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Characterization of Two Novel *PNKP* Splice-Site Variants in a Proband With Microcephaly, Intellectual Disability, and Multiple Malformations

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ABSTRACT

Polynucleotide kinase phosphatase (PNKP), encoded by the *PNKP* gene, is a DNA processing enzyme involved in double-strand break and single-strand break repair pathways, which are essential for genome stability and for the correct development and maintenance of human nervous system. *PNKP* biallelic loss-of-function variants have been associated with a broad spectrum of neurological anomalies, ranging from congenital microcephaly with intellectual disability and seizures (MCSZ), to later onset forms of ataxia-oculomotor apraxia (AOA4) or peripheral neuropathy (CMT2B2). We report the atypical clinical manifestations of a patient with severe microcephaly, short stature, developmental delay, conductive hearing loss, and tracheoesophageal malformation, in the absence of seizures. Whole exome sequencing analysis identified two novel, compound heterozygous splice-site variants in the *PNKP* gene (NM_007254.4): c.1448+1G > A and c.199-8_199-5del. To demonstrate the effect of both variants on the splicing process and prove their pathogenicity, we performed a hybrid minigene assay, which successfully highlighted a deleterious impact on the transcript, particularly regarding the c.199-8_199-5del variant. The uncommon clinical features of the proband and the identification of two newly associated pathogenic variants add further evidence to the allelic and phenotypic heterogeneity of the *PNKP* locus.

1 | Introduction

Polynucleotide kinase phosphatase (PNKP), coded by the *PNKP* gene on chromosome 19q13.33 (MIM: 605610), is a DNA processing enzyme constituted of a forkhead-associated domain (FHA), a phosphatase domain and a kinase domain. PNKP is involved in the repair of both double strand breaks (DSB) and single strand breaks (SSB) of the DNA molecule (Jilani et al. 1999). Whenever SSB or DSB occur, usually as a consequence of oxidative stress or physical damage, 3′-phosphorylated and 5′-hydroxylated nucleotides can be generated at the breakpoints. For the DNA repair process to take place, the enzyme DNA ligase requires the preliminary restoration of properly 3′-hydroxylated and 5′-phosphorylated fragments, which is provided by the 3′-phosphatase and 5′-kinase activities of PNKP (Breslin and Caldecott 2009; Coquelle et al. 2011). Depending on the type of DNA damage, PNKP and DNA ligase are recruited together with different scaffolding enzymes, namely XRCC1 and XRCC4, to assemble into crucial DNA repairing complexes (Aceytuno et al. 2017; Mani et al. 2019; London 2020, 1). PNKP-dependent DNA repair is essential for genome stability, particularly in neural progenitor cells (NPCs), which exhibit a high replication rate in the early development stages and are therefore highly susceptible to the accumulation of DNA damage (Shimada et al. 2015).

NPCs deprived of PNKP have been demonstrated to accumulate substantial DNA damage and chromosomal abnormalities, leading to catastrophic mitotic events and ultimately to cellular death (Reynolds et al. 2012; Shin et al. 2021; Rychlowska et al. 2022).

In human patients, biallelic *PNKP* loss-of-function variants have been associated with a broad spectrum of neurological anomalies, which are distinguished by the severity of their clinical manifestations and the earliness of onset (Dumitrache and McKinnon 2017; Gatti et al. 2019). The three main clinical phenotypes are microcephaly with seizures and developmental delay (MCSZ) (Shen et al. 2010; Nakashima et al. 2014; Furones García et al. 2021; Marcilla Vázquez et al. 2021), ataxia oculomotor apraxia-4 (AOA4) (Bras et al. 2015; Islam et al. 2023) and Charcot–Marie–Tooth disease (CMT2B2) (Leal et al. 2018). Rare instances of primordial dwarfism without seizures or cerebellar manifestations have also been reported, suggesting the existence of another possible distinct phenotype (Nair et al. 2016). Surprisingly, despite being involved in fundamental DNA repair mechanisms, PNKP defects have not been consistently associated with cancer predisposition to date, with only anecdotal exceptions (Garrelfs et al. 2020; Jiang et al. 2022). Pathogenic variants affecting either the kinase or the phosphatase activity of PKNP have been demonstrated to cause different disruptive effects on the enzyme's function, partially explaining the heterogeneity of PNKP-related clinical manifestations (Bermúdez-Guzmán et al. 2020; Kalasova et al. 2020; Neuser et al. 2022). However, a comprehensive genotype–phenotype correlation is still a subject of speculation.

In this work, we describe the functional validation and possible genotype–phenotype correlations of two *PNKP* compound heterozygous splice-site variants identified in a young patient with severe microcephaly, short stature, developmental delay, congenital conductive hearing loss and tracheoesophageal malformation, in the absence of seizures.

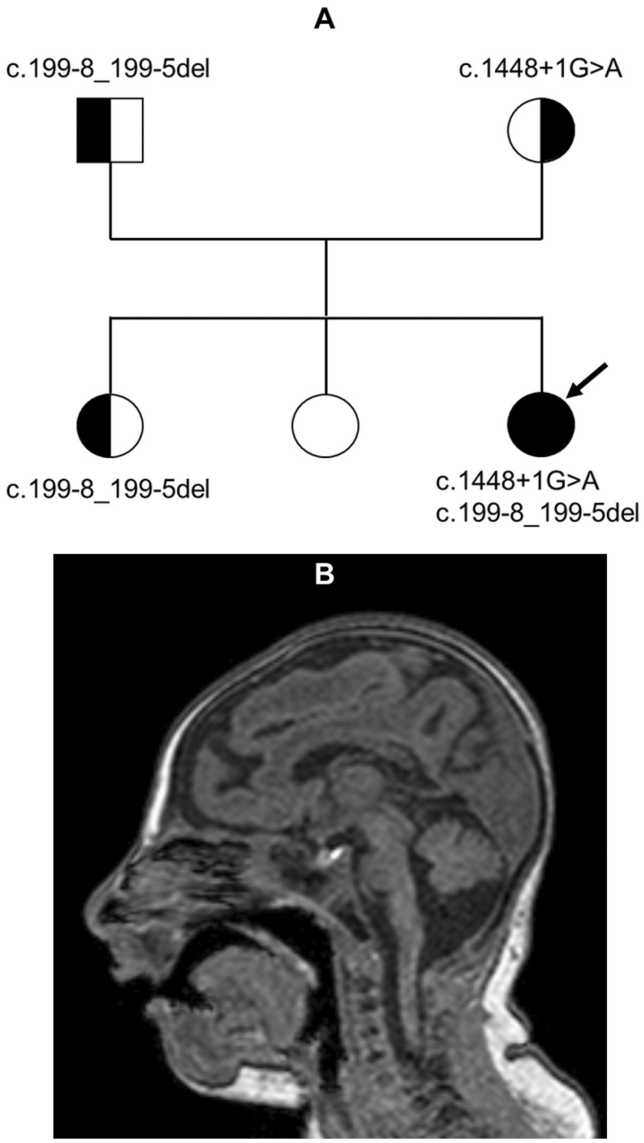

FIGURE 1 | (A) Representation of the family pedigree, showing the segregation of the two identified variants. (B) Cerebral MRI at 37 days old, showing microcephaly with frontal lobes hypoplasia, simplified gyral pattern, hippocampal dysplasia, and cerebellar hypoplasia. Retro/micrognathia can also be noted.

2 | Material and Methods

2.1 | Patient Report

The patient is the third child of healthy, nonconsanguineous parents, with unremarkable family history (Figure 1A). The patient's parents have provided their written consent to the publication of relevant clinical and molecular data as appropriate and according to ethical guidelines.

Pregnancy was complicated by the identification of microcephaly at the second trimester ultrasound, substantiated by prenatal magnetic resonance imaging (MRI), which also raised the suspicion of lissencephaly. Invasive prenatal chromosome analysis was performed, resulting in normal female karyotype. Congenital infections, exposure to teratogens and maternal comorbidities were also investigated and excluded. The child was born at term. Weight, length and head circumference were all reported below the third centile. Because of sialorrhea, an esophageal atresia with tracheoesophageal fistula was detected, for which the patient underwent multiple surgical treatments, complicated by the onset of chronic gastroesophageal reflux disease. Post-natal brain MRI confirmed a diffuse simplified gyral pattern, more evident in the frontal lobes and the hippocampi, associated with dilated ventricular system and cerebellar hypoplasia (Figure 1B,C). Newborn audiological assessments detected severe congenital conductive hearing loss due to bilateral atresia of the external auditory canals, which required bone conduction hearing aids. Cardiac and abdominal ultrasound examination did not reveal major abnormalities. Ophthalmologic assessment was normal.

The patient was referred to our clinic at 12 months of age. Head circumference was 33 cm, corresponding to −9.9 standard deviations (SD) from the mean, with a body length of 65 cm (< −3 SD). Hair was woolly and sparse. She had a sloping forehead, occipital plagiocephaly, epicanthus inversus, long eyelashes,

a hypoplastic bulbous nose with short philtrum, a thin upper lip, and retro/micrognathia. Ears were small (length < 3rd centile) and dysplastic. Chest, abdomen, and limb examination did not reveal significant anomalies, except for four small café-au-lait macules detected in the limbs and the inguinal region. Clinical examination of the parents and healthy siblings was unremarkable.

Since the first evaluation, the patient's growth has stably maintained below −3 SD in weight and between −2 SD and −3 SD in height, while her head circumference always remained in the neighborhood of −10 SD. At her latest evaluation at the age of 9 years and 2 months, she weighed 16.5 kg (< −3 SD), was 120 cm tall (< −2 SD) and her head circumference was 38 cm (−10.43 SD).

Early motor milestones were only slightly delayed, and the child ultimately acquired the ability to walk and run for brief distances, although she still uses the wheelchair for most of her daily living. Neurological assessments did not find any sign of ataxia or dystonia, while the musculature has always been hypotrophic. Language development was compromised: at age 9 years she could use approximatively 50 different words and basic two-word sentences. The child has also never acquired bladder and bowel control, and she was diagnosed with severe intellectual disability. The patient did not suffer from seizures nor exhibited any abnormal electroencephalographic pattern.

2.2 | Molecular Analyses

Genomic DNA (gDNA) from the child and parents' peripheral leukocytes were extracted using standard methods. Array CGH was performed as reported (Rigon et al. 2011). Next generation sequencing of a multigenic panel associated with craniofacial abnormalities was performed using a Haloplex custom kit (Agilent Technologies, Santa Clara, CA, USA) on an Illumina MiSeq sequencer. Exome sequencing was performed with a Paired-End 100 bp protocol on a Hiseq2500 sequencer (Illumina Inc., San Diego, CA, USA); sequencing was preceded by selective enrichment of coding regions of the human genome using SureSelectXT Clinical Research Exome kit (Agilent Technologies). Variants' calling, annotation, selection, and interpretation were performed as previously described (Desbats et al. 2015). Variants were confirmed by Sanger sequencing, following a standard protocol.

2.3 | Hybrid Minigene Assay

A pcDNA3.1 beta-globin (HBB) vector was engineered and amplified as previously described (Forzan et al. 2010). We then proceeded with PCR-driven vector linearization using the CloneAmp HiFi PCR Premix kit (Takara Bio Inc., Kusatsu, Shiga prefecture, Japan) with the vector-specific primers 5′-TTGTACACATATTGACCAAATC-3′ (forward) and 5′-ACATATTAAAACATTACACTTTAAC-3′ (reverse). The original methylated template was degraded by digestion with the enzyme DPNI. A separate PCR was used to amplify fragments containing either PNKP variants or wild type sequences from the genomic DNA of the patient or a wild-type control. The primers used for the reaction were composed of a nucleotide sequence complementary to the vector and a nucleotide sequence specific to the insert. For the c.199-8_199-5del variant, we used the forward primer 5′-ACCTGTAACCGTTCCAAAGCC and the reverse primer 5′-GATAAATGCAGGGATCGTTGTAAG-3′ to amplify the terminal 156 nucleotides of intron 3, the entire exon 4, and the first 139 nucleotides of intron 4. In the case of the c.1448+1G > A variant, three additional adjacent exons (exons 14, 15, 16) were included in the minigene construct due to their proximity, using the forward primer 5′-TCCCATCTCCACCTCTCAACTGG-3′ and the reverse primer 5′-GCTTCTCTGCCATCCTGGAGATC-3′. Wild type controls were similarly produced. Subsequently, recombination cloning between the inserts and the vector was performed with the aforementioned hybrid primers, using a standard protocol. The PCR products were purified and confirmed by direct sequencing. The hybrid minigene vectors were then transformed into MACH-1 bacteria (ThermoFisher Scientific, Watham, MA, USA) for plasmid methylation and, after customary amplification and purification routines, ultimately transfected in HEK293 cells using a Lipofectamin based protocol (ThermoFisher Scientific), as described in previous works (Cassina et al. 2017; Morbidoni et al. 2021). After 24–48 h, total RNA was extracted with TRIzol (ThermoFisher Scientific) and subsequently retrotranscribed using a SuperScript II Reverse Transcriptase (ThermoFisher Scientific) based protocol. The resulting cDNA was amplified using the specific primers 5′-CA GTCTGCCTAGTACATTACTATTTGG-3′ for exon 2 (forward) and 5′-AATCTCTTTCTTTCAGGGCAA-3′ for exon 3 (reverse) of the beta-globin gene. Gel electrophoresis of the PCR products was then performed on a 2% agarose gel intercalated with 0.1% Midori^{Green} Nucleic Acid Stain (Nippon Genetics Europe GmbH, Düren, Germany), allowing for UV staining of the cDNA bands. Following electrophoresis, the cDNA bands were cut from the gel, eluted, and verified by Sanger sequencing.

3 | Results

First-line genetic investigations, which included standard karyotyping, array-CGH, and NGS analysis of a panel of genes associated with craniofacial malformations, were normal. Subsequently, we proceeded with exome sequencing of the family unit (the proband, her parents, and her two healthy sisters, as illustrated in Figure 1A). In the proband, we identified two compound heterozygous variants in the *PNKP* gene (NM_007254.4): c.199-8_199-5del on the paternal allele and c.1448+1G > A on the maternal allele.

The c.1448+1G > A variant is absent from the main variation databases (LOVD, HGMD, ClinVar) and displays a minor allele frequency of zero in the gnomAD database. The variant affects the canonical splice donor site of the last intron of the gene and it is therefore predicted to affect protein synthesis; because the variant met the PVS1 and PM2 ACMG classification criteria (Richards et al. 2015), it was initially classified likely pathogenic (class LP).

The c.199-8_199-5del variant is reported with conflicting interpretations of pathogenicity in the ClinVar database and has been detected in 7/124 235 (approximatively 1/17 700)

heterozygous carriers in the gnomAD database. This intronic deletion affects the polypyrimidinic trait of the acceptor site of intron 3 of *PNKP*. Because *in silico* predictions and epidemiologic data alone were not conclusive in supporting its pathogenicity, the variant was initially considered of unknown significance (class 3 ACMG). To better validate the presumed impact of each separate variant on transcription, a hybrid minigene assay was performed, as described in the methods section. Results are shown in Figure 2.

The c.199-8_199-5del variant assay showed that clones transfected with the mutated vector produced two different transcripts, both shorter than the wild type, demonstrating that the variation abolishes the proper splicing consensus site of

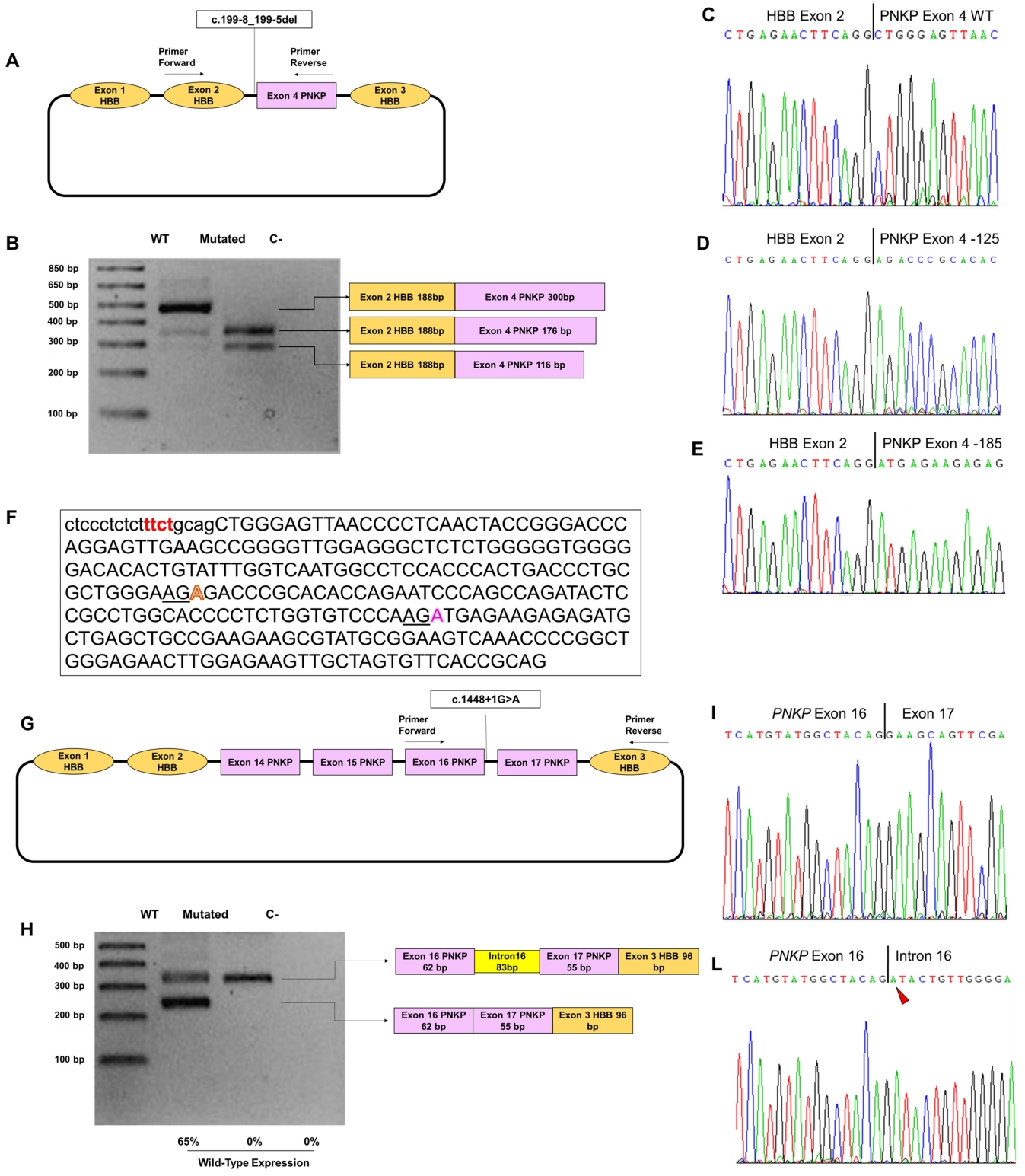

FIGURE 2 | Legend on next page

exon 4, leading to the activation of two exonic cryptic splice sites at the 125th and 185th nucleotide of exon 4. Both sites are predicted to determine the shifting of the reading frame, resulting in the creation of premature stop signals, which in turn results in either synthesis of a truncated protein or nonsense mediated mRNA decay. Unfortunately, no patient RNA was available for evaluating the latter hypothesis. No normal transcripts were detected.

As for the c.1448+1G > A variant, the assay revealed a complete loss of normal transcript in cells transfected with the mutant minigene, which expressed only a longer transcript that retained intron 16. This is predicted to cause the formation of a premature stop codon five residues downstream of exon 16.

In both assays, the splicing of the wild type minigene was not 100% efficient, as demonstrated by the detection of abnormal transcripts within cells transfected with the wild-type construct. However, in both instances the normal transcript was predominant, while being completely absent in the mutant minigenes. This is not unusual in these type of assays, as artificial constructs may lack some control sequences which may be important for the correct processing of the pre-mRNA; however, this could also reflect the physiological presence of non-canonical transcripts even in normal cells (Trevisson et al. 2007).

The results of the assays allowed the classification of both variants as Pathogenic (class 5 ACMG).

4 | Discussion and Conclusions

In the first published series of MCSZ patients, biallelic *PNKP* loss-of-function variants were inevitably associated with severe microcephaly, early-onset epilepsy, and neurodevelopmental delay (Shen et al. 2010). Since then, the knowledge of the clinical manifestations caused by *PNKP* defects has gradually evolved and expanded to include milder or partial forms of the disease (Entezam et al. 2019; Gatti et al. 2019). The discovery of additional, markedly distinct disorders, characterized by later-onset cerebellar (AOA4) or neuropathic manifestations (CMT), further widened the phenotypic spectrum associated with the locus.

The neurological phenotype of our proband exhibited peculiar features, for while falling within the most severe range of microcephaly and cognitive impairment, it was characterized by the notable absence of seizures and cerebellar signs. This is not unprecedented, as there have already been reports of milder MCSZ cases characterized by treatable seizures, and even complete absence thereof (Nair et al. 2016; Gatti et al. 2019). Interestingly, epilepsy-free phenotypes such as the one of our proband, who also displayed growth retardation and short stature, have already been compared to primordial dwarfism syndromes associated with defects in other genes involved in DNA repair, rather than fully manifest MCSZ (Nair et al. 2016). Other notable clinical features in our patient were bilateral external auditory canal atresia and esophageal atresia with tracheoesophageal fistula, whose presence greatly complicated the diagnostic and therapeutic management. To our knowledge, this is the first time that these specific malformations have been reported in a *PNKP*-related case; however, we acknowledge that our current understanding of *PNKP* pathophysiological mechanisms does not allow for an immediate etiologic connection. Therefore, although whole-exome sequencing had not identified a plausible alternative explanation, the hypothesis that these new features might be a so far unreported consequence of the loss of *PNKP* function should still be considered remote compared with that of an independent co-occurrence, of genetic or nongenetic etiology, until further evidence emerges.

The search for an in-depth understanding of the genotype–phenotype correlations related to *PNKP* has proven to be both a complex and compelling endeavor, which several authors have already addressed (Gatti et al. 2019; Bermúdez-Guzmán et al. 2020; Garrelfs et al. 2020; Kalasova et al. 2020; Thuresson et al. 2023). In compliance with the current consensus that the severity of the phenotype positively correlates with the degree of loss of function of *PNKP*, the scientific debate focused on the type and the intragenic position of the causative variations. However, predicting the phenotypic effect based on variant type alone has so far proved challenging, as various combinations of missense, truncating and splicing variants have been reported across the whole spectrum of clinical manifestations (Gatti et al. 2019). Regarding intragenic position, it has been demonstrated that variants compromising the DNA phosphatase activity of the enzyme correlate primarily with neurodevelopmental dysfunction, while a reduced DNA kinase activity enables neurodegeneration. It should also be noted that the vast majority of reported *PNKP* variations affects the kinase domain, while only a small residual proportion has been reported in the FHA and phosphatase domains. However, it has also been suggested that this observation

FIGURE 2 | (A) Representation of the hybrid minigene comprising exon 4 of *PNKP*, its adjacent intronic regions and the c.199-8_199-5del variant, for an expected normal transcript of 465 base pairs. (B) Agarose gel electrophoresis showing two abnormal transcripts of 364 and 304 base pairs in the column where mutated samples were loaded, in the absence of normally processed transcript. (C, D, E) Visualization of the Sanger sequences of the PCR products extracted from the gel electrophoresis bands, highlighting the fusion products of Exon 2 of *HBB* with (C) wild-type *PNKP* exon 4, (D) the exonic cryptic splice site at nucleotide 125 of *PNKP* exon 4 (genomic position GRCh37/hg19: Chr19:50368559), and (E) the exonic cryptic splice site at nucleotide 185 of *PNKP* exon 4 (genomic position GRCh37/hg19: Chr19:50368499). (F) In red, the 4 deleted nucleotides within the polypyrimidine tract of intron 3. Bold letters indicate the 2 cryptic splice sites that are activated in the mutant: In orange the one corresponding to the upper band and in pink the one corresponding to the lower band. Both are preceded by a canonical AG consensus (underlined) and by a pyrimidine-rich region. (G) Representation of the hybrid minigene comprising exon 14, 15, 16, and 17 of *PNKP*, their intronic regions and the c.1448+1G > A variant, for an expected wild-type transcript of 235 base pairs. (H) Agarose gel electrophoresis showing an abnormal transcript of 296 base pairs, which can also be observed in a minority of transcripts from cells transfected with wild-type constructs. (I; L) Visualization of the Sanger sequences of the PCR products extracted from the gel electrophoresis bands, highlighting the fusion products of *PNKP* exon 16 with (I) exon 17 (wild-type) and (L) intron 16 (mutant). WT: Wild-type; C-: Negative control.

could be distorted by survivorship bias, as the complete loss of PNKP activity, particularly regarding its phosphatases domain, may be incompatible with life, as demonstrated in the murine model (Bermúdez-Guzmán et al. 2020). The hypothesis of the indispensability of the phosphatase domain is further supported by the evidence that pathogenic variants reported therein have been described in MCSZ patients only (Shen et al. 2010; Nakashima et al. 2014; Gatti et al. 2019; Marcilla Vázquez et al. 2021; Thuresson et al. 2023).

In our proband, we identified two compound heterozygous *PNKP* variants: c.1448+1G > A is canonical splice site variant, while c.199-8_199-5del is a polypyrimidine tract variant. To explain the milder epileptological and cerebellar phenotype of the patient, we initially considered the hypothesis that the variant causing the most severe effect on the transcript might be the canonical splice site one, while the polypyrimidine tract variant might instead result in a hypomorphic protein, capable of maintaining a residual level of activity. To our surprise, the hybrid minigene assay showed the c.199-8_199-5del variant to cause a more radical and premature truncation of the transcript, thus qualifying it as the most damaging variant of the pair. In this scenario, the revised hypothesis of a less impactful canonical splice site variant, as it would appear to be the case for the c.1448+1G > A variant, which falls within the last intron of the gene and would therefore be expected to escape nonsense mediated mRNA decay, would be consistent with other literature reports indicating that truncating variants of the terminal tract are associated with milder phenotypes (Gatti et al. 2019), maybe due to the persistence of C-terminally truncated proteins resulting in the impairment of kinase domain activity alone. Regrettably, unavailability of suitable patient-derived cells has precluded us to perform further experimental assays to confirm the functional impact of the identified variants. In particular, we acknowledge that direct transcriptomic and proteomic analysis would have allowed us to integrate the results of the hybrid minigene model with more exhaustive functional data. Based on current knowledge, the atypical complexity of our proband's manifestations and the unavailability of a consistent, established genotype–phenotype correlation model in the literature do not allow for a definitive interpretation of the functional effect of the identified variants.

In conclusion, we describe two novel *PNKP* pathogenic variants associated with atypical clinical manifestations, broadening the genotypic and phenotypic spectrum of *PNKP*-related disorders and invigorating the ongoing effort of correlating the characteristics of *PNKP* variants to their clinical consequences.

Author Contributions

Conceptualization: U.S. and L.S. Data curation: U.S. and E.B. Formal analysis: U.S. and M.C. Methodology: E.B. and M.A.D. Resources: O.Z. and L.S. Supervision: O.Z. and M.C. Validation: E.B. and M.A.D. Visualization: U.S. Writing – original draft: U.S. Writing – review and editing: L.S. and M.C.

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Ethics Statement

Ethical review and approval were waived for this study because it retrospectively analyzed data obtained from established diagnostic procedures.

Consent

Informed consent was obtained from all subjects involved in the study.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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