et al., 2021).

To date, *VPS13D* mutations described are exonic autosomal recessive (compound heterozygous or homozygous) missense and/or nonsense, with also duplications and/or deletions reported, scattered in the whole coding region of the gene, leading to a variable loss of function of the protein. Recently, intronic pathogenic and biallelic *VPS13D* variants have been described as well, resulting in altered splicing with loss of function of *VPS13D* (Pauly et al., 2023). Functional studies demonstrated how these variants disrupted mitochondria integrity at different levels, particularly leading to a neuronal disfunction in several key cerebral structures such as cerebellum, basal ganglia and neocortex.

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Clinical phenotypes related to *VPS13D* pathogenic variants appeared distinctly neurologic with variable additional features, and include spastic ataxia and/or paraplegia, hyperreflexia, Babinski and Hoffman signs, movement disorders such as chorea, dystonia, athetosis, and tremor.

Myoclonus, intellectual disability, neurodevelopmental delay, leukoencephalopathy Leigh-like and oculomotor disfunction have been described, as well (Pauly et al., 2023). Rarely, patients with *VPS13D* pathogenic variants presented with an epileptic phenotype (Gauthier, Meijer, Lessel, et al., 2018; Seong et al., 2018). The onset age was particularly variable. Both our patients exhibited seizures, and their phenotype was consistent with those previously reported (Lee et al., 2020), and well controlled by the ASM administered. The pathophysiology of seizures related to *VPS13D* pathogenic variants is likely related to mitochondrial dysfunction, affecting neuronal cells.

Specifically, neurons mainly use an aerobic metabolism to maintain the membrane polarization, mediated by neuronal ion channels. Both mitochondrial impairment and mitophagy, leading to an ATP depletion, probably represent the pivotal cause underlying seizure onset in the affected individuals. Specifically, GABAergic interneurons are more prone to defective OXPHOS with consequent decrease of GABA-mediated inhibition and suppression of hippocampal-mediated inhibitory activity. Other mechanisms likely involve the increase of glutamate release in the synaptic cleft, with resulting neuronal hyperexcitability. Seizure types associated to *VPS13D*-related neurological disorders are variable and included myoclonic, focal and generalized tonic–clonic seizures. The management of seizures includes current available ASMs, with no targeted therapies yet identified. Levetiracetam, zonisamide, lacosamide, gabapentin, rufinamide, and stiripentol are all considered safe options, whereas oxcarbazepine, phenytoin or phenobarbital could worsen

myoclonus, and lamotrigine could exacerbate myoclonic seizures. Valproate should be avoided to its known mitochondrial toxicity (Lopriore et al., 2022).

Interestingly, *VPS13D*-related phenotypes have been differentiated into two groups, related to the age at onset. The first phenotype group is characterized by an early-onset of delated developmental milestones (since the neonatal period or during early infancy), intellectual disability (ID), neurological abnormalities including spastic paraplegia and leigh-like leukoencephalopathy (with evolving brain MRI abnormalities and progressive loss of autonomous deambulation), movement disorders including as chorea/choreoathetosis, dystonia and ataxia. The second phenotype group is characterized by a normal neurodevelopment and usually an adult-onset of the disease, which is associated to a non-progressive spastic paraplegia and/or ataxia with tremor/dystonia (Durand et al., 2022).

Concerning the radiological spectrum related to *VPS13D* pathogenic variants, a wide array of patterns has been described. Brain MRI findings, when available, often delineated a picture of 'leigh-like' syndrome (Gauthier, Meijer, Lessel, et al., 2018). Lesions included the brainstem, affecting the medulla oblongata, pons and midbrain (periaqueductal white matter, tegmentum and/or both). Anomalies were also found in basal ganglia, including the caudate nuclei, putamen, globus pallidus, thalami, cerebral cortex, and dentate nuclei of the cerebellum. More rarely, global atrophy, delayed myelination and, multifocal white matter involvement, have also been reported. Lesions usually appeared as symmetrical hyperintensities on T2-weighted image sequences and evolved. However, follow-up imaging studies showed that some patients presented regression/decreased size or complete resolution of the lesions, whereas others developed MRI anomalies later, suggesting that a normal brain MRI could not exclude the diagnosis (Bonfante et al., 2016).

The affected individuals from the present study underwent a brain MRI at 4 and 5.5 years of age, respectively, when the myelination process is almost completed. However, their normal neuroimaging might be transient, and a follow-up imaging study could be crucial in such patients, to detect potential late-onset lesions.

In our study, the affected boy developed a more severe disorder characterized by choreoathetosis, epilepsy, neurodevelopmental delay, speech impairment, oculomotor dysfunction and congenital hearth defects (cyanotic heart disease TGA with intact septum), while the affected girl had a milder presentation with neurodevelopmental delay, oculomotor abnormalities, seizures and choreoathetosis. WES analyses revealed a segregating homozygous p.Ile1908Thr variant of the *VPS13D* gene in both affected siblings, inherited inherited from their healthy heterozygous parents.

In spite of distinct clinical phenotypes with variable severity level related to *VPS13D* pathogenic variants, no clear phenotype-genotype correlation has been found to date. We could suppose that *VPS13D* pathogenic variants might differentially lead to subcellular processes disruption, resulting in a wide clinical spectrum with a minor or major impairment of neurologic functions.

The early-onset disease in our family, is probably associated with the severe neurodevelopmental impairment that we observed in the affected siblings. Moreover, the affected boy also showed a severe cardiac defect, which has not been associated before to *VPS13D* biallelic variants. Even though the *VPS13D* gene is known to be highly expressed in several tissues, including the heart, the VPS13D protein was not detected in heart undifferentiated cells and in cardiomyocytes (Kopanos et al., 2019; The UniProt Consortium et al., 2023). WES revealed a novel *VPS13D* homozygous non-synonymous variant as the underlying cause of the disease in our two patients. No other pathogenic or plausible variants that could explain the different clinical

phenotypes of our patients (including the cardiac defects), have been detected. However, other potential factors cannot be excluded in the phenotypical outcome of our affected individuals and these include possible environmental factors and/or epigenetic modifiers of the disease.

Functional studies of the homozygous p.Ile1908Thr variant could shed a light on the potential mechanisms related to the disruption of cellular processes, possibly explaining the clinical phenotype observed in this family. In this study we described two patients with a novel *VPS13D* homozygous missense variant related to a broad clinical phenotype, which expands the clinical spectrum of *VPS13D*-related disorders. We also surveyed the literature and delineated clinical phenotypes associated so far with *VPS13D* pathogenic biallelic variants. Importantly, in the last decade a wide array of genes and pathways emerged as crucial in the field of hyperkinetic early-onset movement disorders with associated neurodevelopmental impairment (Dworschak et al., 2021; Epi25 Collaborative). These advances from disease gene discovery studies gave us a deeper knowledge of the rare conditions and the cellular and sub-cellular mechanisms that need to be targeted in the future towards the development of personalized strategies for disease prevention and/or management of neurodevelopmental conditions (Iacomino et al., 2020; Wiessner et al., 2021).

In the field of *VPS13*-related neurological disorders, further research work will be required in order to fully dissect *VPS13D* function and how genetic defects of this protein may affect both mitochondria homeostasis and morphology and brain development. This will shed a new light to clarify in deep genotype-phenotype correlations with prognostic and possibly therapeutic implications.

Conflict of Interest

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