SHORT COMMUNICATION

**A homozygous splice variant in *AP4S1* mimicking NBIA**

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**Abstract**

Adaptor protein complex 4 is a member of adaptor protein complexes functioning in vesicular transport. Using exome sequencing, we identified a homozygous splice site variant in *AP4S1* in a patient with a neurodegenerative disease course initially presenting with marked ataxia. Brain MRI at the age of 13 years showed bilateral alterations in the globus pallidus compatible with an iron accumulation, suggesting neurodegeneration with brain iron accumulation (NBIA). This case adds to the clinical and radiological definition of AP4 deficiency and emphasizes the efficacy of exome sequencing in unclear neurodegenerative paediatric disease. The spectrum of NBIA may be expanded by AP4 deficiency.

**Introduction**

Adaptor protein (AP) complex 4 is one of the five known members of the highly conserved family of heterotetrameric adaptor protein complexes (AP1-5) functioning in vesicular transport by cargo selection and formation of coated vesicles for subsequent delivery to post-Golgi compartments. The structural composition of AP complexes is uniform (each containing two large [β 1-5, and γ, α, δ, ε or ζ], one medium [μ 1-5], and one small [σ 1-5] subunit) with a loss of any subunit likely resulting in destabilisation of the entire complex. All five complexes are widely expressed in eukaryotes. In mammals there is evidence for a tissue-specific expression of different isoforms. In mice, knock out of several AP1 or AP2 subunits is embryonic lethal ([1](#_ENREF_1)) ([2](#_ENREF_2)), while a knock out of the AP4 subunit AP4 β 1 leads to a very mild phenotype ([3](#_ENREF_3)). In humans pathogenic variants in genes coding for structural subunits of complexes AP1-3 and AP5 have been associated in at least 20 families with clinically heterogeneous presentations and patterns of autosomal dominant (,OMIM #600740( *AP2S1)*), autosomal recessive (OMIM #609313 (*AP1S1*), *O*MIM #608233 (*AP3B1*), OMIM #613647 (*AP5Z1*)) as well as X-linked (OMIM #304340 (*AP1S2*)) inheritance. In addition, 15 families with 33 affected individuals have been reported to carry bi-allelic variants in genes encoding subunits of the AP4 complex (OMIM #614066 (*AP4B1*), OMIM #613744 (*AP4E1*), OMIM #612936 (*AP4M1*), and OMIM #614067 (*AP4S1*)) ([4](#_ENREF_4)) ([5](#_ENREF_5)) ([6](#_ENREF_6)) ([7](#_ENREF_7)) ([8](#_ENREF_8)) ([9](#_ENREF_9)) ([10](#_ENREF_10)) ([11](#_ENREF_11)) ([12](#_ENREF_12)). Main clinical features are spastic paraplegia and severe intellectual disability (ID). Due to similar clinical signs and symptoms observed in AP4-defective patients it has been proposed to subsume this group of disorders as AP4 deficiency syndrome ([7](#_ENREF_7), [12](#_ENREF_12)). However, besides spastic paraplegia and severe intellectual disability, which are consistent findings, a number of additional phenotypic features have been reported including but not limited to facial dysmorphism, microcephaly, shy character, stereotypic laughter, and short stature. Microcephaly and stereotypic laughter appear in the majority of cases and short stature is reported in approxemitely half of them. Epilepsy and facial dysmorphic features show up variably ([4](#_ENREF_4), [5](#_ENREF_5)) ([8](#_ENREF_8)) ([12](#_ENREF_12)) ([13](#_ENREF_13)). Neuroimaging findings, so far reported in a total of eight AP4-deficient patients, are rather heterogeneous. Frequent alterations include asymmetrical mild to massive ventriculomegaly, thin splenium of the corpus callosum, delayed myelinisation, and white matter loss. Cerebral and cerebellar atrophy appear occasionally. Tortuosity of vessels, absence of the corpus callosum, involvement of basal ganglia, and prominent cisterns are reported in single cases ([5](#_ENREF_5)) ([6](#_ENREF_6)) ([8](#_ENREF_8)) ([9](#_ENREF_9)). Iron accumulation is not reported in AP4-deficient patients, only brain calcification has been reported in a family with AP1-deficient patients ([14](#_ENREF_14)).

Here we report on a female patient with a homozygous *AP4S1* splice variant associated with a neurodegenerative clinical course and MRI findings compatible with brain iron accumulation, leading to the diagnosis of neurodegeneration with brain iron accumulation (NBIA).

**Materials & Methods**

*Exome sequencing*

Exome sequencing and variant filtering was performed as described previously ([15](#_ENREF_15)), using a SureSelect Human All Exon 50Mb V5 Kit (Agilent, Santa Clara, CA, USA) for enrichment of coding DNA sequences and a HiSeq2000 system (Illumina, San Diego, CA, USA) for sequencing. Reads were aligned to the human reference assembly (hg19) using BWA (version 0.5.8) and single-nucleotide variants (SNVs) and small insertions and deletions were identified with SAMtools (version 0.1.7). The average coverage was 131-fold and 97% of the target region was covered at least 20-fold.

**Results**

*Clinical synopsis*

The female patient was born at 39+3 weeks of gestation after a normal pregnancy and spontaneous vaginal delivery as the first child of healthy, non-consanguineous parents from Kosovo. Birth weight was 3020 g (25-50th WHO percentile,), length 49 cm (50th WHO percentile,) and head circumference 32.5 cm (3-15th WHO percentile). Two younger siblings are healthy and no neurological disorders have been reported in the family. Psychomotor retardation was recognized within the first year of life. The girl achieved sitting at the age of twelve months and started walking at the age of 24 months. Independent walking was possible at the age of 36 months. By this time the neurological examination revealed a developmental deficit with marked ataxia and discreet signs of upper motor neuron involvement with positive Babinski sign and slightly increased muscle tone of the legs. Orthotic care was initiated from the age of four years on. In the course of the disease, motor function decreased significantly with progressive spastic paraplegia. At the age of 6 years the walking distance remained only 20 meters. She lost independent ambulation by the age of nine years and at the age of thirteen years, walking with an anterior or posterior walker was only possible for a few meters and she was almost completely wheelchair-dependent.

At the age of 14 years, her expressive language was poor and she only used words for “mom” and “dad” and protowords for her siblings and a ball. Understanding appeared to be a little better and she was able to follow simple commands. Communication was also done by simple gestures. She had no cerebral seizures. Prolonged attacks of laughter were reported by her parents. Her stature was short with 142 cm at her 14th birthday which is 3 cm below the 3rd percentile; head circumference was normal. She underwent surgery for strabismus. There were no signs of facial dysmorphism.

Laboratory investigations in blood (creatine kinase, lactate, amino acids, copper, coeruloplasmin, transferrine electrophoresis, lipoproteins, very long-chain fatty acids, phytanic acid, alpha-fetoprotein, thyroid markers, acylcarnitines), CSF (amino acids, pterines, lactate, glucose), and urine (complex carbohydrates, sulphite, lactate, organic acids) were normal and there was no evidence of CNS infection with cytomegaly virus, toxoplasmosis, or herpes simplex virus.

*Neuroimaging*

Cerebral MRI at the age of 2.3 years revealed mild delay of myelination and a thin corpus callosum, especially of the splenium. At the age of 13 years, imaging showed no relevant atrophy; myelination was completed. However there were bilateral hypointense signal alterations in T2-weighted sequences in the globus pallidus, compatible with brain iron or copper accumulation (Figure 1). T1-weighed sequences showed no pathological signal in the basal ganglia. CT scan showed no evidence of calcifications.

*Genetic studies*

Genetic testing by Sanger excluded pathogenic variants in *CDKL5*, *MECP2, C19orf12,* and *PLA2G6*. Methylation analysis of the *UBE3A* locus showed normal patterns.

*Exome sequencing identifies a homozygous AP4S1 splice site variant*

Assuming an autosomal recessive mode of inheritance, we searched for genes affected by homozygous or predictively compound heterozygous non-synonymous variants with a minor allele frequency >0.1 % in 6,000 in-house control exomes. This analysis identified 8 genes with *AP4S1* (NM\_007077.4) being the only gene listed in OMIM (\*607243, phenotype key 3) and carrying two predicted loss-of-function alleles. We identified the predicted splice site variant c.138+3\_6delAAGT, p.(?) in a homozygous state. Sanger sequencing confirmed the variant in a homozygous state in the patients with both healthy parents being heterozygous carriers. This variant was absent from 6,000 in-house control exomes and >120,00 alleles of the Exome Aggregation Consortium (ExAC) browser (Cambridge, MA; URL: http://exac.broadinstitute.org) [06/2015]). This variant changes the splice donor site of intron 2 and is predicted to severely impair splicing efficacy. Only very recently Hardies *et al.* published the presumed frameshift variant c.137\_140delAATG, p.(Arg46Phefs\*9) compound heterozygous with a nonsense variant c.289C>T, p.(Arg97\*). They demonstrated that these variants result in an absence of the *AP4S1-*encoded subunit σ4 in patient’s fibroblasts and a concomitant reduction of all other AP4 subunits. These findings are in line with the hypothesis that a loss of σ4 results in the destabilization of the entire heterotetrameric complex. On the basis of the electropherograms provided in the manuscript by Hardies *et al.* we concluded that the presumed variant c.137\_140delAATG, p.(Arg46Phefs\*9) indicates the same change detected in a homozygous state in the patient presented in this manuscript. This notion has been confirmed in a personal communication. When referring to the plus strand we suggest that this 4 bp deletion should read as c.138+3\_6delAAGT, p.(?). Along this line there is functional evidence that the identified splice variant results in virtually absent amounts of AP4S1 protein with detrimental consequences for the assembly of the AP4 complex.

**Discussion**

We present a child with neurodegenerative disorder and MRI findings suggestive of brain iron accumulation, in which exome sequencing revealed a pathogenic homozygous mutation in *AP4S1*, encoding Adapter 4 Protein complex subunit 1.

Up to now, 15 families with bi-allelic variants in genes encoding subunits of the AP4 complex *AP4B1*, *AP4E1*, *AP4M1*, and *AP4S1* have been reported. Spastic paraplegia and severe intellectual disability are consistent findings. Among them, five affected individuals from two families with AP4S1 deficiency have been described. One family with AP4S1 deficiency originating from a consanguineous Israeli–Arab family carried a homozygous nonsense variant in *AP4S1* (c.124C>T, p.(Arg42\*)). Besides progressive spastic paraplegia and severe ID with almost absent speech, they presented with shy character, stereotypic laughter, short stature, microcephaly, and discreet facial dysmorphism, including a prominent and bulbous nose, a wide mouth, and coarse features ([7](#_ENREF_7" \o "Abou Jamra, 2011 #11)). In the second family, two affected siblings from non-consanguineous, caucasian parents carried a nonsense variant c.289C>T, p.(Arg97\*) compound heterozygous with the same c.138+3\_6delAAGT, p.(?) splice variant which we identified in our patient. Both siblings presented with spastic paraplegia, truncal hypotonia, severe mental retardation, and epilepsy with fever-sensitive seizures. Facial dysmorphism was more pronounced but very different from the first family with a broad nasal bridge, hypertelorism with telecantus, arched eyebrows, bulbous nose, short philtrum, wide mouth, full lips, and high palate ([6](#_ENREF_6)).

Information on neuroimaging studies was available of two female siblings of the two families carrying clinically relevant AP4S1 variants. Brain MRI showed a dilation of the lateral ventricles and hypoplasia of the posterior portion of the corpus callosum in one and a non-progressive hydrocephalus with severe reduction of subcortical white matter and absence of the corpus callosum in the other sister.

Our patient showed a thin splenium of corpus callosum and delayed myelination in a first MRI, performed at the age of 28 months. Ventricles were bilaterally symmetrical and showed age-appropriate dimensions. At the age of thirteen years, myelination was completed, ventricles still showed normal size and there was no loss of white matter. However, a prominent pathological finding was the bilateral T2-wheighted hypointensity in the globus pallidus (Figure 2), which in coincidence with the infancy-onset movement disorder with marked ataxia suggested a differential diagnosis of NBIA subtypes such as MPAN or PLAN.

NBIA is a heterogeneous group of disorders characterized by iron accumulation especially within the basal ganglia ([16](#_ENREF_16)). Since 2001 defects in a number of different genes have been associated with the NBIA spectrum. Mutations in *PANK2* (MIM 606157) cause pantothenate kinase-associated neurodegeneration (PKAN or NBIA1) ([17](#_ENREF_17)), defects in *PLA2G6* (MIM 256600) underlie phospholipase A2-associated neurodegeneration (PLAN or NBIA2) ([18](#_ENREF_18)). Depending on the cohort investigated, these two subtypes may represent the most common forms of NBIA ([16](#_ENREF_16)). *C19orf12* (MIM 614297) variants have been identified as the molecular correlate of mitochondrial-membrane protein-associated neurodegeneration (MPAN or NBIA4) ([19](#_ENREF_19)). *FA2H* (MIM 611026) mutations cause fatty acid hydroxylase-associated neurodegeneration (FAHN) ([20](#_ENREF_20)) and *WDR45* (MIM [300894](http://omim.org/entry/300894)) has been associated with X-linked beta-propeller protein-associated neurodegeneration (BPAN or NBIA5) ([15](#_ENREF_15)). More recently, in two patients with NBIA a missense mutation in *COASY* (MIM [615643](http://omim.org/entry/615643)) could be identified (NBIA6) ([21](#_ENREF_21)). Defects in these genes account for about 70% of suspected NBIA cases, while in the remaining 30% the molecular bases remain still to be indentified. “NBIA3” (*FTL* gene) (MIM [606159](http://omim.org/entry/606159)) is sometimes used as a synonyme of adult onset Neuroferritinopathy ([22](#_ENREF_22)). Kufor-Rakeb-Syndrome, Neuroferritinopathy, Woodhouse-Sakati-Syndrome, and Aceruloplasminemia sometimes are specified in the expanded spectrum of NBIA disorders. With clinical exome sequencing being applied routinely the number of NBIA-associated genes will continue to grow (PMID 26497993).

Our case adds to the clinical and radiological definition of AP4 deficiency and provides additional evidence for the association of *AP4S1* variants with neurodegeneration in humans. The spectrum of NBIA may be expanded by AP4 deficiency. Basic pathophysiology of the iron accumulation in the main NBIA subtypes is currently poorly understood. It has been shown that ceramide-mediated apoptosis is dependent on increased cellular iron uptake ([23](#_ENREF_23)). In patients with AP4 deficiency only limited MRI data is available. The few patients who underwent MRI showed a thin corpus callosum (especially in the splenium) and a delayed myelination but no iron accumulation. More pronounced findings like in our patient are not very consistent in other AP4 patients. Possibly the brain iron accumulation in our patient is an epiphenomenon of apoptosis as it was found in other neurodegenerative disorders ([24](#_ENREF_24)). There is an increasing body of evidence that there is a considerable overlap of neuroradiological findings between NBIA disorders and other neurodegenerative disorders such as leukodystrophies, neuronal ceroid lipofuscinosis, and other spastic paraplegias ([24](#_ENREF_24)), which makes diagnosis solely based on clinical and radiologically findings challenging.

The fact that there is an expanding spectrum of clinical and radiological findings in AP4-patients diagnosis supports the importance of broad and unbiased sequencing approaches. Besides the uncommon findings in MRI, our patient showed an unusual clinical presentation with ataxia as the leading symptom at the onset. This emphasizes the usefulness and efficacy of exome sequencing in paediatric patients with unclear neurodegenerative diseases, especially regarding the so far small number of cases in rare diseases but the expanding number of case reports since availability of exome and whole genome sequencing.

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**Figure 1:** MRI scans age 2.3 years (a: T2 Flair; b: DW/SSH; c: T1) and age 13 years: (d: T2 Flair; e: T2 Flair; f: T1).

Thin corpus callosum in the splenium from the age of 3 years on (c;f); bilateral hypointense singal alterations in T2-weighted sequences in the globus pallidus at the age of 13 years (d;e) which was not present in comparable sequences at the age of 3 years (a;b). No relevant atrophy, no ventriculomegaly.