Novel *PPP1R21* mutation in a family with autosomal recessive neurodevelopmental disorder: results of genomics and molecular analysis

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Abstract

Background Neurodevelopmental diseases are a group of disorders affecting the development of the nervous system and brain function. In particular, neurodevelopmental disorder with hypotonia, facial dysmorphism, and brain abnormalities is a novel neurodevelopmental syndrome caused by biallelic *PPP1R21* loss-of-function variants. This study aimed to investigate the molecular etiology of this neurodevelopmental disorder in an Iranian patient from a consanguineous marriage family.

Methods and results After clinical examination and DNA sampling, whole exome sequencing was performed for the patient. The findings were confirmed and segregated via Sanger sequencing and bioinformatics approach in the patient and parents, respectively. We identified the novel loss-of-function mutation of c.1317_1318delAG p.(Asp440Tyrfs*6) in *PPP1R21* gene in our patient suffering from severe developmental delays, mental retardation, facial deformities, muscle weakness, difficulty breathing and feeding, and vision impairment. Through Sanger sequencing, the homozygous and heterozygous statuses of this variant were observed in the patient and the parents, respectively. As well, the bioinformatics approach demonstrated the disease-causing effect and clinical pathogenicity of this mutation.

Conclusions Such findings improve our knowledge of patients with neurodevelopmental phenotypes. In addition, these results can be particularly helpful for prenatal and preimplantation diagnosis and genetic counseling of families with a high risk of infantile intellectual disabilities.

Keywords PPP1R21, Neurodevelopmental disorder, Whole exome sequencing

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Introduction

Neurodevelopmental disorders are clinically and genetically heterogeneous and indicate serious problems in the function of brain and central nervous system. In addition to affecting development and nervous system, this category of disorders severely impairs abilities related to learning, excitement, memory, and emotions. Injuries from neurodevelopmental defects are not usually limited to childhood, and their effects can be present in adulthood [1]. In particular, the neurodevelopmental disorder characterized by hypotonia, facial dysmorphism, and

brain abnormalities is an autosomal recessive neurologic syndrome. It is commonly associated with hypotonia and global developmental delay, leading primarily to severely impaired intellectual development, characteristic coarse facial features, and an inability to walk or sit. Furthermore, brain imaging demonstrate variable abnormalities, including thin corpus callosum, cerebellar hypoplasia, enlarged ventricles, decreased white matter volume, and white matter changes [2].

Mutations in *PPP1R21* (*protein phosphatase 1 regulatory subunit 21*) gene have been reported to cause neurodevelopmental disorder with hypotonia, facial dysmorphism, and brain abnormalities [2–4]. This gene is located on chromosome 2p16.3 and encodes a 3142 amino acid protein [4]. The protein produced by the *PPP1R21* gene has been identified as a regulatory subunit of protein phosphatase-1 (PP1), which plays a role in glycogen metabolism, cell differentiation, and endosome maturation pathway [5]. Various enzyme deficiencies within endosomes can lead to the accumulation of toxins and cause metabolic disorders [6]. Neurological deficits are also frequently observed with metabolic disorders characterized by growth retardation, lethargy, facial dysmorphism, splenomegaly, vision impairment, respiratory problems, vomiting, and feeding difficulties [7].

Advances in molecular genetics testing have significantly improved the landscape of investigating neurodevelopmental disorders. In particular, whole exome sequencing (WES) offers a high diagnostic yield in the detection of novel genes and has emerged as an efficient diagnostic approach for neurodevelopmental patients. A high diagnostic rate, coupled with improvements in variant filtering and interpretation, are additional advantages that have made WES an appealing alternative to the traditional diagnostic tests [8–10].

In this study, our goal was to study the disease-causing etiology in an Iranian patient with a neurological disorder from healthy consanguineous parents. This family also had a deceased affected child with the same phenotypes before. After gathering the clinical features of the patient, the sampling and WES analysis were performed. The findings were confirmed and segregated in the patient and parents, respectively, using Sanger sequencing and bioinformatics approach.

Materials and methods

Case presentation

In this investigation, we studied a 13-year-old boy with a neurodevelopmental disorder from an Iranian consanguineous family. In early infancy, the patient was found to have hypotonia, poor feeding, respiratory distress episodes, recurrent chest infections, seizures, and muscle weakness. After a few months, he had signs of developmental delay and mental retardation.

Of note, his parents were healthy regarding neurodevelopmental phenotypes. The family had an older affected girl with the same phenotypes who passed away a few month after birth. No other family history with similar symptoms was observed. The patient was referred by the Genetic Counseling Center in Kermanshah, a Western city in Iran, to the Dr. Alibakhshi Genetics laboratory for genetic evaluations and clinical exome sequencing. Written informed consent was obtained from the parents for further analysis.

DNA extraction

DNA was extracted from the peripheral blood of the patients and their parents using Qiagen Flexi Gene DNA Kit (Qiagen, Hilden, Germany). The purity and quantity of the extracted DNAs were investigated by Thermo Scientific™ Nano Drop™ One Micro volume UV–Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA) and through running on the 2% agarose gel. We applied WES analysis for the patient (Fig. 1, IV-1).

Whole exome sequencing pipeline

To create the DNA library, DNA was fragmented into 150–300 bp DNA fragments by ultrasonic processor and the target capture was carried out at Macrogen Company, Germany using the SureSelect Human All Exon V7 kit (Agilent Technologies, Santa Clara, CA, USA). The generated library was sequenced on the Illumine HiSeq 2500 platform with an average coverage depth of 175X.

The sequence read quality assessment was done by generating quality control (QC) step with FastQC and IlluQC softwares. Burrows-Wheeler Alignment (BWA) algorithm was used with default parameters for alignment to the human genome assembly GRCh38 (hg38). Samtools was used to convert the SAM (Sequence Alignment Map) file to the BAM (Binary Alignment Map) file. Picard-Tools was used to sort the input BAM file by coordinate and to remove the duplications. Then, BQSR (Base Quality Score Recalibration) tool was utilized to recalibrate the base qualities of the input BAM or CRAM files. Variant calling was performed with HaplotypeCaller algorithm from Genome Analysis Toolkit version 4.0 (GATK4) package [11, 12].

Annotations of the variants were performed with ANNOVAR based on various public and in-house databases. The allele frequency was searched in 1000 Genomes Project (1000GP), Exome Aggregation Consortium (ExAC), Genome Aggregation Database (gnomAD), dbSNP, and Iranome. Being focused on the minimum allele frequency (MAF) of the variants, a MAF < 1% was considered, and the remaining variants were prioritized
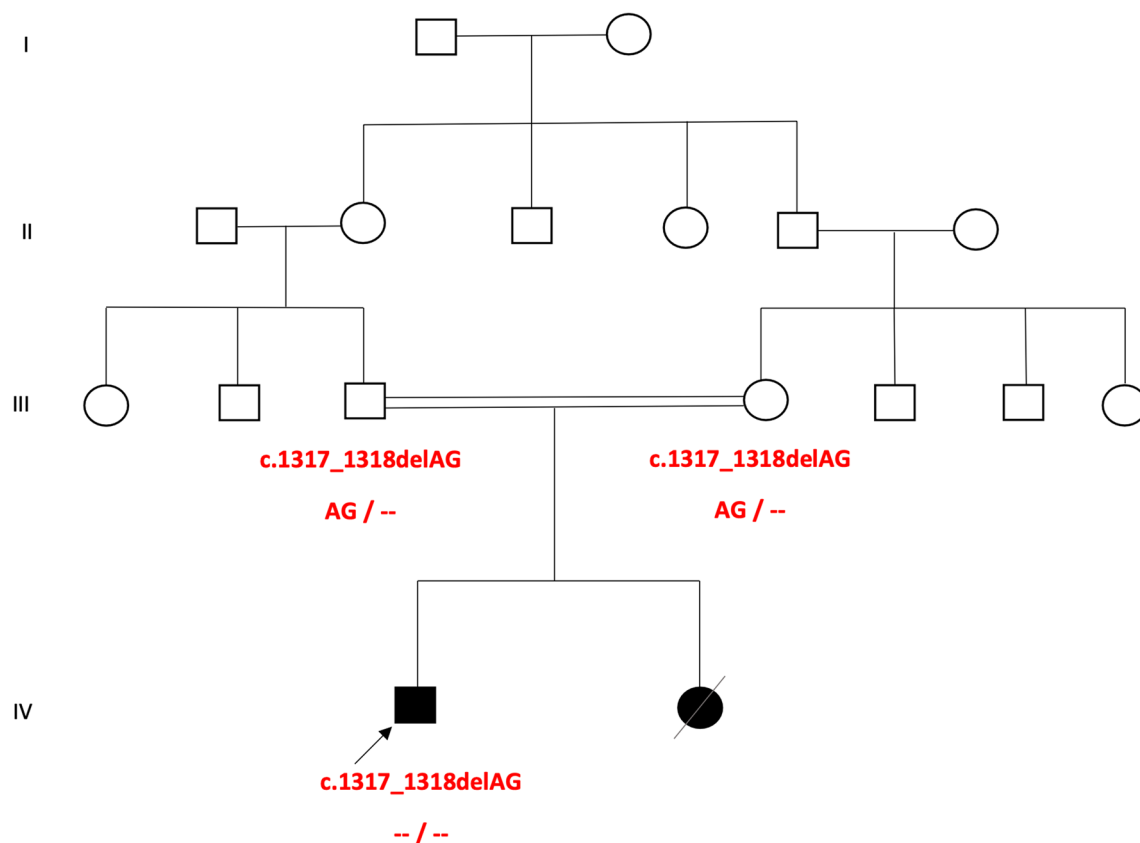
Fig. 1 Pedigree of the family. The c.1317_1318delAG; p.(Asp440Tyrfs*6) mutation in the *PPP1R21* gene was observed homozygous in the patient. This mutation was heterozygous in the parents

based on other filtering parameters such as being located in exonic regions and/or being homozygous, in addition to selecting the variants in phenotype-related genes. These steps were taken into account mainly because of the likely autosomal recessive nature of the disorder phenotype and consanguinity in the family. Afterward, clinical significance of variants was evaluated using ClinVar, VarSome, Franklin, Ensemble, PolyPhen-2, SIFT, and MutationTaster databases. The clinical criteria of the American College of Medical Genetics (ACMG) guidelines were implemented throughout all these steps [13]. The functional protein association network was assessed through in-silico analysis using the online STRING server [14]. The local cluster network of STRING database with at least medium confidence (0.400) option was performed for the functional network of PPP1R21 protein. The full STRING network type was selected to indicate both functional and physical protein associations.

Variant confirmation and segregation
Sanger sequencing was used to confirm and segregate the identified variant(s) in the patient and his parents. Primers were designed and checked by using Primer3

(bioinformatics.nl/cgibin/primer3plus/primer3plus. cgi) and Primer-BLAST NCBI, respectively. Primer sequences and polymerase chain reaction conditions are available upon request.

Results
Clinical data
The patient (Fig. 1, IV-1) was a 13-year-old boy with unaffected first-cousin parents. At birth, he had normal growth parameters. However, he began to suffer from feeding difficulties, respiratory distress, brain anomalies, and hypotonia, and consequently was admitted to neonatal intensive care unit for two weeks. Physical examination revealed developmental delay (including jerking movements of the arms and legs that cannot be controlled), distinctive facial features, epilepsy and tonic seizures, chest infections, and mental abnormalities. Phenotypes of the patient included thick eyebrows, hypertelorism, short nose, upslanted palpebral fissures, thick lips, broad nasal bridge, upturned nasal tip, broad and low-hanging columella, low-set ears, high-arched palate, coarse facies, and flat occiput. His developmental delay consisted of slower-than-normal development of motor,

cognitive, social, and emotional skills. Additionally, the child exhibited pronounced hypotonia, often described as a 'strong floppy' condition, and the recurrent chest infections were attributed to respiratory issues.

Brain MRI showed white matter atrophy in parieto-occipital lobes with abnormal periventricular T2 hyper-intensity returned from the deep white matter, and foreshortening and thinning of a corpus callosum (Fig. 2).

Interestingly, his deceased sister (Individual IV-2 in the pedigree, Fig. 1) was reported to have the same clinical symptoms and phenotypes. Her clinical course included the presence of muscle weakness, seizures, hypotonia, feeding difficulties, respiratory distress, developmental delay, and mental retardation. Unfortunately, no DNA sample was available from her and no molecular examination was possible.

Genetic findings and bioinformatics analysis

WES was performed on the patient (Fig. 1, IV-1). After quality control and alignment steps, a number of 72,695 variants were called by GATK4. Subsequently, annotation of these variants was performed with ANNOVAR. In particular, the homozygous alterations (21,815 variants) were considered because of apparently recessive mode of inheritance. In the filtering process, by excluding the variants with allele frequency of greater than 1%, only 2071 homozygous variants remained. Then, synonymous and benign variants were excluded, which ended up with 314 variants. Next, we chose only variants located in the patient's phenotype-related genes (19 variants). The details of these filtering steps are shown in Fig. 3.

Variants were interpreted based on clinical significances and *in-silico* predictions. We detected one rare homozygous frameshift variant (Fig. 4), which was a deletion of two nucleotides, c.1317_1318delAG;
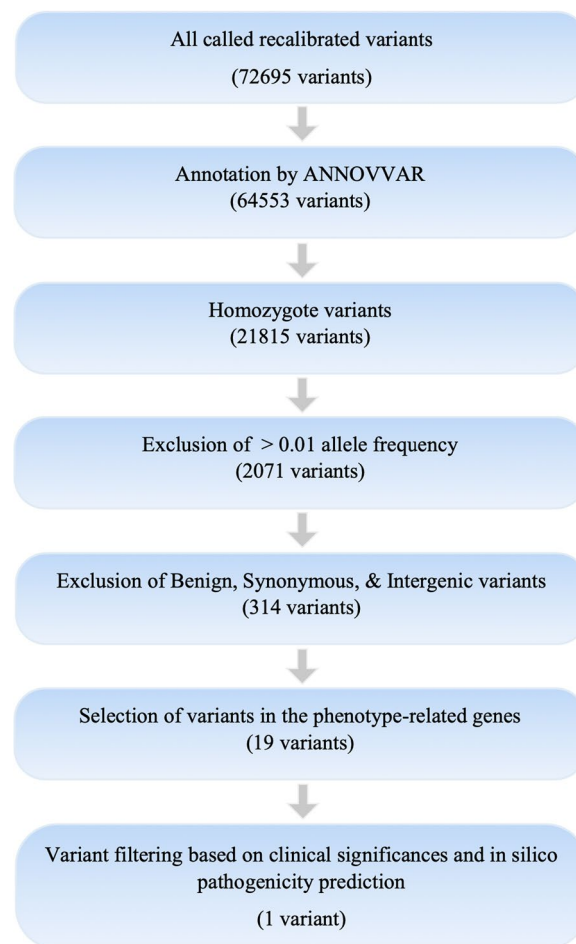
Fig. 3 Flowchart of variant filtering process performed through whole exome sequencing (WES) data analysis. After annotation, variants were filtered based on described parameters, and finally one disease-causing variant in *PPP1R21* gene was found
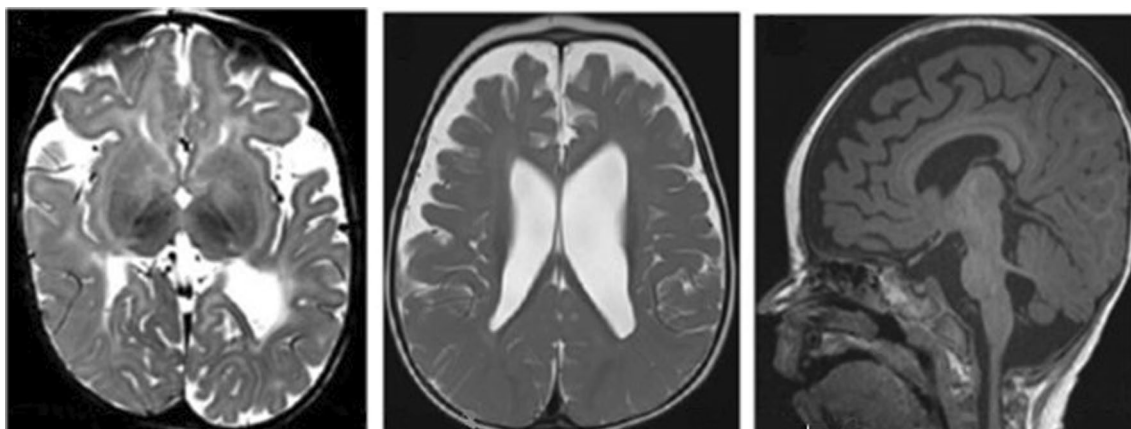
Fig. 2 Brain MRI of the patient (right to left sagittal T1, axial T2 and coronal T2 images) showing mild ventricular hypoplasia with prominent CSF spaces, reduced white matter volume, hypoplasia of corpus callosum, and cavum septum pellucidum
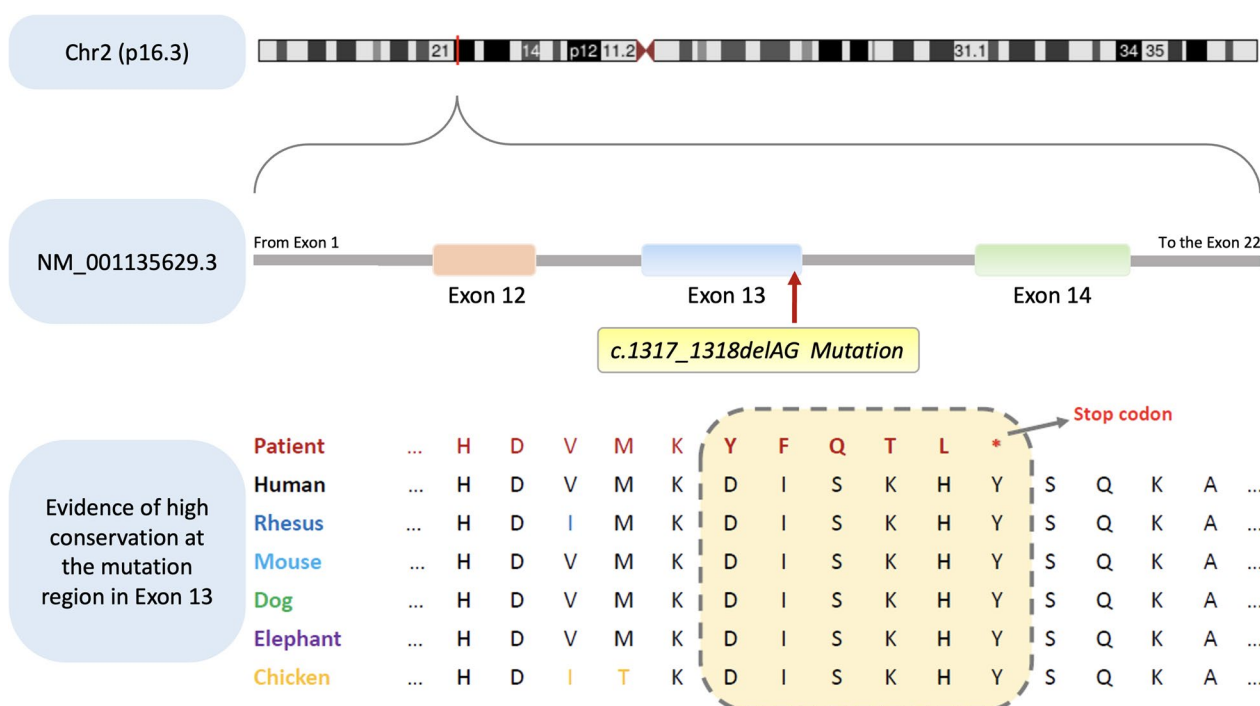
Fig. 4 A schematic section of the *PPP1R21* gene, and the location of the identified pathogenic variant (c.1317_1318delAG) in human genome (GRCh38/hg38 assembly). Conservation of the region containing the mutation across different species is shown as well

p.(Asp440Tyrfs*6) in exon 13 of *PPP1R21* gene (NM_001135629.3). According to the American College of Medical Genetics (ACMG) guidelines [15], this loss-of-function variant is classified as a pathogenic mutation. The ACMG evidences in support of its pathogenicity are as follows:

PVS1 This mutation is a null (frameshift) variant in *PPP1R21* gene, for which loss-of-function is a known mechanism of disease. The identified variant p.(Asp440Tyrfs*6) would lead to a change in the protein structure by disturbing normal splicing and creating a stop codon at amino acid (AA) 445 instead of AA 781 in wild type sequence, causing nonsense-mediated mRNA decay and complete loss of function;

PM2 This *PPP1R21* variant has not been yet described and was not found in any population database including gnomAD, dbSNP, ExAC, and Iranome databases;

PS4 The variant was seen in affected family members and was not detected in the normal controls;

PP4 Patient's phenotype and family history is highly specific for a neurodevelopmental disease with a single genetic etiology;

PM4 This variant reduces the length of the protein;

PP1 The variant is segregated with the disease in affected family members; and

*PP3* In silico prediction algorithms, including SIFT, PolyPhen2, MutationTaster, Franklin, and VarSome, strongly support the pathogenic and deleterious effects of this mutation.

Sanger sequencing confirmed the homozygous status in the patient and the heterozygous status in his parents (Fig. 5). Thus, the parents were obligate carriers. In conclusion, results showed the co-segregation of the c.1317_1318delAG variant in *PPP1R21* gene with the disease in the family.

The protein cluster network of STRING database for PPP1R21 protein was performed, and it showed the highest predicted association scores with *C2orf44*, *PIBF1*, *TMEM247*, *CRYZL1*, *STON1*, *PCDHGA10*, and *STRN* genes. Likewise, multispecies alignment for this variant indicated high conservation within species (Fig. 6).

Discussion

To the best of our knowledge, this is the first study in Iranian population and is among the few investigations on disease-causing variants of *PPP1R21* gene worldwide in neurodevelopmental disorders [2–4]. We identified the pathogenic homozygous mutation of c.1317_1318delAG in *PPP1R21* gene in a patient with autosomal recessive neurodevelopmental disorder, and its heterozygous status in the parents was confirmed as well. The *PPP1R21* belongs to a family of proteins with coiled-coil domains and is evolutionary conserved regarding several developmental processes. This mutation is novel and has not

Heterozygous AG deletion

Heterozygous mutation of c.1317_1318delAG in exon 13 of the *PPP1R21* gene in the father

Heterozygous AG deletion

Heterozygous mutation of c.1317_1318delAG in exon 13 of the *PPP1R21* gene in the mother

Homozygous AG deletion

Homozygous mutation of c.1317_1318delAG in exon 13 of the *PPP1R21* gene in the patient
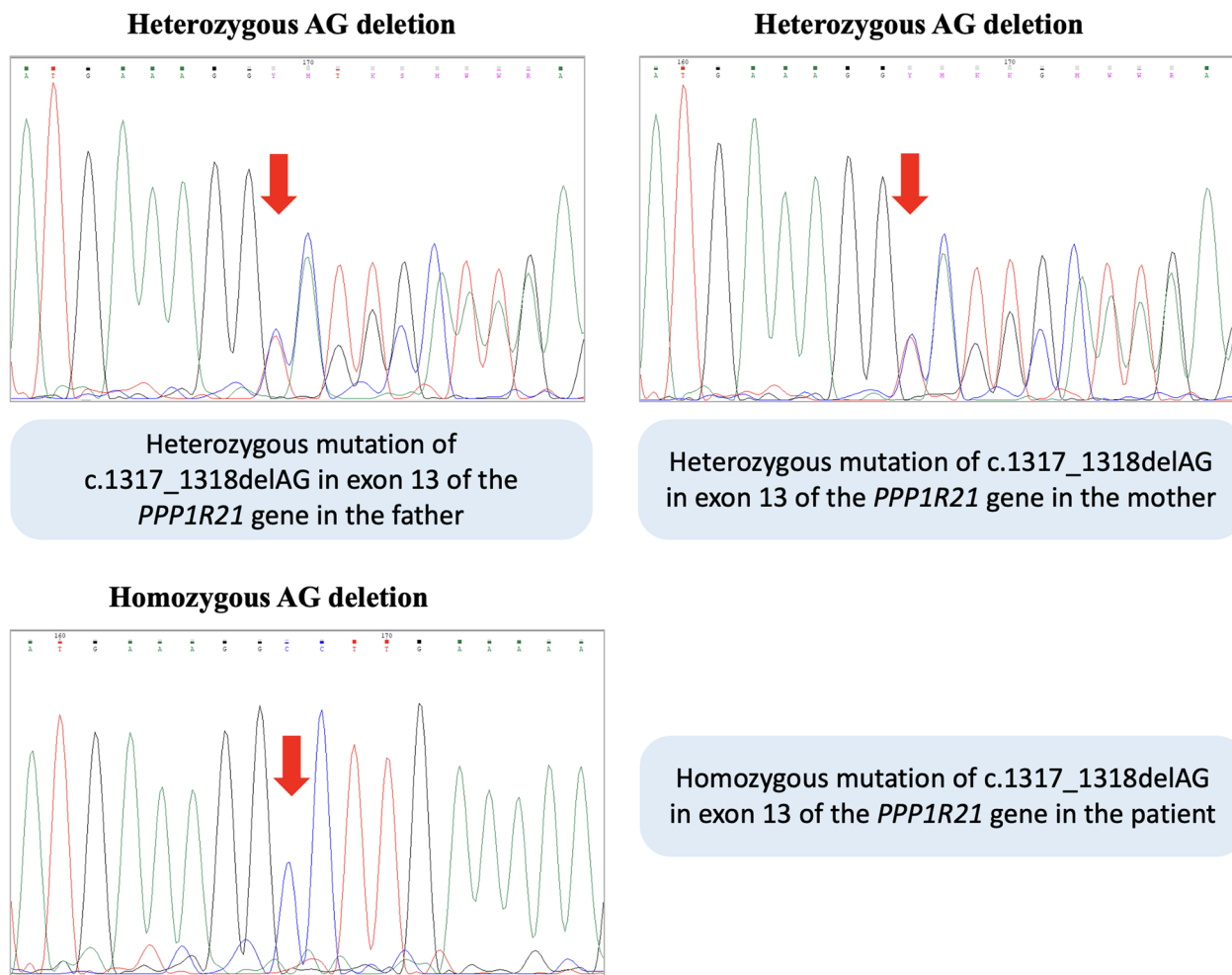
Fig. 5 Sequencing chromatogram of genomic DNA showing c.1317_1318delAG; p.(Asp440Tyrfs*6) mutation in *PPP1R21* gene. The patient was homozygous for this variant, and the parents were heterozygous

been reported in population and literature databases so far. The aggregated prediction of computational tools demonstrated a deleterious role for this variant too. Moreover, the deceased sister of the patient was mentioned to have the same clinical phenotypes, suggesting another evidence for the inherited basis of this neurodevelopmental disease in the family.

Neurodevelopmental disorders are a subset of nervous system disorders that specifically impact the central nervous system, leading to impairments in the growth, development, and function of the brain [1, 16]. Examples of neurodevelopmental disorders include learning disabilities, communication disorders, attention deficit, autism spectrum disorder, mental disabilities, movement problems, and developmental delays. These disorders start early in life and last a lifetime [17]. Despite the progress that has been made in recent years, significant gaps remain in understanding the heterogeneity of

neurodevelopmental diseases and their basic molecular mechanisms [18]. The next-generation sequencing (NGS) methods have been successfully used in sequencing a variety of protein coding and non-coding regions of human genome associated with neurodevelopmental disorders [19]. This extensive coverage has improved the sensitivity of mutation detection where conventional DNA sequencing has not been able to achieve such precise genetic diagnosis. Among all NGS methods, WES is recognized as the initial clinical approach for patients. It is particularly effective for identifying many genetic diseases, especially monogenic disorders [20].

Protein phosphorylation is the most common post-translational modification and also is known as the principal regulatory mechanism in eukaryotic intracellular processes. This reversible action is regulated by protein kinases and protein phosphatases. The PPP1R21 protein serves as the regulator of protein phosphatase 1 (PP1),
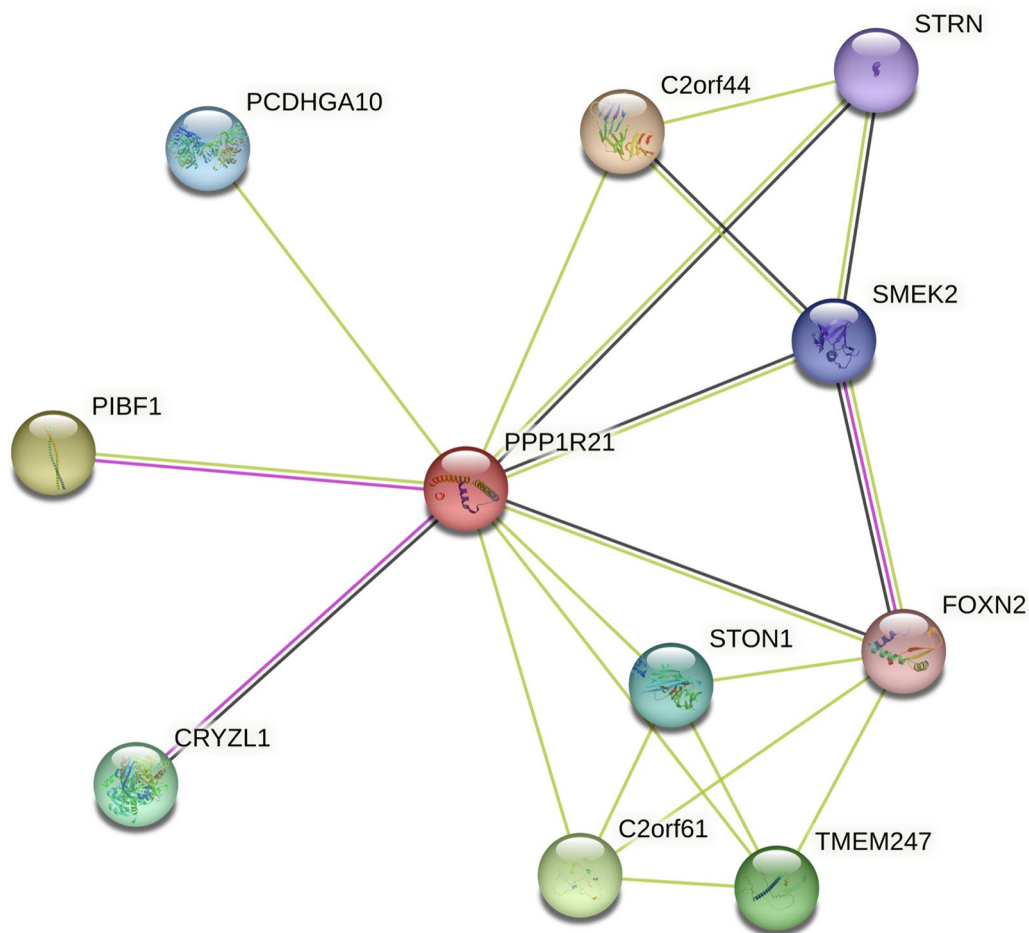
Fig. 6 The *in-silico* analysis of the functional protein association network was assessed via online STRING server for *PPP1R21* interactions

which belongs to a family of highly conserved serine/threonine-specific protein phosphatases. PP1 acts as a catalyst in protein dephosphorylation events across a variety of cellular functions [3, 21]. Thus, mutations in *PPP1R21* lead to defects in its interaction with PP1, resulting in a range of human diseases, particularly those characterized by intellectual disability phenotypes such as microcephaly and short stature disorders [4, 22, 23]. According to the results of functional protein association network, the PPP1R21 protein had a strong network with genes related to developmental delay and intellectual disability phenotypes (*CRYZL1*, *PCDHGA10*, *STON1*, *STRN*, and *PIBF1*), cancers (*C2orf44*), and gastrointestinal disorder (*TMEM247*) (Fig. 6). In a similar manner, Hentschel et al. [24] through proteomic signature of *PPP1R21*-mutant fibroblasts elucidated the dysregulation of a variety of critical proteins involved in neurological diseases via cross-link interactions.

More specifically, mutations in *PPP1R21* gene could cause endolysosomal functional defects. Therefore,

accumulation of its metabolites results in autophagy pathway impairment and severe neurodevelopmental outcomes. It has been well-established that autophagy is predominantly important in post-mitotic and metabolically active cells, including neurons, and is essential for the normal development and function of the central nervous system. Interestingly, a modified autophagy pathway has been demonstrated to be linked with brain malformations mainly involving white matter [25]. This particularly includes the defective axon guidance that eventually leads to the abnormal interhemispheric axon tracts, including the corpus callosum [26]. Also, loss of autophagy has been reported to cause axonal outgrowth defects, again supporting the paramount role of this pathway for maintaining required brain connectivity [2]. In complete agreement with these evidences, the brain imaging in our patient represented reduced white matter volume, hypoplasia of corpus callosum, ventricular hypoplasia, and cavum septum pellucidum.

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Rehman et al. (2018) worked on functional analysis and physiological role of *PPP1R21*. They identified four previously unreported homozygous truncating *PPP1R21* alleles in four families with neurodegenerative diseases and found that PPP1R21 protein was absent in fibroblasts of an affected individual. Furthermore, their results point toward a defect within the endosomal-lysosomal compartment, indicating that *PPP1R21* is essential for proper functioning of the early endosome compartment [4].

There are similarities between the attitudes expressed by Anazi et al. (2017), Rehman et al. (2018), Suleiman et al. (2018), Loddo et al. (2020), and Hentschel et al. (2023) studying neurodevelopmental disorder cases [2, 3, 6, 9, 24]. The clinical features of patients described in these studies are very similar to what we observed in two patients in our study, including global developmental delay, dysmorphic facial features, movement disorder, respiratory problems, feeding difficulties, hepatomegaly, and vision impairment. Brain MRI exhibited several overlapping common findings. These included a prominent dysmorphic ventricular system with an irregular outline to the bodies of the lateral ventricles, reminiscent of PVL; loss of white matter; increased T2 hyperintensity in the deep white matter; and thinning of the corpus callosum. The similarities in the clinical and neuroimaging features in these studies strongly support that biallelic loss of *PPP1R21* is responsible for the observed phenotype. Our study confirms these findings and further supports the vital role of this gene. Finally, as a follow-up investigation for this study, we suggest performing transgenic and knockout animal models for this novel mutation in PPP1R21 gene to more thoroughly confirm these findings. Such experiments would provide strong validation of our results across biomedical contexts and also pave the way for future interventional strategies leading to improved targeted therapeutics and genetic counseling.

Conclusion

In conclusion, we reported the novel loss-of-function mutation of c.1317_1318delAG p.(Asp440Tyrfs*6) in *PPP1R21* gene, resulting in a truncated protein. Such findings can improve our knowledge of patients with neurodevelopmental phenotypes. In addition, these results can be particularly helpful for prenatal and pre-implantation diagnosis and genetic counseling of families with a high risk of infantile intellectual disabilities.

Abbreviations

1000GP	1000 Genomes project
ACMG	American College of Medical Genetics
BAM	Binary alignment map
BQSR	Base quality score recalibration
BWA	Burrows-Wheeler alignment
CNS	Central nervous system
ExAC	Exome aggregation consortium
GATK4	Genome analysis toolkit version 4
gnomAD	Genome aggregation database
MAF	Minor allele frequency
MRI	Magnetic resonance imaging
NGS	Next-generation sequencing
PP1	Protein phosphatase-1
PPP1R21	Protein phosphatase 1 regulatory subunit 21
QC	Quality control
SAM	Sequence alignment mapping
WES	Whole-exome sequencing

Declarations

Ethics approval and consent to participate
This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Kermanshah University of Medical Sciences, Kermanshah, Iran. Written informed consent was obtained from all individual participants included in the study.

Consent for publication
Consent to publish from the parents of the affected child has been taken.

Competing interests
The authors have no relevant financial or non-financial interests to disclose.

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