Novel deep intronic mutation in PLA2G6 causing early-onset Parkinson’s disease with brain iron accumulation through pseudo-exon activation

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Abstract
PLA2G6 is the causative gene for a group of autosomal recessive neurodegenerative disorders known as PLA2G6-associated neurodegeneration (PLAN). We present a case with early-onset parkinsonism, ataxia, cognitive decline, cerebellar atrophy, and brain iron accumulation. Sequencing of PLA2G6 coding regions identified only a heterozygous nonsense variant, but mRNA analysis revealed the presence of an aberrant transcript isoform due to a novel deep intronic variant (c.2035-274G > A) leading to activation of an intronic pseudo-exon. These results expand the genotypic spectrum of PLAN, showing the paramount importance of detecting possible pathogenic variants in deep intronic regions in undiagnosed patients.

Keywords PLA2G6 · PLAN · NBIA · Early-onset Parkinson’s disease · Pseudo-exon activation · Deep intronic variant

Introduction
PLA2G6-associated neurodegeneration (PLAN) is a heterogeneous group of rare autosomal recessive neurodegenerative disorders caused by mutations in the PLA2G6 gene [1]. This gene encodes for iPLA2β, a group VIA calcium-independent A2 phospholipase, involved in phospholipids metabolism essential for maintaining cell membrane integrity. Depending on age of onset and clinical features, PLAN can be classified in three subtypes, including infantile neuroaxonal dystrophy (INAD), atypical neuroaxonal dystrophy (ANAD), and PARK14 autosomal recessive early-onset Parkinson’s disease (EOPD). INAD and ANAD typically occurred in childhood, often associated with cerebellar cortical atrophy and iron deposition in the brain, a condition known as neurodegeneration with brain accumulation type II (NBIA2) [2]. Contrariwise, EOPD onset is in early adulthood, typically associated with dystonia, rapid cognitive decline, psychosis, dystarthis, and pyramidal tract signs [3]. However, it has been increasingly reported that PLAN can manifest with intermediate phenotypes partially matching those classically associated with this disorder, thus preventing the identification of a precise genotype–phenotype correlation [4–6].

In general, genetic disorders causative mutations have been prevalently identified in exons and in RNA donor or acceptor splice sites. However, despite next generation sequencing (NGS) has revolutionized genetic testing, a considerable proportion of patients with a clinical diagnosis for a recessive condition have only one heterozygous mutation, suggesting the presence of not detected deep intronic variations. Intronic point mutations can activate pseudo-exons, such as intronic sequences flanked by apparently good-to-consensus acceptor and donor-site signals that are never recognized by the splicing machinery [7].

Here, we report the first case of PLAN caused by a combination of nonsense and deep intronic variants in PLA2G6 gene.
Materials and methods

This study was approved by the Ethics Committee of the Besta Institute with an informed, written consent. For sequencing of PLA2G6 transcript, PCR products were processed with Nextera XT DNA sample kit (Illumina) [8]. Real-time quantitative PCR (qPCR) was performed in a CFX-96 system (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad), with a primer pair specific for PLA2G6; the ACTB gene was used as reference. In silico predictors were used to assess the effect of variants on splicing (SpliceAI, HSF, NetGene2, NNSplice, varSEAK, MaxEntScan) [9]. The c.2035-274G>A variant was submitted to the Leiden Open Variation Database (DB-ID: PLA2G6_000182).

Results

Clinical reports

The patient is a 43-year-old lady born to non-consanguineous parents and with no family history of neurological diseases. Her symptoms started at age 32 years with gait ataxia and scanned speech, and, since age 36, a progressive asymmetric parkinsonian syndrome, with rigidity, hypokinesia, resting and postural tremor predominantly on the right, and cognitive decline. Response to L-Dopa was poor. She later developed behavioral abnormalities—irritability and occasional aggressiveness—treated with quetiapine. She progressively lost walking ability and developed dysphagia and severe akinesia.

At 43 years, examination revealed severe cognitive decline in memory, linguistic, frontal-executive, and visual-spatial skills; pursuit saccadization, vertical upward gaze palsy, marked hypomimia, almost absence of spontaneous speech, which was scanned and monotonous, dysphagia, and drooling; severely hypokinetic gait, with double support; marked axial and limb rigidity, with trocheal sign, and generalized akinesia; oromandibular and bilateral upper limb resting tremor; increased deep tendon reflexes; and bilateral Babinski sign.

Brain MRI showed T2 GRE hypointensity of the pallida, substantia nigra and head of the left caudate nucleus, cerebellar atrophy, T2 hyperintensity of cerebellar cortex and dentate nuclei, and clava hypertrophy, and diffuse cerebral atrophy with hyperintensity of the hippocampi (Fig. 1).

EEG showed some slow abnormalities in the temporal regions, more pronounced on the left side, photosensitivity,
Fig. 2 Molecular studies. a Schematic representation of the PLA2G6 gene (upper) and protein (lower), and location of variants identified in this study. Protein consists of seven ankyrin repeats (numbered circles), a proline-rich motif (P), a glycine-rich nucleotide binding motif (G), a lipase motif (PNPLA), and a binding site for calmodulin (Calmod). Numbers shown below are the amino acid positions. b Amplicons spanning exons 14–15 of the patient (P) and a healthy control (C) show a different-sized PCR product in the patient sample. c Sanger sequencing of PLA2G6 cDNA shows inclusion of a 118-nucleotide intronic sequence between exons 14 and 15 (boxed) in patient. d Sanger sequencing of the PLA2G6 gene show compound heterozygous variants. The c.109C>T variant is inherited from the father, the c.2035-274G>A variant is from the mother. e Schematic of PLA2G6 exons 14–15 showing the c.2035-274G>A variant, which substitutes a less favored G (WT) at the +4 position for a highly favored A (MUT), strengthens a naturally occurring cryptic donor splice site to activate spliceosomal inclusion of the intron 14 pseudo-exon (boxed region). f Schematic representation of PLA2G6 gene and protein resulting from pseudo-exon inclusion. g Sashimi plots of PLA2G6 cDNA sequencing data show the presence of intron 14 pseudo-exon in patient, representing about 43% of total transcript. h Relative PLA2G6 mRNA expression in control (C1, C2) and patient (P) fibroblasts. Mean of three independent experiments ± SD is shown. ***p < 0.001 (Student’s t test)
and photomyoclonic response, without clinical evidence of seizures. There was latency prolongation of the central components of somatosensory evoked potentials.

**Molecular investigations**

Sequencing of PLA2G6 coding and flanking intron sequences revealed heterozygosity for the nonsense variant c.109C>T in exon 2 of the NM_003560.4 transcript (Fig. 2a), which introduces a premature stop codon at position 37 of the iPLA2β protein (p.Arg37*). This variant is reported as pathogenic in ClinVar database (accession: RCV000023318.6) and already associated with PLAN [10, 11]. However, we could find neither a second variant in the coding regions or adjacent introns nor the presence of exon deletion or duplication in PLA2G6 by MLPA analysis. Therefore, we isolated RNA from patient’s fibroblasts and retrotranscribed it into cDNA to search for a second variant. Using intron-spanning primers pairs, we amplified an abnormal band of higher molecular weight in the patient that was not detected in controls (Fig. 2b). Sequencing of patient’s PLA2G6 transcript revealed the inclusion of a 118 nt region belonging to intron 14 (Fig. 2c). Then, we performed Sanger sequencing to explore the noncoding regions flanking the included sequence and identified a heterozygous deep intronic variant at position chr22: hg19:38,509,935 (c.2035-274G>A) (Fig. 2d, e), which was absent in the Genome Aggregation Database (gnomAD). In silico algorithms predicted the c.2035-274G>A variant generates a pseudo-exon activation, gaining a novel splicing donor site leading to a pseudo-exon inclusion by activating a preexisting cryptic acceptor splice site. This case, in which only one coding variant was detected at first, highlights that the existence of putative dominant variants in PLA2G6 should be reexamined [12]. Moreover, different studies reported that a consistent fraction of cases, ranging from 8 to 45%, were heterozygous for a single PLA2G6 mutant allele, missing the second mutation [10, 13–15]. Our patient’s presentation is consistent with the rare phenotype observed in young adults, with atypical findings such as the T2 GRE hypointensity in the left caudate nucleus head and T2 hyperintensity in both hippocampi. Residual PLA2G6 activity may explain later symptom onset. To the best of our knowledge, the molecular mechanism here identified has never been described in genetic forms of Parkinson’s disease. Furthermore, this case underlines the importance of cDNA analysis for detection of mutations in the intronic sequence of candidate genes in instances where exon sequencing and MLPA fail to provide a conclusive diagnosis. Although whole genome sequencing is entering faster and faster in the clinical diagnostic, RNA analysis offers a valid alternative for selected cases, although it may present limitations linked to sample availability, tissue-specific gene expression, or mutations inducing complete nonsense-mediated mRNA decay.

| Prediction tool | Acceptor gain site | Donor gain site |
|-----------------|-------------------|----------------|
|                 | Wild-type score   | Mutant score   |
|                 | Wild-type score   | Mutant score   |
| SpliceAI        | n.a              | 0.67           |
|                 | n.a              | 0.64           |
| HSF            | 85.21            | 85.21          |
|                 | 79.82            | 90.1           |
| NetGene2       | 0.22             | 0.22           |
|                 | -                | 0.64           |
| NNSplice       | 0.66             | 0.66           |
|                 | -                | 0.61           |
| varSEAK        | n.a              | n.a            |
|                 | -48.06           | +29.04         |
| MaxEntScan     | 7.49             | 7.49           |
|                 | 1.95             | 5.97           |

*n.a.* not available

**Discussion**

It has been estimated that approximately half of the patients affected by rare genetic diseases remains without a definite molecular diagnosis, about 10% of which is due to pathogenic variants located deep within introns [7]. Here, we reported for the first time a deep intronic mutation in the PLA2G6 gene, causing the creation of a new donor splice site leading to a pseudo-exon inclusion by activating a preexisting cryptic acceptor splice site. This case, in which only one coding variant was detected at first, highlights that the existence of putative dominant variants in PLA2G6 should be reexamined [12]. Moreover, different studies reported that a consistent fraction of cases, ranging from 8 to 45%, were heterozygous for a single PLA2G6 mutant allele, missing the second mutation [10, 13–15]. Our patient’s presentation is consistent with the rare phenotype observed in young adults, with atypical findings such as the T2 GRE hypointensity in the left caudate nucleus head and T2 hyperintensity in both hippocampi. Residual PLA2G6 activity may explain later symptom onset. To the best of our knowledge, the molecular mechanism here identified has never been described in genetic forms of Parkinson’s disease. Furthermore, this case underlines the importance of cDNA analysis for detection of mutations in the intronic sequence of candidate genes in instances where exon sequencing and MLPA fail to provide a conclusive diagnosis. Although whole genome sequencing is entering faster and faster in the clinical diagnostic, RNA analysis offers a valid alternative for selected cases, although it may present limitations linked to sample availability, tissue-specific gene expression, or mutations inducing complete nonsense-mediated mRNA decay.
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Declarations

Conflict of interest  The authors declare no competing interests.

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