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Mutations in *CSPP1*, *TMEM67*, *PLP1*, and *GAN* associated with pediatric neurological disorders in Iran

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Abstract

Background & Objective: Neurological disorders significantly impact patients' mental, personality, and movement functions, with a rising prevalence globally, especially in low-income and middle-income countries. This study aims to evaluate gene mutations in pediatric neurological disorders patients to contribute to our understanding of these disorders' genetic basis. *Methods:* In the current survey, all patients with maternal signs of neurological disorders who were referred to the neurology department during 2023 to 2024 were evaluated. DNA samples from patients were enriched using the Agilent SureSelect Human All Exon Kit V6, and subsequent sequencing took place on an Illumina HiSeq 4000 platform based on the manufacturer's procedures. *Results:* In the current cross-sectional study, 13 patients with maternal neurological disorders including 6 males (46%) and 7 females (54%) were evaluated. Our results identified inherited neurological disorders, including Joubert syndrome, Pelizaeus-Merzbacher disease, and giant axonal neuropathy-1. Our data identified a novel missense mutation in exon 8 of *PLP1* gene (NM_001128834.3: c.772A>C; p.Met258Leu) with X-linked recessive inheritance in a patient with Pelizaeus-Merzbacher disease. Gene variants, including *CSPP1* frameshift mutation in exon 20 (NM_001382391.1: c.2259_2260delAA; p.Glu755GlyfsTer30), and autosomal recessive homozygous *TMEM67* mutation in exon 8 (NM_153704.6: c.725A>G; p.Asn242Ser) were detected in patients with Joubert syndrome. Finally, in a patient with giant axonal neuropathy-1, a homozygous *GAN* mutation (NM_022041.4: c.1177T>C; p.Cys393Arg) was detected.
Conclusion: Our findings can be useful in understanding the pathophysiology of neurological disorders. Also, this study indicated the importance of genetic analysis in utilizing the treatment strategy in patients with neurological disorders.

Keywords: Joubert syndrome, Pelizaeus-Merzbacher disease, giant axonal neuropathy-1, mutations, exome-sequencing

INTRODUCTION

Neurological disorders (NDs) significantly impact the mental, personality, and movement functions, as well as the lifestyle of patients. NDs stand as the second leading cause of death worldwide. Recent investigations highlight an increasing frequency of disabilities attributable to NDs, particularly in low-income and middle-income countries.[1,2] These findings underscore the imperative for enhanced screening and prevention programs to reduce NDs. A recent study unveiled that Norway exhibits a higher prevalence of hereditary NDs than other European nations.[3] Within the spectrum of NDs, myotonia congenita and limb-girdle muscular dystrophy displayed higher rates, while Charcot–Marie–Tooth polyneuropathy prevalence surpassed that of most European studies.[3]

A significant body of literature underscores the importance of genetic testing in diagnosing and preventing NDs.[4-7] It is crucial to conduct genetic

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assessments for pediatric patients exhibiting developmental delays, intellectual, and functional disabilities.[8] However, despite advancements in genetic assays, there remains unequal utilization of genetic tests for diagnosing NDs.[9] Conversely, due to the considerable overlap in phenotypes among NDs[10], understanding the underlying pathogenic mechanisms is essential for genotype-based treatments.[10-13] Furthermore, most genetic studies related to NDs focus on conditions such as multiple sclerosis, Alzheimer's, Parkinson's disease, and amyotrophic lateral sclerosis, leading to heterogeneous data concerning specific genetic syndromes or mutations.[14] In this regard, the present investigation aims to evaluate pediatric patients with NDs for gene mutation assessment.

METHODS

Subjects and clinical assessment

Patients were systematically recruited from the neurology department of Golestan Hospital, Ahvaz. Inclusion criteria comprised individuals with confirmed NDs via neuroimaging suspected of harboring a genetic disease encompassing unusual ischemic changes, leukodystrophy, other hereditary white matter disorders, and irrespective of family history. Patients with acquired NDs were excluded. This study was performed after obtaining permission from the Research Council and approval of the Medical Ethics Committee of Ahvaz University of Medical Sciences (IR.AJUMS.HGOLESTAN.REC.1402.208).

DNA extraction

After obtaining written informed consent, venous blood samples were collected from both affected and non-affected individuals. Genomic DNA extraction was carried out using a standard desalting protocol, a widely adopted method.

Exome-sequencing

Exome-sequencing exclusively targeted the probands. DNA samples from patients were enriched using the Agilent SureSelect Human All Exon Kit V6, and subsequent sequencing took place on an Illumina HiSeq 4000 platform following recommended procedures. The average read depth exceeded 100×, with at least 20× coverage achieved for 98.0% of the targeted genomic regions. Mutation filtering and annotation involved preprocessing sequenced reads, aligning reads to the human reference genome (GRCh37.p13/hg19), variant calling following GATK Best Practices pipeline guidelines, and final annotation using the ANNOVAR tool. For analysis of the sequencing results, we utilized international publicly available mutation and polymorphism databases such as the 1000 Genomes Project (https://www.internationalgenome.org/) and gnomAD (https://gnomad.broadinstitute.org/). Only variants with a frequency below 1% were selected.

PCR and Sanger sequencing

PCR primer design utilized OLIGO 7 (Table 1). PCR was conducted with 100 ng of genomic DNA, 12.5 µl of Master Mix from Amplicon Co., and 10 pmol of each primer, resulting in a total PCR volume of 25 µl. The amplification proceeded through an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, culminating in a final extension at 72°C for 5 min. Sanger sequencing outcomes were aligned with the reference genomic sequence and analyzed using ChromasPro 2.1.3. Confirmation of identified mutations was obtained through parental analysis and bi-directional Sanger sequencing, with purified DNA fragments undergoing direct sequencing using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an Applied Biosystems 3500 DNA Analyzer. The resulting sequences were analyzed using Chromas and DNA Baser v4 software.

RESULTS

Genetic analysis and clinical data

Thirteen patients with NDs, comprising 6 males

Table 1: Primers utilized for gene amplification

Gene	Exon	Forward Primer	Reverse Primer
CSPP1	20	AGGTCACACGGTCTGTTTGG	AGTCTTCAGTCAGCCTACATCT
TMEM67	8	TGGAACAGACCTGCACAAAG	GCCAAGGAAGATTCTGTCTCC
PLP1	8	CAGAGATGTCTCAGGGACTGC	GCATTTTCCATTCAGGGACTGTG
GAN	7	CAGAGATGTCTCAGGGACTGC	GCATTTTCCATTCAGGGACTGTG

(46%) and 7 females (54%), underwent an extensive genetic analysis, uncovering mutations in *CSPP1*, *TMEM67*, and *GAN* genes alongside a novel mutation in *PLP1*.

CSPP1 mutation

Clinical presentation

In a consanguineous Iranian family, the 11-year-old son displayed pronounced clinical features of Joubert Syndrome, evident in early childhood. These included hypotonia, global developmental delay, abnormal eye movements (oculomotor apraxia), and characteristic molar tooth signs on brain imaging. Notably, the daughter remained asymptomatic.

Genetic analysis

Exome-sequencing revealed a homozygous *CSPP1* (NM_001382391.1): c.2259_2260delAA; p.Glu755GlyfsTer30 mutation in exon 20 (rs587777139), causing a frameshift mutation. Sanger sequencing confirmed homozygosity in the affected son, with carrier status in the unaffected parents (heterozygous state) (Figure 1).

Functional implications

The frameshift mutation (c.2259_2260delAA; p.Glu755GlyfsTer30), located in exon 20 of 31, position 18-19 of 150 (coding, Nonsense-mediated mRNA decay [NMD]), introduces a premature termination codon, resulting in the truncation of approximately 20% of the CSPP1 protein. This disruption contributes to Joubert syndrome, affecting vital cellular processes. Understanding the hereditary aspect clarifies the autosomal recessive inheritance pattern, emphasizing the importance of both parents carrying a single mutated copy. This inheritance pattern indicates that both parents are carriers, highlighting the significance of the mutation's transmission in familial contexts. The interpretation of this genetic variant leans towards a pathogenic classification, and this determination aligns with the established standards and guidelines provided by the American College of Medical Genetics and Genomics (ACMG). Furthermore, this variant

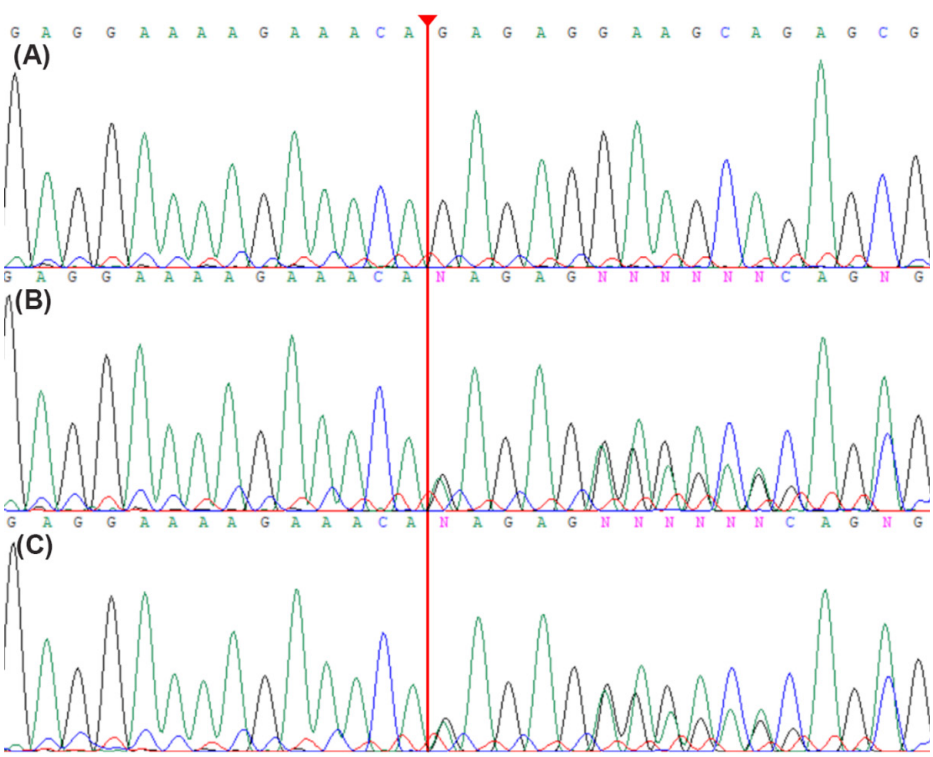

Figure 1. Illustrates the discovery of a mutation in the *CSPP1* gene and the genetic screening conducted on the examined family. Direct sequencing of the patient's DNA (A) uncovered a homozygous mutation (c.2259_2260delAA; p. Glu755GlyfsTer30), while his unaffected parents (B, C) were identified as carriers of the detected mutation in the heterozygous state.

is situated within the third potential coiled-coil domain.[15]

TMEM67 mutation

Clinical presentation

In another consanguineous Iranian family with a Joubert syndrome history, the 7-year-old son and 8-year-old daughter exhibited symptoms, while the 10-year-old daughter remained unaffected.

Genetic analysis

Exome-sequencing identified a homozygous *TMEM67* (NM_153704.6): c.725A>G; p.Asn242Ser mutation in exon 8 (rs775883520). Subsequent Sanger sequencing validated the mutation's presence, confirming its absence in the healthy daughter. Both parents were carriers in the heterozygous state (Figure 2).

Functional implications

The missense mutation in *TMEM67* (asparagine to serine) can potentially disrupt local protein structure and interactions. *TMEM67*, vital for ciliary processes, experiences altered function due to this mutation, leading to characteristic brain abnormalities in Joubert syndrome. Understanding the hereditary transmission provides insights into the autosomal recessive inheritance pattern, elucidating the importance of both parents contributing a mutated allele. This hereditary context underscores the complexity of genetic factors in disease manifestation. The interpretation of this genetic variant leans towards a pathogenic

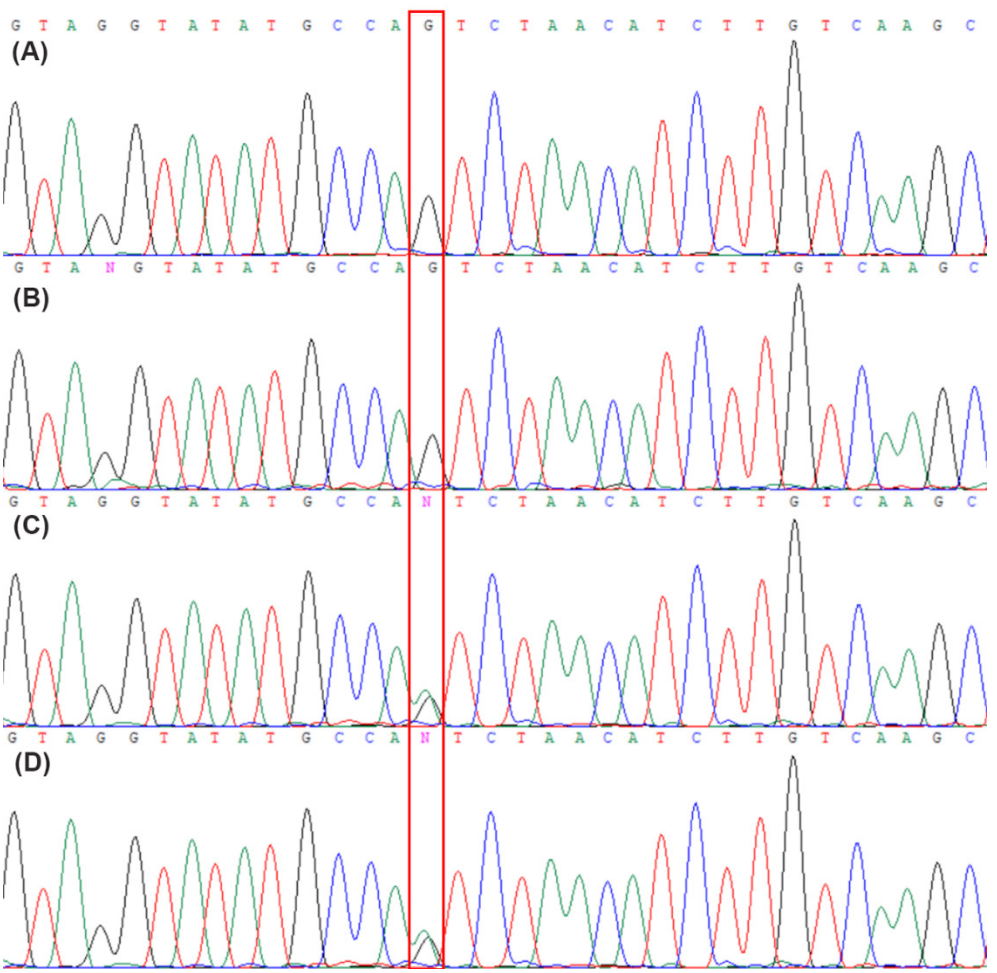

Figure 2. Illustrates the detection of a mutation in the *TMEM67* gene and the genetic screening conducted on the investigated family. Direct sequencing of the DNA from the son (A) and daughter (B) revealed a homozygous mutation (c.725A>G; p.Asn242Ser) in both individuals, while the healthy parents (C, D) were identified as carriers of the detected mutation in the heterozygous state.

classification, and this determination aligns with the established standards and guidelines provided by the ACMG. The functional domain affected by this variant is the D domain.[16]

PLP1 mutation

Clinical presentation

The 5-year-old son exhibited delayed motor milestones, hypotonia, abnormal eye movements, cognitive impairment, and progressive spasticity, consistent with Pelizaeus-Merzbacher disease.

Genetic analysis

Exome-sequencing revealed a novel homozygous *PLP1* (NM_001128834.3): c.772A>C; p.Met258Leu mutation in exon 8, a missense mutation. Sanger sequencing confirmed X-linked recessive inheritance, with the mother as a heterozygous carrier, the affected son as homozygous, and the father exhibiting a normal genotype (Figure 3).

Functional implications

The missense mutation, replacing methionine with leucine, disrupts the PLP1 protein's structure within the D domain.[17] As a major central nervous system myelin component, the altered PLP1 protein leads to abnormal myelination, underpinning Pelizaeus-Merzbacher disease. Understanding the X-linked recessive inheritance pattern clarifies the role of the mother as a carrier and the father's contribution to the unaffected state. Explanation of the mutation's transmission from carrier parents to an affected offspring sheds light on its inheritance. The ACMG classification for this mutation is uncertain significance.

GAN mutation

Clinical presentation

In a consanguineous Iranian family, the 12-year-old daughter exhibited symptoms of giant axonal neuropathy-1, including progressive muscle weakness, sensory impairment, gait abnormalities, and developmental delay.

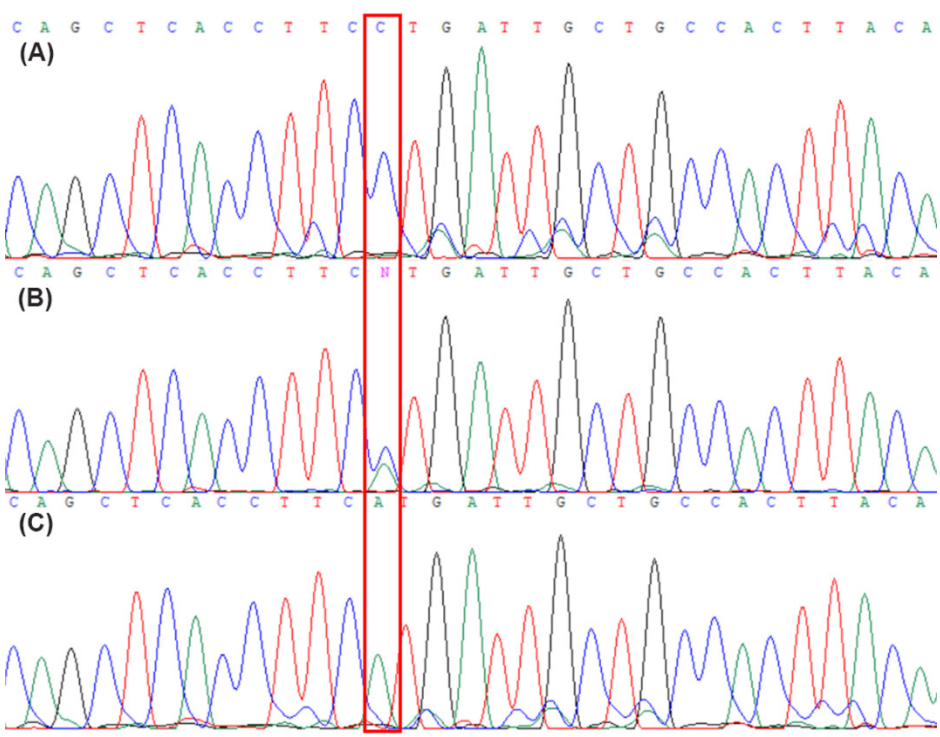

Figure 3. Illustrates the discovery of a new mutation in the *PLP1* gene and the genetic screening performed on the investigated family. Direct sequencing of the patient's DNA (A) unveiled a novel homozygous mutation (c.772A>C; p. Met258Leu), while his healthy mother (B) and father (C) were identified as carriers of the mutation in the heterozygous state and exhibited a normal (wild-type) genotype, respectively.

Genetic analysis

Exome-sequencing identified a homozygous *GAN* (NM_022041.4): c.1177T>C; p.Cys393Arg mutation (RCV003051387.2) in exon 7. Sanger sequencing confirmed homozygosity in the affected daughter and heterozygosity in the unaffected parents (Figure 4).

Functional implications

The substitution of cysteine with arginine due to the mutation disrupts disulfide bond formation in the GAN protein's BACK domain.[18] This alteration impacts protein stability and structure, contributing to the manifestation of giant axonal neuropathy-1 by affecting neuronal function and integrity. Understanding the hereditary transmission emphasizes the autosomal recessive inheritance pattern, elucidating both parents need to contribute a mutated allele. Genetic analysis provides crucial information on the impact of the mutation on protein stability and structure, unraveling the molecular basis for the observed clinical manifestations and guiding potential therapeutic interventions. The discussion on heredity extends to how the mutated allele is transmitted through generations, shedding light on the complexities of familial genetic factors in disease etiology. The ACMG classification for this mutation is uncertain significance.

DISCUSSION

Given the wide variety of genotype-phenotype correlations in NDs, the present survey aims to investigate the spectrum of NDs and their associated gene mutations. Our data identified four patients with genetic NDs, including two with Jubert syndrome (NM_001382391.1 (*CSPP1*): c.2259_2260delAA; p.Glu755GlyfsTer30) (NM_153704.6 (*TMEM67*): c.725A>G; p.Asn242Ser), one with giant axonal neuropathy-1 (NM_022041.4 (*GAN*): c.1177T>C; p.Cys393Arg), and one with Pelizaeus-Merzbacher disease. Among the identified mutations, we discovered a novel missense gene defect (NM_001128834.2 (*PLP1*):

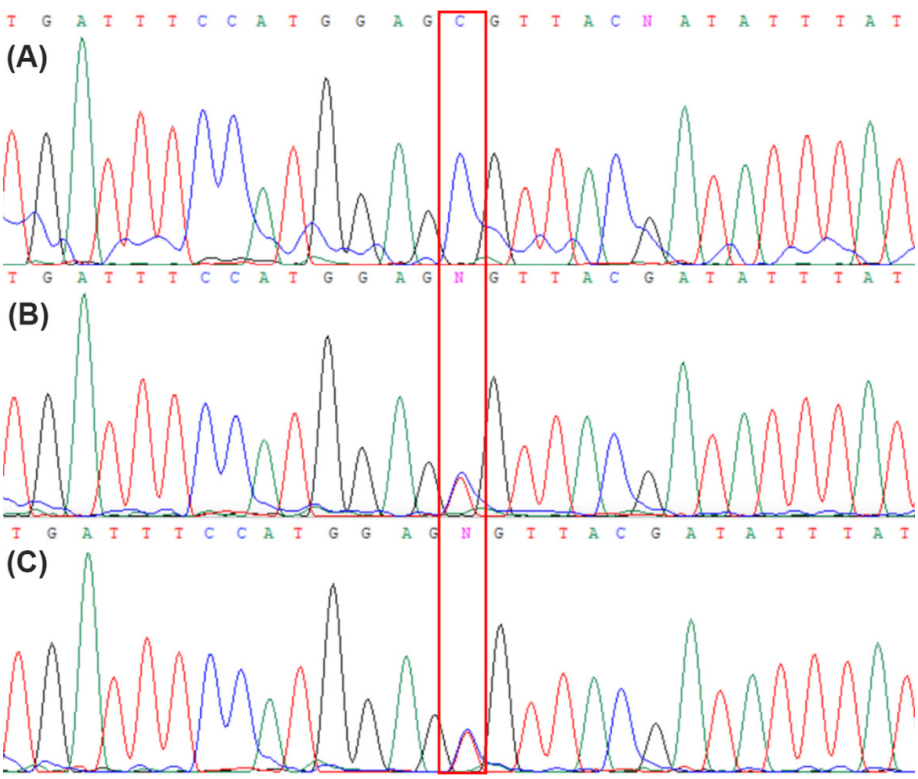

Figure 4. Illustrates the discovery of a mutation in the *GAN* gene and the genetic screening of the family under investigation. The direct sequencing of the patient's DNA (A) uncovered a homozygous mutation (c.1177T>C; p. Cys393Arg), while her unaffected mother (B) and father (C) were found to carry the mutation in a heterozygous state.

c.772A>C; p.Met258Leu) in a patient diagnosed with Pelizaeus-Merzbacher disease.

Pelizaeus–Merzbacher disease is a rare disorder occurring in 1 in 7500 births and is associated with extensive NDs.[19,20] A spectrum of clinical severity exists in Pelizaeus-Merzbacher disease that varies widely; hence, genetic analysis is essential for its diagnosis. The pelicans-Merzbacher disease is characterized by congenital hypomyelination involving the *PLP1* gene. The most frequent gene mutation is *PLP1* duplication, resulting in high expression of *PLP1* with normal function.[21,22] On the other hand, there are rare Pelizaeus-Merzbacher disease patients with null *PLP1* mutations.[23,24] These patients experience a milder phenotype and, as a result, may not be diagnosed until they are up to 40 years old due to the lack of cellular stress and oligodendrocyte apoptosis.[23]

Here, we identified a novel missense mutation in exon 8 of *PLP1* (NM_001128834.2: c.772A>C; p. Met258Leu) in a 5-year-old boy. In a similar investigation, three novel mutations were detected in exon 3B of *PLP1*, including c.354_355delAG; p.G120PfsTer83, c.398A>C; p.H133P, c.435G>A; p.W145Ter.25 In terms of their phenotypes, hypotonia, progressive spasticity, and cognitive impairment were consistent with our cases. This suggests that patients with intermediate phenotypes and unknown mutations may be suspected of harboring mutations in exons 3B and 8. Additionally, it has been revealed that, besides exons, any alterations in the non-coding regions of *PLP1* can contribute to the development of Pelizaeus-Merzbacher disease. Yamamoto-Shimojima et al. reported a novel mutation in intron 3 of *PLP1* (NM_000533.5: c.453+59_+259del), which leads to the severe Pelizaeus-Merzbacher disease phenotype (26). Additionally, at the distal end of *PLP1*, some enhancers are crucial for *PLP1* expression. In this context, it has been demonstrated that a microdeletion in Xq22.2 (approximately 24.5 kb) results in transcriptional repression of *PLP1*, associated with hereditary spastic paraplegia 2.27 In a study by Lee *et al*., a case of Pelizaeus-Merzbacher disease was diagnosed after 25 years; the patient had a homozygous mutation in exon 1 of the *PLP1* gene, initially misdiagnosed as cerebral palsy.[28] Accordingly, these findings demonstrate the importance of gene sequencing in patients with neurological deficiency.

Joubert syndrome is a rare autosomal recessive disorder, estimated to occur in 1 in 90,000 births[29-31] However, some researchers argue that the frequency of Joubert syndrome might be higher due to the oversight of the molar tooth sign.[32-34] In the present survey, we encountered an 11-year-old boy exhibiting difficulty walking, abnormal eye movements, and cognitive impairment. Gene analysis revealed a homozygous frameshift mutation in exon 20 of *CSPP1*. In a study by Luo *et al*., four mutations were identified in *CSPP1*, highlighting the diagnostic significance of *CSPP1* mutations in treating Joubert syndrome.[35] Defects in the *CSPP1* gene lead to a reduction in the number of primary cilia and/or shortening of primary cilia[36] *CSPP1* encodes two protein isoforms, CSPP1 and CSSPL1, with the former being crucial for encoding a centrosome protein. Disruption in CSPP1 function results in a delay in cell cycle progression[15] *CSPP1* mutations give rise to a classical form of Joubert syndrome. In contrast to *ASCC1*, prenatal diagnosis renders the *CSPP1* phenotype undetectable at 10 weeks of gestation. Therefore, evaluation of *CSPP1* through gene sequencing should be considered for suspected patients.[37] Additionally, we identified another male patient with Joubert syndrome caused by a *TMEM67* mutation, with his sister also affected. Unlike other forms of Joubert syndrome, a genotype-phenotype correlation has been established for *TMEM67*.[38] Patients with Joubert syndrome carrying *TMEM67* mutations are predisposed to liver fibrosis. In our patient, liver dysfunction was observed.

The last patient was identified as having giant axonal neuropathy-1 with *GAN* gene involvement. Giant axonal neuropathy-1 is a progressive chronic peripheral neuropathy; however, lesions can be detected in the central nervous system, especially in the brainstem and cerebellum. In a study by Chakravorty *et al*., pediatric patients with sensory polyneuropathy were evaluated for genetic deficits. Their results identified two patients diagnosed with giant-axonal neuropathy, one with a homozygous pathogenic variant (c.851+1G>A), and the other in a heterozygous manner in the *GAN* gene (c.805C>T; p.Arg269Trp, and c.732delT; p.Ile244MetfsX33).[39] In a study conducted by Dorsey *et al*., a reduction in the downregulation of giant axonal neuropathy-1 was observed following the treatment of HIV with nucleoside reverse transcriptase inhibitors, which can cause neuropathy.[40]

In conclusion, our study significantly advances the understanding of Joubert syndrome, Pelizaeus-Merzbacher disease, and giant axonal neuropathy-1 by uncovering key genetic mutations and shedding light on their intricate clinical presentations. The discerned mutations in *CSPP1*,

TMEM67, *PLP1*, and *GAN* not only deepen our understanding of the molecular mechanisms underlying these disorders but also offer valuable insights into potential avenues for targeted therapeutic interventions. Genetic analysis proves pivotal in unraveling the complexities of these NDs, emphasizing the crucial role of hereditary factors in their manifestation. This comprehensive approach not only enhances our comprehension of the diseases at a molecular level but also provides a solid foundation for developing precise and effective treatment strategies, aligning with the broader goal of advancing precision medicine for neurological conditions.

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DISCLOSURE

Date availability: The current study has generated and/or analyzed datasets available in the [PubMed, Web of Science, Scopus, EM Base] repository.

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Conflict of interests: None.

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