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## Novel variants in *PNPLA6* causing syndromic retinal dystrophy

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### Abstract

*PNPLA6*-related disorders include several phenotypes, such as Boucher–Neuhäuser syndrome, Gordon Holmes syndrome, spastic paraplegia, photoreceptor degeneration, Oliver-McFarlane syndrome and Laurence-Moon syndrome. In this study, detailed clinical evaluations and genetic testing were performed in five (4 Chinese and 1 Caucasian/Chinese) syndromic retinal dystrophy patients. Genotype-phenotype correlations were analyzed based on review of the literatures of previously published *PNPLA6*-related cases. The mean age of patients and at first visit were 20.8 years (11, 12, 25, 28, 28) and 14.2 years (4, 7, 11, 24, 25), respectively. They all presented with severe chorioretinal dystrophy and profoundly decreased vision. The best corrected visual acuity (BCVA) ranged from 20/200 to 20/2000. Systemic manifestations included cerebellar ataxia, hypogonadotropic hypogonadism and hair anomalies. Six novel and three reported pathogenic variants in *PNPLA6* (NM\_001166111) were identified. The genotypes of the five cases are: c.3134C>T (p.Ser1045Leu) and c.3846+1G>A, c.3547C>T (p.Arg1183Trp) and c.1841+3A>G, c.3436G>A (p.Ala1146Thr) and c.2212–10A>G, c.3436G>A (p.Ala1146Thr) and c.2266C>T (p.Gln756\*), c.1238\_1239insC (p.Leu414Serfs\*28) and c.3130A>G (p.Thr1044Ala). RT-PCR confirmed that the splicing variants indeed led to abnormal splicing. Missense variants p.Thr1044Ala, p.Ser1045Leu, p.Ala1146Thr, p.Arg1183Trp and c.3846+1G>A are located in Patatin-like phospholipase (Pat) domain. In conclusion, we report the phenotypes in five patients with *PNPLA6* associated syndromic retinal dystrophy with variable systemic involvement and typical choroideremia-like fundus changes. Ocular manifestations may be the first and the only findings for years. All of our patients carried one severe deleterious variant (stop-gain or splicing variant) and one milder variant (missense variant). Retinal involvement was significantly correlated with severe deleterious variants and variants in Pat domain.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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## Keywords

*PNPLA6* gene; syndromic retinal dystrophy; Boucher-Neuhäuser syndrome; Oliver-McFarlane syndrome; Choroideremia-like

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## 1. Introduction

Patatin-like phospholipase domain containing 6 (*PNPLA6*) is a highly conserved phospholipase also known as neuropathy target esterase (NTE), which deacetylates intracellular phosphatidylcholine to produce glycerophosphocholine (Zaccheo *et al.*, 2004; Sogorb *et al.*, 2016; Glynn, 2003). The *PNPLA6* gene maps to chromosome 19p13.2, and five different transcripts have been identified, with the longest transcript, transcript variant 1 (NM\_001166111), encoding a protein of 1,375 amino acids. *PNPLA6* plays an important role in multiple systems and is speculated to be involved in phosphatidylcholine metabolism, neuronal development, intercellular membrane trafficking, axon maintenance and is the target in organophosphate-induced delayed neuropathy (OPIDN) (Synofzik *et al.*, 2014; Richardson *et al.*, 2013).

*PNPLA6* was first linked to disease in 2008 (Rainier *et al.*, 2008), and has been identified in patients diagnosed with Boucher–Neuhäuser syndrome, Gordon Holmes syndrome, spastic paraplegia, retinal degeneration, Oliver–McFarlane syndrome and Laurence–Moon syndrome (Synofzik *et al.*, 2014; Hufnagel *et al.*, 2015; Kmoch *et al.*, 2015; Synofzik *et al.*, 2015). *PNPLA6*-related disorders have variable clinical phenotypes and span a phenotypic continuum characterized by variable combinations of cerebellar ataxia, chorioretinal dystrophy, hypogonadotropic hypogonadism, peripheral neuropathy, hair anomalies, short stature, and intellectual disability. No obvious genotype–phenotype correlation has been proposed in patients with *PNPLA6* variants and all the associated diseases are very rare. Overlapped phenotypic features exist among different symptoms and therefore it is challenging to make a diagnosis based on clinical manifestations (Synofzik *et al.*, 2015).

To the best of our knowledge, only about 65 cases with biallelic *PNPLA6* variants have been reported worldwide ([hgmd.org](http://hgmd.org)) (Zheng *et al.*, 2018; D’Amore *et al.*, 2018; Coutelier *et al.*, 2018; Stone *et al.*, 2017; Teive *et al.*, 2018; Hufnagel *et al.*, 2015; Tarnutzer *et al.*, 2015; Kmoch *et al.*, 2015; Koh *et al.*, 2015; Synofzik *et al.*, 2014; Topaloglu *et al.*, 2014; Fogel *et al.*, 2014; Deik *et al.*, 2014; Yoon *et al.*, 2013; Rainier *et al.*, 2008; Patsi *et al.*, 2018; Wiethoff *et al.*, 2017; Langdahl *et al.*, 2017; O’Neil *et al.*, 2019; Salgado *et al.*, 2019; DeNaro *et al.*, 2018; Rainier *et al.*, 2011). Most previous studies on *PNPLA6*-related disorders were performed in the western population, and only one Chinese patient was available (Zheng *et al.*, 2018). Chorioretinal dystrophy is a common clinical feature shared by Boucher–Neuhäuser syndrome (BNS), Oliver–McFarlane syndrome (OMS) and Laurence–Moon syndrome (LMS). We find that vision impairment is often the initial symptom. However, an awareness of the *PNPLA6*-related disorders is low among ophthalmologists and the *PNPLA6* syndromes are prone to be misdiagnosed because of their rarity and complexity. Frequently, previous reports lacked detailed description of ocular manifestations in *PNPLA6*-related disorders. In this study, we report the detailed clinical

features, especially ocular features, in four Chinese patients and one Caucasian/Chinese patient with biallelic *PNPLA6* variants. Additionally, we provide an up to date review of all published *PNPLA6*-related cases to date.

## 2. Materials and Methods

### 2.1. Recruitment of subjects

Participants were enrolled at Peking Union Medical College Hospital (PUMCH), Beijing, China and from the Oregon Health & Science University (OHSU) – Casey Eye Institute, Portland, Oregon, USA. This study was approved by the Institutional Review Board of PUMCH and OHSU and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from each participant.

### 2.2. Clinical evaluations

Detailed medical history and family history were obtained for all affected patients. Full ophthalmological examinations, including best corrected visual acuity (BCVA), intraocular pressure, detailed slit-lamp examination, dilated indirect ophthalmoscopy, fundus photography (Topcon, Tokyo, Japan and Optos Inc., Marlborough, MA, USA), static perimetry (Octopus, Interzeag, Schlieren, Switzerland or Humphrey, Zeiss, Dublin, California, USA) or kinetic perimetry, optical coherence tomography (OCT; Heidelberg HRT II, Heidelberg, Germany), fundus autofluorescence imaging (Heidelberg HRT II, Heidelberg, Germany and Optos Inc., Marlborough, MA, USA), full-field electroretinography (ERG; Roland Consult, Wiesbaden, Germany). Magnetic resonance imaging (MRI) of the brain and hormonal studies were conducted for all patients.

### 2.3. Genetic testing

Peripheral blood samples were collected, and genomic DNA was extracted from all affected subjects and unaffected available family members with the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. A customized retinal disease gene target capture with next-generation sequencing was performed first in two patients as previously described (Jiang *et al.*, 2015). And multiplex ligation-dependent probe amplification (MLPA) was performed in the same two patients with SALSA multiplex ligation-dependent probe amplification probe mix P366-A2 *CHM-RP2-RPGR* (Lot A2-0614; MRC-Holland, Amsterdam, the Netherlands) according to the manufacturer's instructions. The detailed procedure of MLPA was described previously (Zhou *et al.*, 2017). Subsequently, all patients' DNA underwent whole exome sequencing (WES). Starting with 200ng high quality genomic DNA, libraries were constructed using KAPA HyperPlus Kits (Kapa Biosystems) by following the manufacture's instruction. The post-PCR library was then used for exome capture using the Fulgent WESPlus panel, an enhanced design based on IDT xGen Exome Research Panel v1.0 (Fulgent Genetics), and xGen Hybridization and Wash Kit (Integrated DNA Technologies). Enriched samples were sequenced with 2×150bp on an Illumina NovaSeq6000 (Illumina Inc).

## 2.4. Bioinformatics analysis

After sequencing, the reads were obtained and aligned to assembly hg19 of the human genome using NextGENe V2.3.4. Base quality recalibration and local realignment were performed by the Genome Analysis Tool Kit. Variants were called using NextGENe V2.3.4. Variant frequency data were obtained from public and internal control databases including the Exome Aggregation Consortium (ExAC) database, dbSNP database, ClinVar database, the Human Gene Mutation Database (HGMD) and 1000 Genomes Project. Variants with a frequency higher than 0.5% were filtered out. Annotate Variation (ANNOVAR) was used to annotate protein-altering changes and dbNSFP (contains SIFT, PolyPhen-2, LRT, Mutation Taster) was used to predict the pathogenicity about the deleteriousness of variants.

## 2.5. Sanger sequencing and segregation analysis

Sanger sequencing was performed to validate the variants identified by WES. Polymerase chain reactions (PCR) primer pairs were designed on Primer 3 software (<http://bioinfo.ut.ee/primer3-0.4.0/>) for each exon of interest. After PCR amplification, the amplicons were sequenced on an Applied Biosystems 3730XL Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequences were assembled and analyzed using Lasergene SeqMan software (DNASTAR, Madison, WI, USA). All available family members were Sanger sequenced in order to perform segregation tests.

## 2.6. RNA analysis

Total RNAs were extracted from the periphery blood samples of the patients and unaffected control subjects using the QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Single-stranded cDNA was synthesized from RNA using GoScript Reverse Transcription System (Promega, Madison, USA) according to manufacturer's protocol. RT-PCR primers were designed on Primer 3 software to amplify the segments encompassing splicing variants. After PCR amplification, the amplicons were sequenced on an Applied Biosystems 3730XL Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequences were assembled and analyzed using Lasergene SeqMan software (DNASTAR, Madison, WI, USA).

## 2.7. Statistical analysis

All patients with biallelic *PNPLA6* variants in the reported literatures and this study were enrolled to take genotype-phenotype analysis. Chi-square test and Fisher's exact test were applied to compare the differences between ocular involved patient group and the without ocular phenotype group using the SPSS version 19.0. *P* value < 0.05 was considered statistically significant.

# 3. Results

## 3.1. Clinical findings

A total 3 males and 1 female from 4 unrelated Chinese families and 1 female with mixed Caucasian/Chinese background are recruited for this study (Figure 1A). The detailed clinical findings of 5 patients were summarized in Table 1. The mean age of patients was 20.8 years

(range, 4–28). The mean age at first visit was 14.2 years (range, 7–25). P1, P2, P3 and P5 were followed for 5, 4, 3 and 21 years, respectively. All patients had night blindness and vision impairment since childhood. BCVA ranged from 20/200 to 20/2000. Color fundus photography revealed severe chorioretinal dystrophy with scattered pigment clumps or areas of chorioretinal dystrophy with visible sclera (Figure 2A). Fundus autofluorescence (FAF) photographs displayed extensive hypofluorescence (Figure 2B). OCT indicated disorganization and thinning of outer retina, outer retinal tubulations, loss of external limiting membrane, ellipsoid and interdigitation zone, thinning of the retinal pigment epithelium (RPE) and choriocapillaris (Figure 2C). Full-field ERGs were recorded in four patients (P1, P2, P4, P5) and three patients (P1, P2, P3) showed extinguished scotopic and photopic responses and P5 had remarkably reduced cone and rod responses at age 9. Three patients (P2, P3, P5) were able to take visual field test and two (P2, P3) presented with severe visual field defects (Figure 3). Visual fields of P2 indicated tunnel vision (OD) and residual 5° central and upper nasal quadrants (OS). Visual fields of P3 remained about 2° (OD) and 5° (OS) using a size III, white stimulus. Visual fields in P5 between the ages of 20 and 25 demonstrated intact responses to a V4e target peripherally but variably responses centrally, often demonstrating a scotoma to this target OU. Obvious systemic symptoms were observed in four patients (P1, P2, P3, P5) during the follow-up as described below.

P1 was 7 years old when he first presented complaining of decreased visual acuity and nyctalopia. He was diagnosed possibly having choroideremia or X-linked retinitis pigmentosa, but no causative variants were identified in *CHM*, *RPGR* or *RP2*. At 11 years old, he was diagnosed with short stature (131cm). Hormonal testing revealed a deficiency of growth hormone (GH), insulin like growth factor 1 (IGF1) and testosterone; parathyroid hormone (PTH) was high and thyroid stimulating hormone (TSH) was normal. The radiological bone age was also delayed, and MRI of the brain showed small pituitary gland. In addition, he was observed to have long eyelashes, eyebrows and sparse hair. During the 5-year follow-up, the visual acuity decreased from 20/200 (OD) and 20/100 (OS) to 20/2000 (OD) and 20/630 (OS).

Patient P2 presented at 24 years old with complaint of blurred vision and night blindness since childhood. The fundus showed extensive chorioretinopathy and the diagnosis of choroideremia was made. However, panel capture sequencing and MLPA did not find a disease-causing variant in *CHM*. He developed a tremor of the hands and head at 26 years old. Further investigation revealed he had delayed secondary sexual development with testosterone deficiency at 20 years old and testosterone replacement therapy was initiated. MRI of the brain indicated atrophy of the cerebellar hemispheres. During the 4-year follow-up, the visual acuity 20/200 (OU) was stable.

Patient P3 was referred at age 25 with a diagnosis of retinitis pigmentosa and reported vision loss and night blindness for more than 10 years. Her past medical history included primary amenorrhea. Hormonal testing revealed luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol (E2) and prolactin (PRL) were significantly lower than normal. Ultrasound of the abdomen indicated hypoplasia of uterus. During the 3-year follow-up, there was no significant change in vision (20/500, OU).

P4 reported a three-year history of nyctalopia and decreased visual acuity and with a rapid loss of visual acuity over the previous 3 months. At presentation, his visual acuity was 20/200 (OD) and 20/250 (OS). After *PNPLA6* compound heterozygous variants were confirmed, hormonal testing was obtained and showed testosterone was 1.68ng/ml, slightly lower than normal (1.75–7.81ng/ml), while other hormones were normal.

P5 presented at the age of 4 with a history of nyctalopia. Full field ERGs revealed a rod-cone dystrophy and a diagnosis of early onset severe retinal dystrophy (EOSRD) was made. At age 12, she was diagnosed with short stature (below the 5<sup>th</sup> percentile) and at age 14 with hypogonadotropic hypogonadism and started at on estrogen replacement therapy. As of age 25 she had not manifested any neurological symptoms. Genetic testing revealed compound heterozygous variants in *PNPLA6*.

### 3.2. Genetic findings

We performed WES and obtained high quality data (mean coverage: 120×) for exonic regions. After filtering and prioritization, WES data revealed that biallelic pathogenic variants in *PNPLA6* were identified in all five patients (annotation according to GenBank: NM\_001166111). There was one insertion, one nonsense, three splicing and four missense variants found in five patients. Compound heterozygous c.3134C>T (p.Ser1045Leu) and c.3846+1G>A, c.3547C>T (p.Arg1183Trp) and c.1841+3A>G, c.3436G>A (p.Ala1146Thr) and c.2212–10A>G, c.3436G>A (p.Ala1146Thr) and c.2266C>T (p.Gln756\*), c.1238\_1239insC (p.Leu414Serfs\*28) and c.3130A>G (p.Thr1044Ala) were identified in P1, P2, P3, P4 and P5, respectively. Sanger sequencing was performed to validate the variants and confirm the co-segregation of variants with disease phenotype in the pedigrees (Figure 1A). Three variants (c.3134C>T, p.Ser1045Leu; c.3547C>T, p.Arg1183Trp; c.1238\_1239insC, p.Leu414Serfs\*28) were reported previously (Synofzik *et al.*, 2014; Stone *et al.*, 2017; Kmoch *et al.*, 2015), and six variants (c.2266C>T, p.Gln708\*; c.3436G>A, p.Ala1146Thr; c.3130A>G, p.Thr1044Ala; c.1841+3A>G; c.3846+1G>A and c.2212–10A>G) were novel. All of the missense variants are well conserved across different species (Figure 1B). To further confirm that the identified *PNPLA6* splicing variants were *bona fide* splicing-disrupting, RT-PCR and Sanger sequencing were performed. For the splicing variant c.3846+1G>A, a 117bp deletion of *PNPLA6* mRNA (r.3731\_3847del) was noted (Figure 4A). For the splicing variant c.1841+3A>G, a 185bp insertion of *PNPLA6* mRNA (r.1841\_1842ins1841+1\_1841+185) was detected (Figure 4B). The mRNA of P3 was unavailable. SpliceSiteFinder-like, MaxEntScan, Human Splicing Finder all predicted the splicing variant c.2212–10A>G would influence splice. The results of genetic analysis were summarized in Table 2.

### 3.3. Genotype–phenotype analysis

In total, 70 affected subjects (65 reported in the literature and 5 in this study) from 49 pedigrees harboring biallelic pathogenic *PNPLA6* variants were ascertained from previous literature and this study. Among 49 families, 34 (34/49, 69.4%) had chorioretinal dystrophy and 15 (15/49, 30.6%) had no reported ocular phenotype. The genotype/phenotype correlation of these *PNPLA6* cases are summarized in Table 3. A total 98 alleles and 71 different variants are also analyzed. The variants are diverse and widely distributed in the

gene. Only 2 variants, c.3084\_3085insGCCA and p.Gly1129Arg are considered as frequent variants, accounting for 9.18% (9/98) and 5.10% (5/98), respectively. The novel variant p.Ala1146Thr identified in this study, accounts for 20% (2/10) in this study, and was the most frequent allele.

Among 71 variants, there are 53 missense variants (53/71, 74.65%), which is the most common variant type, followed by splicing variants (6/71, 8.45%), small insertion and indel variants (6/71, 8.45%), nonsense variants (4/71, 5.63%), and gross insertion and deletion variants (2/71, 2.82%). We classified splicing, nonsense, small and gross insertion and deletion variants as severe allele and missense variant as mild allele. Based on this, we categorized the patients into two groups: those with one or two severe alleles and those with only mild alleles. We found that patients with chorioretinal dystrophy carried more severe deleterious variants (22/34, 64.7%) than patients without chorioretinal dystrophy (5/15, 33.3%),  $P < 0.05$  by Chi-square test. Only one patient carrying two severe deleterious alleles presented with spastic paraplegia.

Among 53 missense variants, 32 (32/53, 60.4%) of them are located in patatin-like phospholipase (Pat) domain (amino acid interval, 964–1269) and 6 (6/53, 11.3%) in cyclic nucleotide monophosphate (cNMP) binding domain (amino acid interval, 195–316/512–622/635–743). Among 18 severe variants, 3 (3/18, 16.7%) are located in Pat domain and 8 (8/18, 44.4%) in cNMP binding domain. More subjects with chorioretinal dystrophy (9/34, 26.5%) harbor biallelic variants in both domains (one variant in Pat domain and the other one in cNMP binding domain) than that of subjects without chorioretinal dystrophy (0/15, 0%), but the result was not statistically significant ( $P = 0.071$  by Chi-square test). We also discovered that patients with chorioretinal dystrophy carried significantly more variants (32/34, 94.12%) in Pat domains (1 variant in Pat domain) than those without retinal phenotypes (8/15, 53.3%) ( $P < 0.01$  by Chi-square test). Another interesting finding is that 3 splicing variants were identified in our 4 Chinese patients, but only 4 splicing alleles were found previously in 44 families.

#### 4. Discussion

PNPLA6 belongs to a family of nine patatin-like phospholipase domain-containing proteins. It is mainly expressed in the brain and involved with neural development. Deficits are associated with neurodegeneration. It is also expressed in several non-neuronal tissues such as testes and kidney (Sogorb *et al.*, 2016; Winrow *et al.*, 2003; Hufnagel *et al.*, 2015). *PNPLA6* is expressed in the retina, crystal lens, pituitary, cerebellum and ventricular zones of the brain (Hufnagel *et al.*, 2015). Knockdown of *pnpla6* expression resulted in developmental abnormalities and motor neuron defects in zebrafish (Hufnagel *et al.*, 2015; Song *et al.*, 2013). Swiss cheese protein (the orthologue of vertebrate PNPLA6) was essential for membrane lipid homeostasis and cell survival in both neurons and glia of the adult *Drosophila* brain. It was proposed that NTE might play an analogous role in vertebrates (Mühlig-Versen *et al.*, 2005). Furthermore, it was found that *PNPLA6* was expressed in horizontal, amacrine and photoreceptors cells, which was crucial for photoreceptor maintenance in adult *Drosophila*. PNPLA6 is localized mostly in the plasma membrane of the inner segments of mouse photoreceptors, suggesting its role in the

formation of distinct plasma membrane domains in the photoreceptors or maintenance of photoreceptor integrity(Kmoch *et al.*, 2015).

All of our patients manifested vision loss and night blindness at very young age without any obvious systemic abnormalities. They were diagnosed with non-syndromic retinal degeneration (EOSRD, choroideremia or retinitis pigmentosa) until systemic involvement became apparent or genetic testing was performed. We followed four patients (P1, P2, P3, P5) for a number of years and found that they had varying degrees of progressive vision loss. In our series, all patients presented with a distinct chorioretinal atrophy. However, the retinal appearance of *PNPLA6*-related disorders can be diverse, with the full spectrum ranging from a mild retinal pigment epitheliopathy to severe chorioretinal atrophy(Zheng *et al.*, 2018; Salvador *et al.*, 1995; DeNaro *et al.*, 2018; Teive *et al.*, 2018). The degeneration can be confined to the macula, mid periphery(Tarnutzer *et al.*, 2015), or involve the entire retina and mimic advanced choroideremia(Yu *et al.*, 2008; Kmoch *et al.*, 2015; O'Neil *et al.*, 2019; Synofzik *et al.*, 2014; Deik *et al.*, 2014). Since systemic features many not present later in life, it is important to consider the possibility of *PNPLA6*-related disorders in patients with choroideremia-like changes, especially when genetic testing is inconclusive.

For patients found to have *PNPLA6*-associated syndrome, a full systemic work up is critical. This should include a referral to an endocrinologist and neurologist. Four patients (P2, P3, P4, P5) were diagnosed as Boucher–Neuhäuser syndrome (BNS) and P1 was diagnosed as Oliver-McFarlane syndrome (OMS) based on their clinical assessments and genetic data. In our series, three adult patients (P2 ,P3, P5) exhibited hypogonadotropic hypogonadism. However, hormone replacement therapy can be effective if hormone abnormalities are detected in adolescence(Boehm *et al.*, 2015).

The *PNPLA6*-related disorders are a complex group of diseases including several syndromes. Possible genotype-phenotype correlation had been proposed in previously, but not confirmed (Synofzik *et al.*, 2014; Kmoch *et al.*, 2015). Synofzik et al observed that variants in the N-terminal side of the Pat domain might associate with spasticity (spastic paraplegia and spastic ataxia), while variants towards the C-terminal end of the Pat domain might associate with cerebellar ataxia and/or hypogonadism (Boucher-Neuhäuser syndrome, Gordon Holmes syndrome, spastic ataxia) based on 7 families(Synofzik *et al.*, 2014). Meanwhile, Kmoch et al noticed that biallelic variants in Pat and cNMP binding domains could cause a more severe impairment of *PNPLA6* function and lead to photoreceptor death based on 7 families(Kmoch *et al.*, 2015).

However, the above putative phenotype-genotype correlation was obtained from limited research cases. After dissecting the genotypes and phenotypes of all *PNPLA6* patients, we bring out some perspectives regarding connections between genotype and phenotype. First, our data suggests that variant position may influence phenotype. Patients with chorioretinal dystrophy tended to have *PNPLA6* variants located in both domains (one in Pat domain and the other one in cNMP binding domain) as Kmoch et al detected(Kmoch *et al.*, 2015). However, this finding was not statistically significant ( $P=0.071$ ). Nevertheless, we demonstrate that Pat domain plays the key role instead of Pat domain plus cNMP binding domain. One allele or biallelic variants in Pat domain is highly correlated with the



chorioretinal dystrophy ( $P<0.01$ ). Second, variant type may also relate to phenotype. More *PNPLA6* patients with severe deleterious variants presented with chorioretinal dystrophy ( $P<0.05$ ). However, one patient with two severe deleterious variants presented only with spastic paraplegia without retinopathy (Yoon *et al.*, 2013), which is contrary to this conclusion from a dosage standpoint. In addition, we presume that ethnical specific alleles may exist. The c.3084\_3085insGCCA (9/87, 10.34%) and p.Gly1129Arg (5/87, 5.75%) are recurrent alleles in Caucasians. While in Chinese, variant p.Ala1146Thr (2/11, 18.18%) is the most common one and may be a mutational hot spot. Furthermore, it seemed that splicing variants were much more frequent in Chinese (3/11, 27.27%) than that of Caucasians (4/87, 4.60%) ( $P<0.05$  by Fisher's exact test). As the Chinese sample size is very limited, more data are needed to draw a conclusion. Notwithstanding the genotype-phenotype correlations, intra-familial phenotypic variation was reported. For example, two siblings in their 40s, with the same biallelic variants, one had BNS with chorioretinal dystrophy and the other one didn't show any evidence of chorioretinal dystrophy (Synofzik *et al.*, 2014).

Most of the identified *PNPLA6* variants (35/71, 49.3%) were located within the Pat domain, critical for the esterase activity of NTE. This domain has been shown to de-esterify phosphatidylcholine, a major component of biological membranes, into its constituent fatty acids and glycerophosphocholine (Synofzik *et al.*, 2014). Several studies indicated the loss of NTE enzymatic activity of the Pat domain was closely correlated to the *PNPLA6*-related disorders (Synofzik *et al.*, 2014; Hufnagel *et al.*, 2015; Hein *et al.*, 2010a; Hein *et al.*, 2010b). Structural modeling and analysis revealed that the location of variants in the Pat domain was associated with NTE activity (Synofzik *et al.*, 2014; Hufnagel *et al.*, 2015). In addition, it was suggested that the onset and severity of disease was related to the hydrolase activity of the Pat domain, contributing to the phenotypic heterogeneity (Hufnagel *et al.*, 2015). These data support our finding that chorioretinal dystrophy is highly correlated with the variants in Pat domain. In addition, Chang *et al.* revealed that *PNPLA6* exhibited dynamic interactions with the endoplasmic reticulum and lipid droplets that depended on the interplay of two functional regions, the amino-terminal region and the carboxyl-terminal catalytic region. Variants that disrupt this interplay may contribute to *PNPLA6*-related disorders by affecting protein positioning (Chang *et al.*, 2019).

In summary, *PNPLA6*-related disorder is a group of diseases with miscellaneous and overlapping clinical features. Choroideremia-like retinal changes are the characteristic ocular phenotype, which can appear as the only finding at an early age. Chorioretinal dystrophy correlates with variant type and variants in Pat domain. Whole exome sequencing is a useful tool to elicit the genetic diagnosis. This paper is the most detailed report and review regarding *PNPLA6*-related chorioretinal dystrophy so far. Our findings expand the clinical and genetic spectrum related to the rare inherited diseases.

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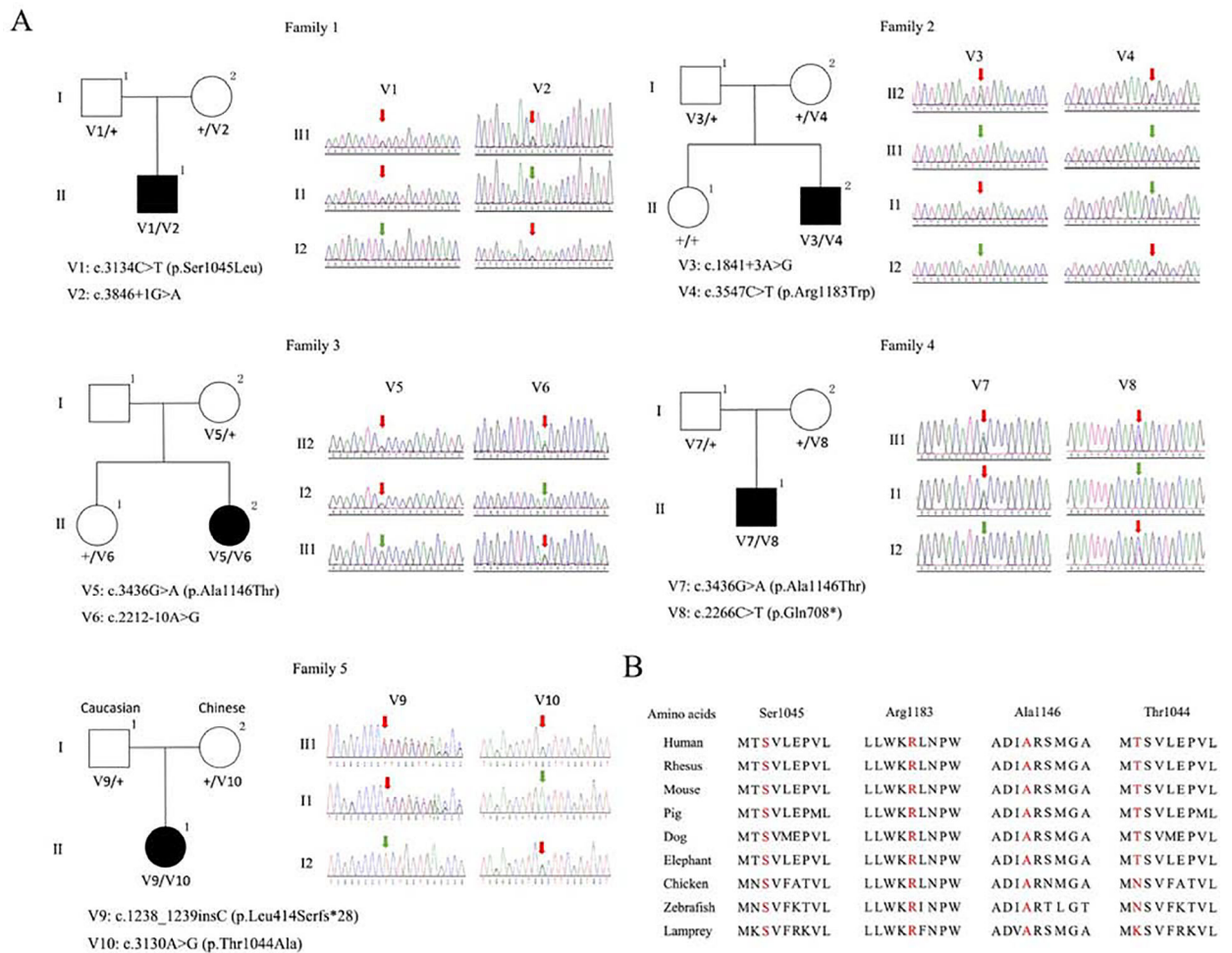
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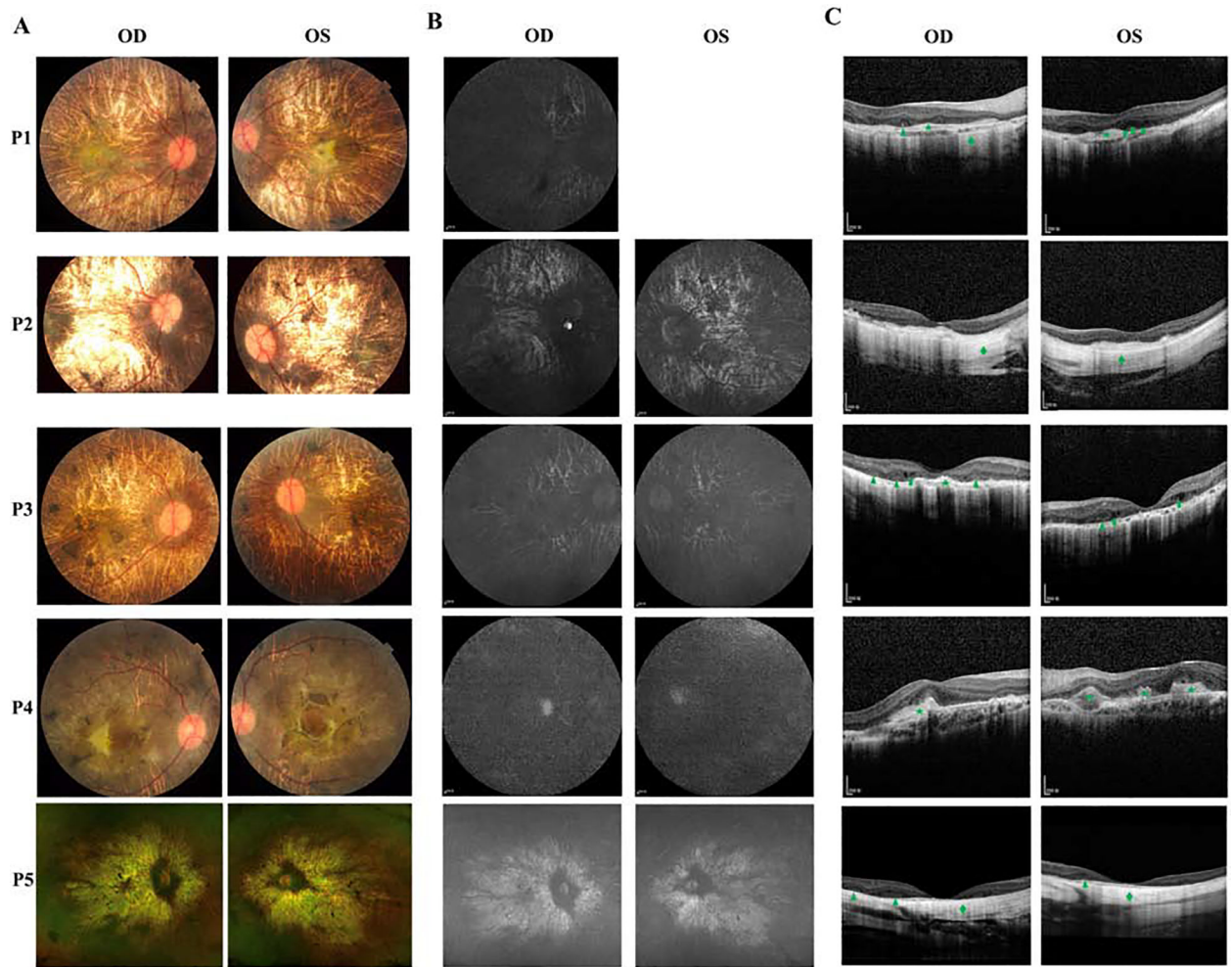
The paper described phenotype and genetic defects of 5 *PNPLA6*-related syndromic retinal dystrophy patients from 5 unrelated families.

Choroideremia-like retinal changes are the characteristic ocular phenotype of *PNPLA6*-related syndromic retinal dystrophy.

Genotype-phenotype analysis found chorioretinal dystrophy correlates with variant type and variants in Pat domain.

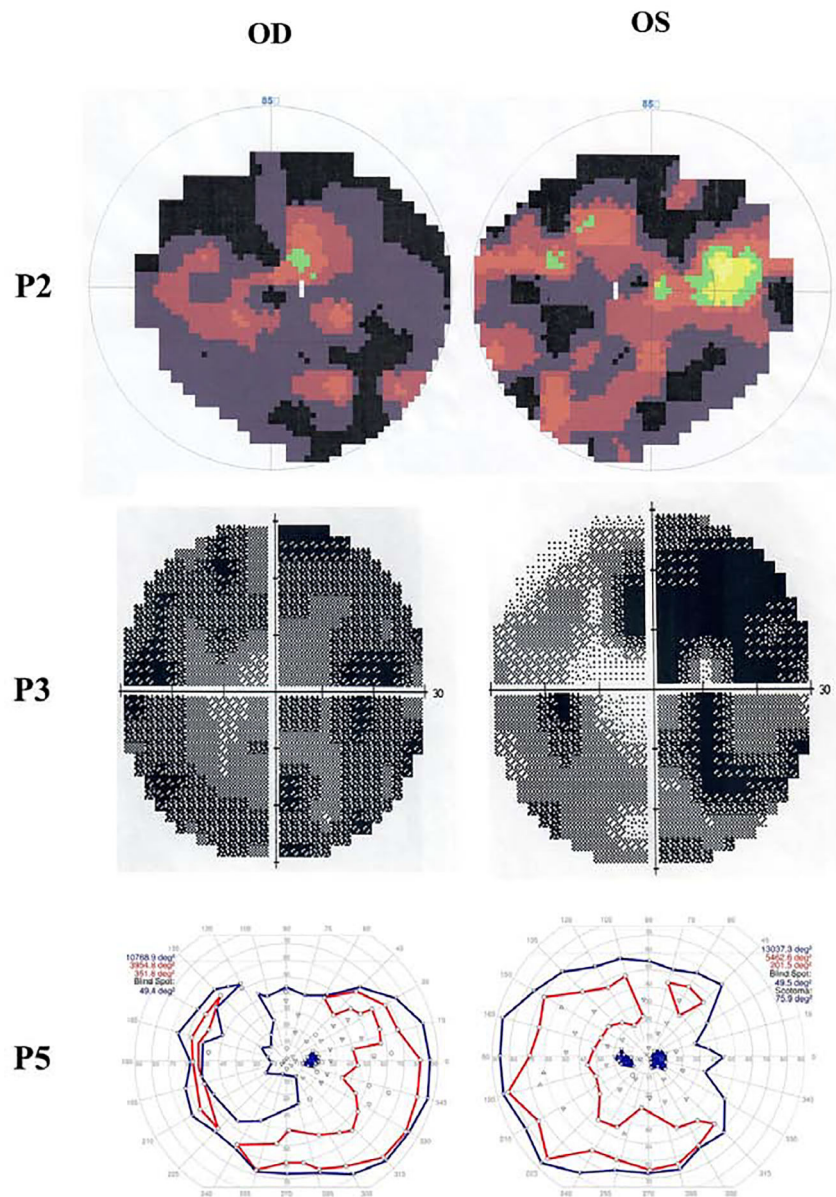
**Figure 1.**

**A:** Pedigrees and Sanger sequencing chromatograms of *PNPLA6*-related disorders patients in this study. A total of five unrelated patients with *PNPLA6* biallelic variants were identified. Variant annotations were based on GenBank: NM\_001166111. Squares indicate men; circles indicate women; black indicates patients; red arrows indicate the variants; green arrows indicate the normal nucleotide. **B:** Conservative analysis of four amino acids affected by missense variants. The Thr1044, Ser1045, Ala1146 and Arg1183 are highlighted and evolutionarily conserved.



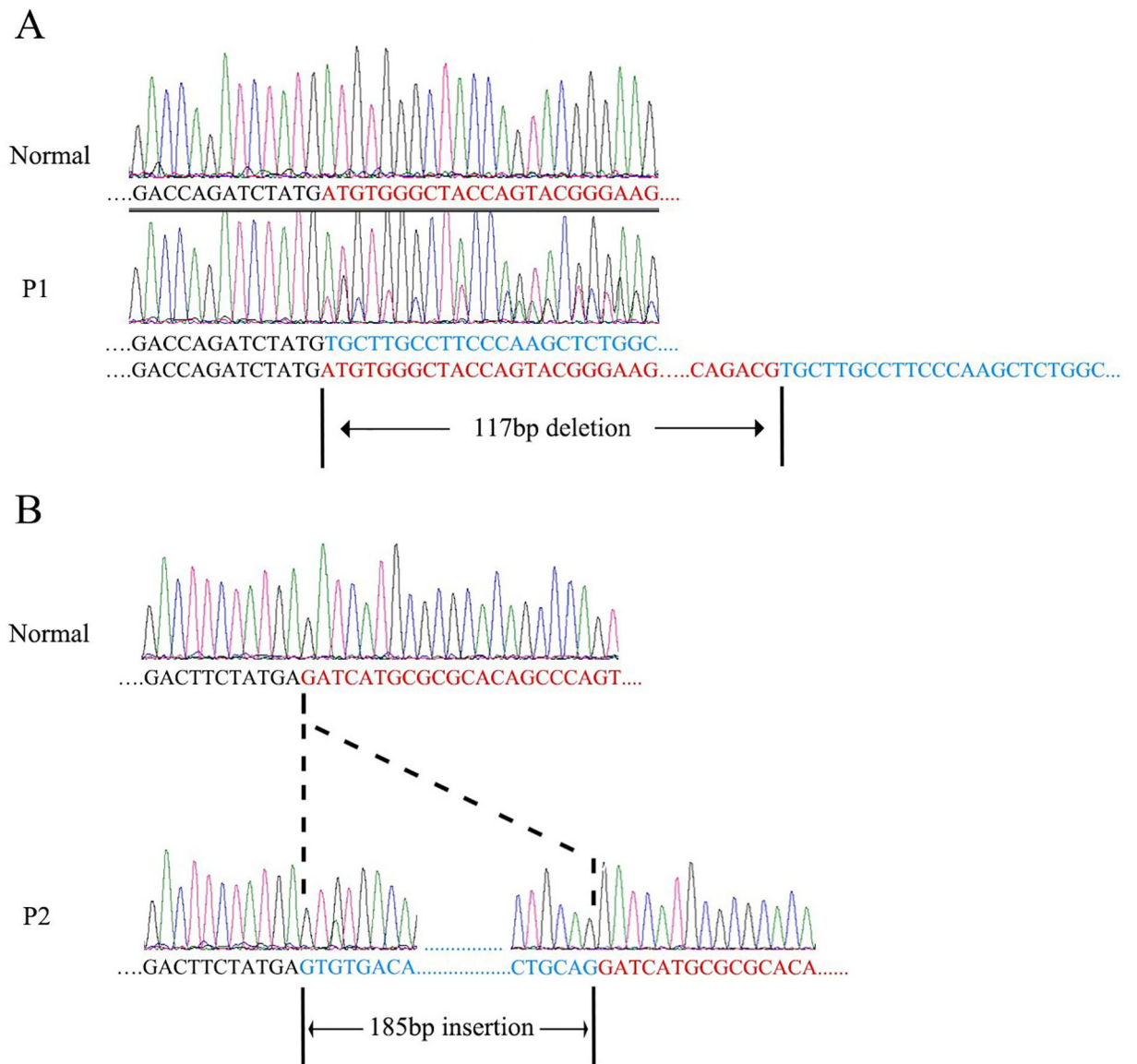
**Figure 2.**

**A:** Color fundus photographs of five patients. P1, P2, P3 and P5 all presented extensive chorioretinal dystrophy and visualization of sclera with pigment clumps. P4 presented chorioretinal dystrophy, scattered pigmentation and fibrosis patches. **B:** The FAF photographs of five patients. All displayed generalized remarkably decreased autofluorescence. **C:** OCT images of five patients. All revealed disorganization and thinning of outer retina, loss of external limiting membrane, ellipsoid and interdigitation zone, thinning of the RPE and choriocapillaris. P1 and P3 had outer retinal hyporeflexive cysts (arrow). P1, P3, and P5 displayed outer retinal tubulations (arrowhead). P1, P3 and P4 indicated hyperreflective retinal fibrosis (star). P1, P2 and P5 showed hyperreflective scleral signals (diamond).



**Figure 3.** Visual field images of P2, P3 and P5. P2 displayed tunnel vision (OD) and residual 5° central and upper nasal quadrants (OS). P3 remained about 2°(OD) and 5°(OS) tunnel visual field. P5 had intact responses to V4e target peripherally but decreased responses to the III4e and smaller targets centrally (OU).





**Figure 4.** RT-PCR and chromatograms of P1 (A) and P2 (B). **A:** A 117bp region was deleted in mRNA compared with normal control. **B:** A 185bp region was inserted in mRNA compared with normal control.

**Table 1.**

Summary of the clinical manifestations of five patients

Patient	P1	P2	P3	P4	P5
<b>Gender</b>	M	M	F	M	F
<b>Ethnicity</b>	Han Chinese	Han Chinese	Han Chinese	Han Chinese	Caucasian/Chinese
<b>Age (years)</b>	12	28	28	11	25
<b>Age at initial visit (years)</b>	7	24	25	11	4
<b>Phenotype</b>	OMS	BNS	BNS	BNS	BNS
<b>BCVA(OD,OS)</b>	20/2000,20/630	20/200,20/200	20/500,20/500	20/200,20/250	20/600,20/1000
<b>Night blindness</b>	+	+	+	+	+
<b>Chorioretinopathy</b>	+	+	+	+	+
<b>ERG</b>	No recordable responses	No recordable responses	NA	No recordable responses	Remarkably reduced
<b>Ataxia</b>	-	+	-	-	-
<b>MRI of brain</b>	small pituitary gland	Atrophy cerebellum	-	-	NA
<b>Hypogonadotropic hypogonadism</b>	+	+	+	+	+
<b>Testosterone</b>	Low	Low	Normal	Slightly low	NA
<b>GH deficiency</b>	+	NA	NA	-	NA
<b>Short stature</b>	+	-	-	-	+
<b>Delayed bone age</b>	+	NA	NA	NA	NA
<b>Trichomegaly</b>	+	-	-	-	-
<b>Sparse hair</b>	+	-	-	-	-

M, male; F, female; OMS, Oliver-McFarlane syndrome; BNS, Boucher-Neuhäuser syndrome; BCVA, best corrected visual acuity; MRI, magnetic resonance imaging; ERG, electroretinography; GH, growth hormone; NA, not available;

Table 2.

Summary of the variants analysis of five patients

Patient	Diagnosis	DNA change	Protein/mRNA change	Variant type	SIPT	Polyphen2	LRT	Mutation Taster	Novel
P1	OMS	c.3134C>T	p.Ser1045Leu	Missense	-	-	-	-	N
		c.3846+1G>A	r.3711_3847del	Splicing	-	-	-	-	Y
P2	BNS	c.3547C>T	p.Arg1183Trp	Missense	-	-	-	-	N
		c.1841+3A>G	r.1841_1842ins1841+1_1841+185	Splicing	-	-	-	-	Y
P3	BNS	c.3436G>A	p.Ala1146Thr	Missense	T	PD	D	DC	Y
		c.2212-10A>G	NA	Splicing	-	-	-	-	Y
P4	BNS	c.3436G>A	p.Ala1146Thr	Missense	T	PD	D	DC	Y
		c.2266C>T	p.Gln756*	Nonsense	-	-	-	DC	Y
P5	BNS	c.1238_1239insC	p.Leu414Serfs*28	Insertion	-	-	-	-	N
		c.3130A>G	p.Thr1044Ala	Missense	T	B	D	DC	Y

OMS, Oliver-McFarlane syndrome; BNS, Boucher-Neuhäuser syndrome; T, tolerated; B, benign; PD, probably damaging; D, deleterious; DC, disease causing; Y, yes; N, no; NA, not available. The splicing variant c.2212-10A>G would influence splice predicted by SpliceSiteFinder-like, MaxEntScan, Human Splicing Finder. All the variants are described according to *PNPLA6* transcript variant (NM\_001166111)

**Table 3.**

List of all available *PNPLA6* variants and associated phenotypes

Reference	Total case	Eye involved	Phenotype	Variant 1	Effect 1	Variant type	Domain 1	Variant 2	Effect 2	Variant type	Domain 2
Zheng et al, 2018	1	Y	BNS	c.3386G>T	p.G1129V	Missense	Pat	c.3534G>C	p.W1178C	Missense	Pat
Tarnutzer et al, 2015	1	Y	BNS	c.288T>G	p.Y96*	Nonsense	-	c.865C>G	p.R289G	Missense	cNMP
Tarnutzer et al, 2015	1	Y	BNS	c.343-2A>T	-	Splicing	-	c.4075C>T	p.R1359W	Missense	-
Synofzik et al, 2014	2	Y	BNS	c.2212-1G>C	p.V738Qfs*98	Splicing	cNMP	c.3328G>A	p.V1110M	Missense	Pat
Synofzik et al, 2014	1	N	Sporadic ataxia	c.3084_3085insGCCA	p.R1031Qfs*38	Insertion	Pat	c.3299T>G	p.V1100G	Missense	Pat
Synofzik et al, 2014	1	N	HSP	c.787G>A	p.V263I	Missense	cNMP	c.2519G>A	p.G840E	Missense	-
Synofzik et al, 2014	1	Y	BNS	c.3134C>T	p.S1045L	Missense	Pat	c.3365C>T	p.P1122L	Missense	Pat
Synofzik et al, 2014	2	Y	BNS	c.1732G>T	p.G578W	Missense	cNMP	c.3197T>C	p.F1066S	Missense	Pat
Synofzik et al, 2014	1	N	GHS	c.3084_3085insGCCA	p.R1031Qfs*38	Insertion	Pat	c.4084C>G	p.R1362G	Missense	-
Synofzik et al, 2014	4	Y	BNS	c.3173C>T	p.T1058I	Missense	Pat	c.3173C>T	p.T1058I	Missense	Pat
Kmoch et al, 2015	1	Y	OMS	c.1571T>C	p.L524P	Missense	cNMP	c.3373G>A	p.D1125N	Missense	Pat
Kmoch et al, 2015	1	Y	OMS	c.2116C>T	p.Q706*	Nonsense	cNMP	c.3385G>C	p.G1129R	Missense	Pat
Kmoch et al, 2015	3	Y	OMS	c.343-2A>T	-	Splicing	-	c.3373G>A	p.D1125N	Missense	Pat
Kmoch et al, 2015	1	Y	LCA	c.3084_3085insGCCA	p.R1031Qfs*38	Insertion	Pat	c.1076C>T	p.T359I	Missense	-
Kmoch et al, 2015	1	Y	OMS	c.3322C>T	p.R1108W	Missense	Pat	c.3385G>C	p.G1129R	Missense	Pat
Kmoch et al, 2015	1	Y	OMS	c.1238_1239insC	p.L414Sfs*28	Insertion	-	c.3385G>C	p.G1129R	Missense	Pat
Fogel et al, 2014	1	N	Cerebellar ataxia	c.1484C>T	p.P495L	Missense	-	c.3742C>G	p.Q1248E	Missense	Pat
Coutelier et al, 2018	1	N	Spastic ataxia	c.2633G>A	p.G878D	Missense	-	c.2633G>A	p.G878D	Missense	-

Reference	Total case	Eye involved	Phenotype	Variant 1	Effect 1	Variant type	Domain 1	Variant 2	Effect 2	Variant type	Domain 2
Couteller et al, 2018	1	N	Cerebellar ataxia	c.1483C>A	p.P495T	Missense	-	c.1857G>T	p.Q619H	Missense	cNMP
Rainier et al, 2008	2	N	MND	c.3178A>G	p.M1060V	Missense	Pat	c.3178A>G	p.M1060V	Missense	Pat
Rainier et al, 2011	2	N	MND	c.3084_3085insGCCA	p.R1031Qfs*38	Insertion	Pat	c.2813G>A	p.R938H	Missense	-
Yoon et al, 2013	1	N	Spastic paraplegia	c.1816_1818delinsTA	p.Arg606*	Indels	cNMP	del exons 16-17	-	Deletion	cNMP
Hufnagel et al, 2015	2	Y	OMS/LMS	c.3296G>A	p.R1099Q	Missense	Pat	c.3526G>A	p.G1176S	Missense	Pat
Hufnagel et al, 2015	1	Y	OMS/LMS	c.3084_3085insGCCA	p.R1031Qfs*38	Insertion	Pat	c.3385G>A	p.G1129R	Missense	Pat
Hufnagel et al, 2015	1	Y	OMS/LMS	c.1973+2T>G	-	Splicing	cNMP	c.3644T>C	p.V1215A	Missense	Pat
Hufnagel et al, 2015	1	Y	OMS/LMS	Dup exons 14-20	-	Insertion	cNMP	c.3644T>C	p.V1215A	Missense	Pat
Hufnagel et al, 2015	1	Y	OMS/LMS	c.3084_3085insGCCA	p.R1031Qfs*38	Insertion	Pat	c.3385G>A	p.G1129R	Missense	Pat
Hufnagel et al, 2015	4	Y	OMS/LMS	c.3084_3085insGCCA	p.R1031Qfs*38	Insertion	Pat	c.2176G>C	p.G726R	Missense	cNMP
D'Amore et al, 2018	1	N	Spastic paraplegia	c.3585C>G	p.D1195E	Missense	Pat	c.2389G>A	p.V797M	Missense	-
Teive et al, 2018	1	N	GHS	c.4081C>T	p.R1361*	Missense	-	c.3373G>A	p.D1125N	Missense	Pat
Teive et al, 2018	1	Y	BNS	c.3531G>A	p.W1177*	Missense	Pat	c.2441T>C	p.L814P	Missense	-
Koh et al, 2015	1	Y	BNS	c.3523_3524insTGTCGG	p.1175_1176insVS	Insertion	Pat	c.2923A>G	p.T975A	Missense	Pat
Koh et al, 2015	1	Y	BNS	c.3534G>C	p.W1178C	Missense	Pat	c.3534G>C	p.W1178C	Missense	Pat
Deik et al, 2014	1	Y	BNS	c.3134C>T	p.S1045L	Missense	Pat	c.3519C>G	p.S1173R	Missense	Pat
Stone et al, 2017	1	Y	Retinal dystrophy	c.3334G>A	p.A1112T	Missense	Pat	c.3547C>T	p.R1183W	Missense	Pat
Topaloglu et al, 2014	2	N	GHS	c.3524C>G	p.S1175C	Missense	Pat	c.3524C>G	p.S1175C	Missense	Pat
Topaloglu et al, 2014	2	Y	BNS	c.1270_1271insG	p.D424Gfs*18	Insertion	-	c.3439C>T	p.R1147C	Missense	Pat
Topaloglu et al, 2014	2	N	GHS	c.2494_2495insI3	p.G832Vfs*27	Insertion	-	c.4075C>T	p.R1359W	Missense	-

Reference	Total case	Eye involved	Phenotype	Variant 1	Effect 1	Variant type	Domain 1	Variant 2	Effect 2	Variant type	Domain 2
O'Neil et al, 2019	1	Y	BNS	c.3084_3085insGCCA	p.R1031Qfs*38	Insertion	Pat	c.3548G>A	p.R1183Q	Missense	Pat
Patsi et al, 2018	1	Y	OMS	c.3620C>A	p.S1207Y	Missense	Pat	c.3620C>A	p.S1207Y	Missense	Pat
#Wiethoff et al, 2017	2	N	Cerebellar ataxia	c.3929A>T/ c.3847G>A	p.D1310V/ p.V1283M	Missense/ Missense	-/-	c.3929A>T/ c.3847G>A	p.D1310V/ p.V1283M	Missense/ Missense	-/-
Langdahl et al, 2017	2	Y	BNS	c.3084_3085insGCCA	p.R1031Qfs*38	Insertion	Pat	c.4076G>A	p.R1359Q	Missense	-
Salgado et al, 2019	4	N	GHS	c.4081C>T	p.R1361*	Missense	-	c.2404G>C	p.E802Q	Missense	-
DeNaro et al, 2018	1	Y	BNS	c.1431T>A	p.C477*	Nonsense	-	c.3034G>A	p.G1012S	Missense	Pat
<b>This study</b>	1	Y	OMS	c.3846+1G>A	r.3711_3847del	Splicing	Pat	c.3134C>T	p.S1045L	Missense	Pat
<b>This study</b>	1	Y	BNS	c.1841+3A>G	r.1841_1842ms1841 +L_1841+185	Splicing	cNMP	c.3547C>T	p.R1183W	Missense	Pat
<b>This study</b>	1	Y	BNS	c.2212-10A>G	-	Splicing	cNMP	c.3436G>A	p.A1146T	Missense	Pat
<b>This study</b>	1	Y	BNS	c.2266C>T	p.Q756*	Nonsense	-	c.3436G>A	p.A1146T	Missense	Pat
<b>This study</b>	1	Y	BNS	c.1238_1239insC	p.L414Sfs*28	Insertion	-	c.3130A>G	p.T1044A	Missense	Pat
<b>Total</b>	<b>70</b>										

cNMP: cyclic nucleotide monophosphate (cNMP) binding domain (amino acid interval, 195–316/512–622/635–743); Pat: patatin-like phospholipase domain (amino acid interval, 964–1269); BNS: Boucher–Neuhäuser syndrome; HSP: hereditary spastic paraplegia; GHS: Gordon Holmes syndrome; OMS: Oliver–McFarlane syndrome; LMS: Laurence–Moon syndrome; LCA: Leber congenital amaurosis; MND: motor neuron disease; Y: Yes; N: No

#: two homozygous missense variants in this consanguineous family; All the variants are described according to *PNPLA6* transcript variant (NM\_001166111)