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RESEARCH

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Novel pyrroline-5-carboxylate reductase 2 (*PYCR2*) mutation in an Iranian patient with hypomyelinating leukodystrophy: findings of molecular and *in silico* investigations

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Abstract

Background Hypomyelinating leukodystrophy (HLD) is a specific group of leukodystrophies and is characterized by progressive postnatal growth delay that represents a type of clinically overlapping but genetically heterogeneous diseases with autosomal recessive inheritance. Loss-of-function mutations in *PYCR2* are one of the main causes of HLD type 10 (HLD10), which is identified by cerebral hypomyelination, inadequate growth, brain atrophy, and movement abnormality. This study aimed to investigate the molecular etiology of HLD10 disorder in an Iranian patient from a consanguineous marriage family.

Results The DNA samples were extracted from the patient, a 9-year-old girl, and her parents. Whole-exome sequencing was conducted for these samples and the results were eventually confirmed and segregated via Sanger sequencing. Our findings demonstrated a novel homozygous frameshift mutation in *PYCR2* gene, c.135dup (NM_013328.4). The heterozygous state of this variant was confirmed in parents. Additionally, this mutation was predicted to exhibit damaging effects through protein sequence alteration.

Conclusions Such findings are of importance for understanding the underlying pathogenicity mechanisms and for improving genetic counseling knowledge of HLD patients for families.

Keywords Hypomyelinating leukodystrophy, *PYCR2*, Whole-exome sequencing

Background

Hypomyelinating leukodystrophy (HLD) is a specific group of leukodystrophies and is characterized by progressive postnatal growth delay that represents a type of clinically overlapping but genetically heterogeneous diseases with autosomal recessive inheritance [1]. These patients mainly suffer from symptoms such as cerebral hypomyelination and severe psychomotor developmental delayed with absent speech. In addition, the hyperreflexia, appendicular hypertonia with hyperextensibility of the wrists and ankles, severe muscle wasting, axial

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hypotonia, and failure to thrive are other reported characteristics of HLD10. This disorder affects one out of every 200,000 to 500,000 individuals and is classified as a rare disease [2]. So far, multiple types of HLD have been recognized based on distinct genes that carry pathogenic variants [1, 3–6].

Hypomyelinating leukodystrophy type 10 (HLD10) is caused by loss-of-function mutations in Pyrroline-5-Carboxylate Reductase 2 (*PYCR2*) and its phenotype is identified by cerebral hypomyelination, inadequate growth, brain atrophy, and movement abnormality [7]. Although three isozymes of *PYCR*-family have been discovered in human (*PYCR1-3*), only *PYCR1* and *PYCR2* contribute to human abnormalities. Dysfunction of *PYCR2* results in proline deficit as it is responsible for encoding the mitochondrial enzyme pyrroline-5-carboxylate reductase 2 engaged in synthesis of proline, which is vital for central nervous system (CNS) [7–10]. Importantly, defects in proline synthesis have major deleterious effects on health, resulting in intellectual disability, skin and joint hyperelasticity, osteopenia, and cataracts [11].

Recent nucleotide sequencing technologies with next-generation sequencing (NGS) have enabled us to identify both known and novel HLD-responsible genes and variants [2]. NGS has revolutionized medical genetics by improving the chances of receiving a molecular diagnosis for rare genetic diseases. Whole-exome sequencing (WES), as one of the widely used NGS approaches in research and clinical setups, has demonstrated an unprecedented success rate in the recognition of disease-causing genes in projects such as discovery of the molecular basis of a recognizable syndrome in a homogeneous group of patients and also in pan-genomic sequencing of large heterogeneous cohorts [12]. Moreover, it has been successfully used, as an edge-cutting approach, to study the protein-coding and significant flanking regions of the genome contributing to more than 85% of the monogenic disorders. This high-throughput technique helps to reveal the disease-causing variants with low cost and high resolution in comparison with other diagnostic molecular methods such as single locus-based and panel-based sequencings in a wide range of inherited diseases [13].

In conclusion, since to the best of our knowledge no molecular investigation has been performed on patients with HLD10 in Iran, our goal was to conduct WES analysis for an Iranian patient with intellectual disability and failure to thrive in order to identify the disease-causing variant(s). The results were confirmed and assessed by Sanger sequencing and *in silico* approach, respectively.

Methods

Case presentation

In this study, a clinical examination was carried out on a 9-year-old girl from an Iranian family with consanguineous marriage. The pedigree of the family is shown in Fig. 1 and was drawn based on National Society of Genetic Counselors (NSGC) recommendations [14]. The patient was referred to the hospital for further evaluations and the phenotypes were CP, growth delay, chronic constipation, abnormality of movement without support, hydrocephalus, and tooth abnormalities. All general tests of the patient, including Complete Blood Count, Urine Analysis, Calcium, Phosphorous, Alkaline phosphatase, Total Vitamin D, and cytogenetic banding analysis, were conducted. Written informed consents were obtained from parents, and the study was approved by the Ethics Committee of the Pasteur Institute of Iran.

DNA extraction and whole-exome sequencing

Peripheral blood was collected from the patient for the extraction of genomic DNA. This process was performed using DNA extraction kit (QIAGEN, Hilden, Germany). The DNA quantity and purity were examined by spectrophotometric measurement of absorbance at 260 and 280 through Thermo Scientific™ NanoDrop™ One Microvolume UV–Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA). The DNA samples were analyzed using electrophoresis for verification on 1% agarose gel, and the samples were stored at −80 °C freezer before sequencing analysis [12].

The obtained DNA was sequenced via WES analysis. The coding exons including genes with known clinical implication were captured by SureSelect Human All Exon V7 kit (Agilent Technologies, Santa Clara, CA, USA). Genomic DNA was fragmented to 151 bp and libraries were produced with Illumina NovaSeq 6000 System (San Diego, CA) in order to generate paired-end reads with the average coverage depth of 100X. The quality assessment of preliminary FASTQ was done by providing quality control (QC) with FastQC, as well as NGS QC Toolkit softwares. Burrows–Wheeler Aligner (BWA) algorithm was applied for alignment of the reads to human genome reference (GRCh37/hg19). Outcomes were, then, assembled in a Sequence Alignment Mapping (SAM) file format. PicardTools was employed for Post-Alignment steps, along with SamTools and Genome Analysis Toolkit version 4 (GATKv4) packages in order to remove duplications. Afterward, the variant calling procedure, comprising both SNV and INDEL variants, was conducted via HaplotypeCaller for production of variant call format (VCF) files of the patient. The quality of attained VCF files was evaluated by commands of variant quality score recalibration

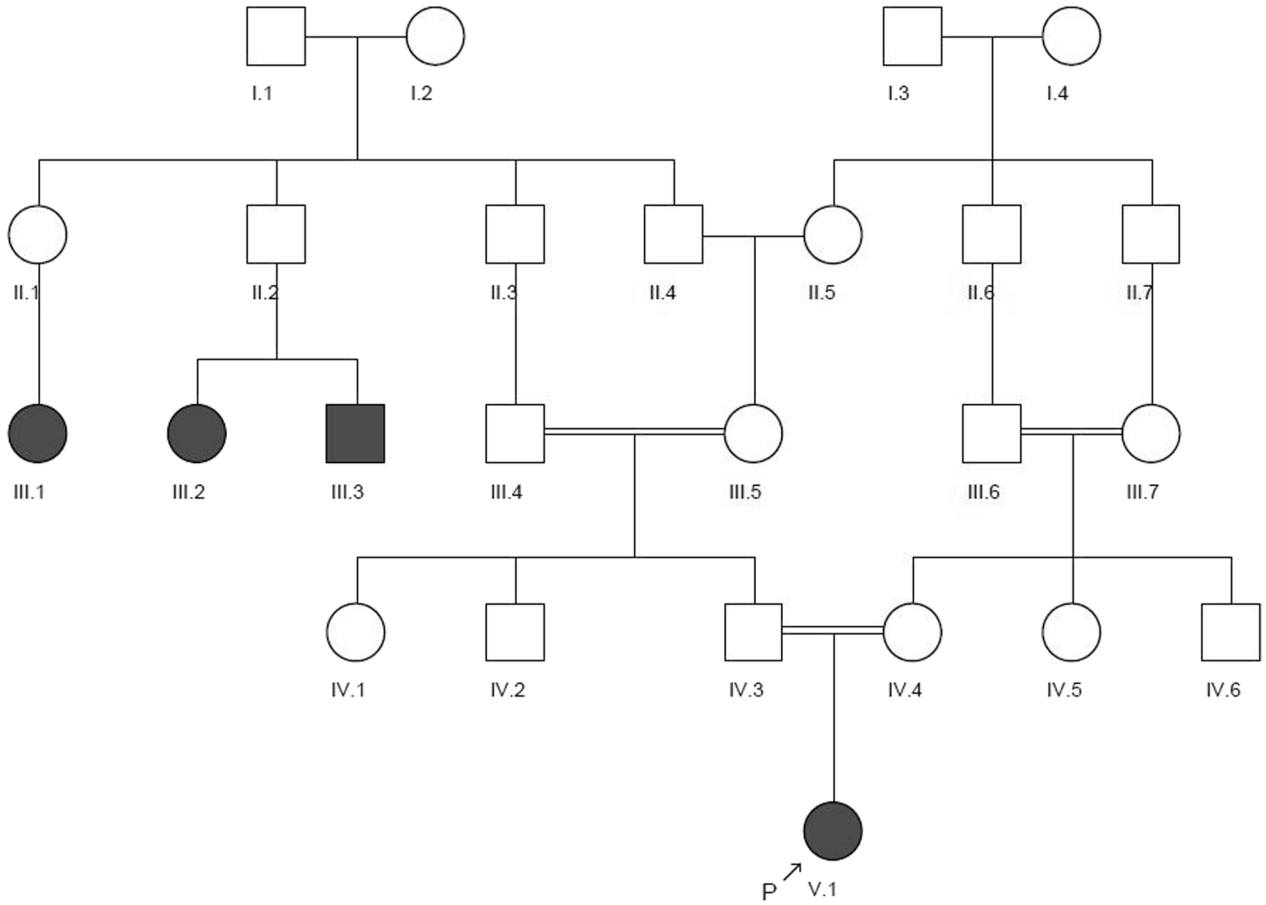

Fig. 1 Pedigree of the family described in this study. The studied patient (proband, V.1) is shown with the arrow. In addition, the III.1, III.2, and III.3 individuals had microcephaly phenotypes

(VQSR) tool. In the next stage, the annotation of variants was performed through the ANNOVAR software utilizing a variety of databases for further filtering of variants, such as 1000 Genome Project (internationalgenome.org), dbSNP (ncbi.nlm.nih.gov/SNP), Genome Aggregation Database (gnomAD, gnomad-old.broadinstitute.org), Exome Aggregation Consortium (ExAC) (exac.broadinstitute.org), ClinVar, and InterVar. Additionally, several effective computational programs and online software tools were used for prediction of functional effect and damaging impact of the variant, including MutationTaster, SIFT, PolyPhen2, PANTHER, PMut, Human Splicing Finder, SNAP, and PROVEAN. PyMOL was applied for visualization of protein structures. Finally, using the Iranome database (http://www.iranome.ir), filtering of Iranian population variants was performed.

In line, the variant filtering and prioritization strategies including total read depth of more than 5X and minor allele frequency (MAF) < 5% were utilized. In-house command lines recruiting compound heterozygous and homozygous variants were applied given the pedigree and phenotype description. Then, by filtering out the synonymous, intronic, and intergenic variants, in addition to selecting the variants in phenotype-related genes, the remaining variants were investigated regarding their pathogenicity. As a supplementary evaluation, the sum of *in silico* predictions was taken into account in order to consider a pathogenic interpretation for variants. Importantly, the comprehensive classification of variant pathogenicity was adjusted based on American College of Medical Genetics (ACMG) guidelines [15].

Variant confirmation and segregation

Sanger sequencing was used to validate and segregate the detected final variants in the patient and family members, respectively. PCR primers were considered to amplify sequence of the *PYCR2* gene. Primers were designed by Primer3 software and checked via Primer-BLAST NCBI (Table 1).

Table 1 The primer sequences used for amplification of region containing the *PYCR2* variant

Gene name	Primer sequences	Type	LEN	TM	GC%	Product size
PYCR2	TGCTTTCATCACATAATCACCTCA	(Forward)	24	58	37	505
	TCCGGTGGGGTAGATGAGAC	(Reverse)	20	60	60	

Results

The cytogenetic analysis was performed on peripheral blood lymphocytes and a normal female 46,XX karyotype was found. Gas chromatographic-mass spectrometric screening for Organoacidophathies in urine was performed and no metabolites specific for organoacidopathy were detected. The magnetic resonance imaging (MRI) in the patient suggested hypomyelination, central brain atrophy, severe non-communicating hydrocephalus, and mega cisterna magna, which are aligned with HLD10 phenotypes. Also, WES in affected individual was performed and a total of 56,179,476 reads were generated. Specifically, 98% of these FASTQ file formats had Phred quality scores of > 20 for nucleotides, and 95% of reads had Phred quality scores > 30 (detailed parameters are shown in Table 2). As a whole, 98% of reads passed the filtering steps in the described pipeline. Then, after variant calling step, a VCF file with 178,343 variants was achieved (Table 3).

Table 2 The statistical parameters of FASTQ files of the patient before and after performing filtering steps in whole-exome sequencing (WES) pipeline

	Before FASTQ filtering	After FASTQ filtering
Total reads	56,179,476	55,159,718
Total bases	8,483,100,876	7,830,438,155
Q20 bases	8,285,622,224	7,743,137,555
Q30 bases	7,961,919,235	7,492,159,554
Q20 rate	0.976721	0.988851
Q30 rate	0.938562	0.9568
Read1 mean length	151	142
Read2 mean length	151	141
GC content	0.520673	0.52038

Table 3 The quality control parameters of variant calling format (VCF) file of the patient

Unique variant alleles	178,343
SNPs (mismatches)	154,981
InDels	23,362
TS/TV ratio	2.19351
Deamination ratio	1.05873
Insertion alleles/deletion alleles	0.87722
Inserted bases/deleted bases	0.768349

After implementation of filtering strategies and investigation of phenotype-related gene panels, the novel homozygous frameshift variant of c.135dup (NM_013328.4) in *PYCR2* gene was found. The c.135dup (p.R46Q*36) in exon 2 is identified as a pathogenic variant in ACMG classification. Based on this guideline, the discovered variant had a very strong evidence known as PVS1, meaning null variant that can result in a loss-of-function mechanism in *PYCR2* gene, involved in Leukodystrophy, hypomyelinating 10. Other introduced criteria for this variant were PM2 and PP3, which relatively refer to significantly low frequency in population databases of Exome Sequencing Project, 1000 Genomes, or ExAC, and multitude computational evidence for prediction of damaging effect of variant on the gene or its product, respectively. Accordingly, in terms of frequency, this variant was not observed in gnomAD (Exome, Genome, and Aggregated) and other relevant databases, such as TOPMed, GME Variome, Iranome, ExAC, 1000 Genomes, ESP6500, 4.7KJPN, and GenomeAsia. One *in silico* database, namely phyloP, predicted this variant as disease-causing, and no benign predictions were found for this variant in any *in silico* tools.

Moreover, no report related to this mutation was found in ClinVar. Subsequently, the results of Sanger sequencing in the patient confirmed the homozygous c.135dup variant in *PYCR2* gene, and both of her parents were heterozygous for this genetic alteration (Fig. 2).

Discussion

In the current study, we conducted WES on an Iranian 9-year-old female patient with hypomyelinating leukodystrophy type 10 (OMIM 616420), and characterized a novel homozygous variant of c.135dup (p.R46Q*36) in *PYCR2* gene, which was confirmed in the patient and segregated in her parents who were heterozygous for this mutation. ACMG criteria classified this mutation as a disease-causing variant, and the *in silico* tools used in the present research predicted the devastating effects of this variant. As exhibited in Fig. 3, when this mutation happens (shown as yellow spot in the schematic description), the red regions will be lost and a truncated protein (containing only green regions) without functional domains will be produced in the patient. No allele frequency was reported in population databases and, likewise, ClinVar

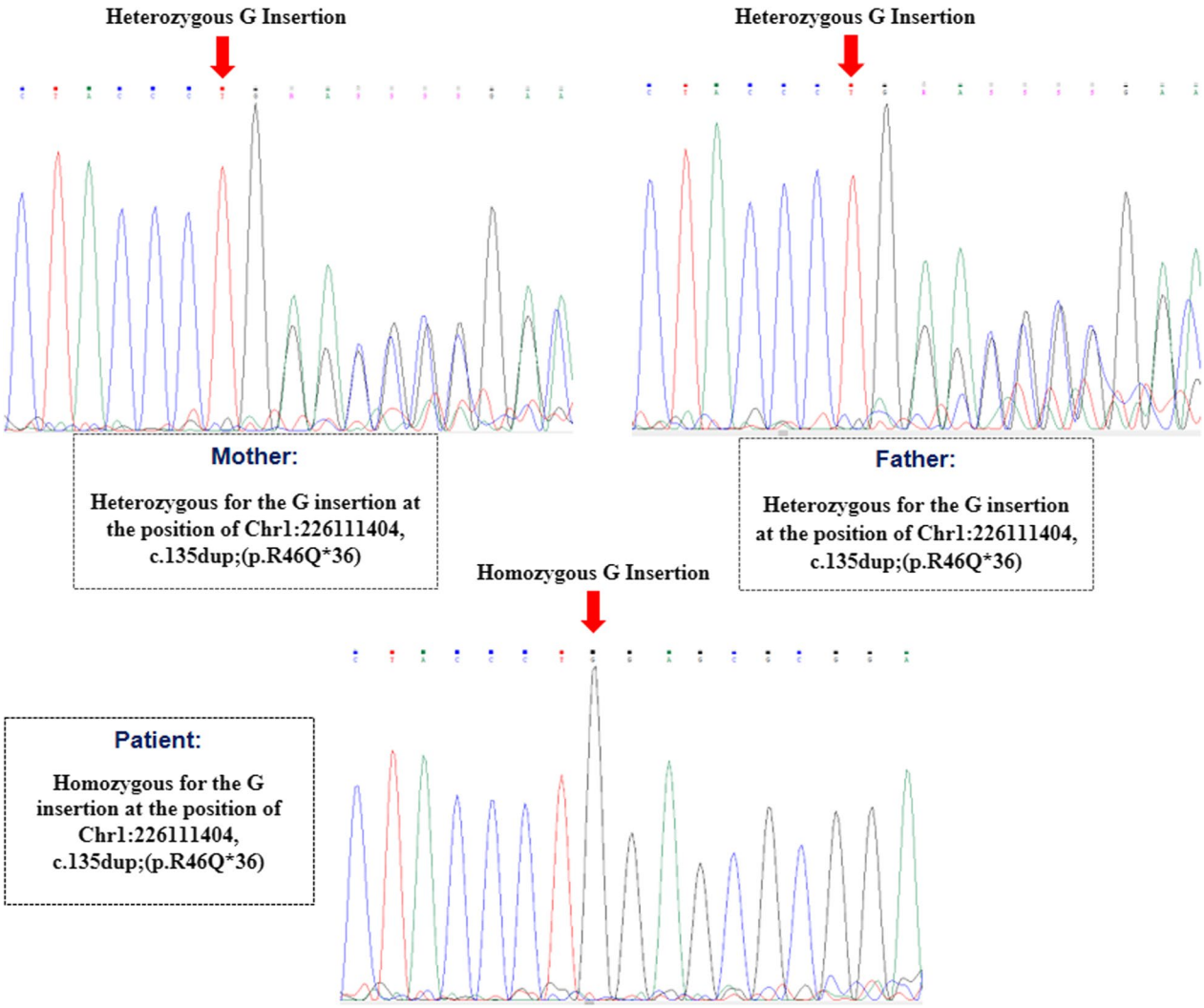

Fig. 2 Sanger sequencing results of the identified *PYCR2* mutation from the whole-exome sequencing analysis in the Iranian patient with hypomyelinating leukodystrophy 10 (HLD10). The homozygous *PYCR2* mutation in the patient (c.135dup) and the heterozygous status of this mutation in the parents were demonstrated, respectively

did not provide any evidence associated with this mutation. Furthermore, its position, 226111403 on chromosome 1, is highly conserved in human according to UCSC genome browser (Fig. 4).

An extensive review of literature from various databases did not reveal any more cases of *PYCR2*-associated HLD 10 beyond other studies in Iran, and to the best of our knowledge, this is the first investigation in *PYCR2* gene and HLD10 in Iranian population. Loss-of-function mutations in *PYCR2* gene result in HLD10, which is identified by hypomyelination, global growth delay, microcephaly, facial dysmorphism, axial hypotonia, and movement disability. It has also been described that these patients could not survive for a long time [9, 11, 16]. HLDs are a class of rare hereditary neuropathies that their related genes can be determined through latest nucleotide sequencing approaches such as NGS technologies; however, no specific therapeutic method has been developed yet [17]. MRI pattern recognition in the recent years has also been beneficial for diagnosis of different hypomyelinating diseases and diminished white-matter volume. Nevertheless, this procedure is combined with crucial limitations in diagnosis. WES has successfully displayed differential diagnostic efficiency, particularly in rare disease-causing variants, and has a significant potential in identification of de novo mutations [18–20].

PYCR2 gene, as a member of Pyrroline-5-carboxylate reductases, is involved in the biosynthesis of proline from glutamate. There have been numerous reports associated with pathogenic effects of *PYCR2* mutation [21]. *PYCR1* and *PYCR2* are 84% similar regarding their protein structure, and biochemically they share notable

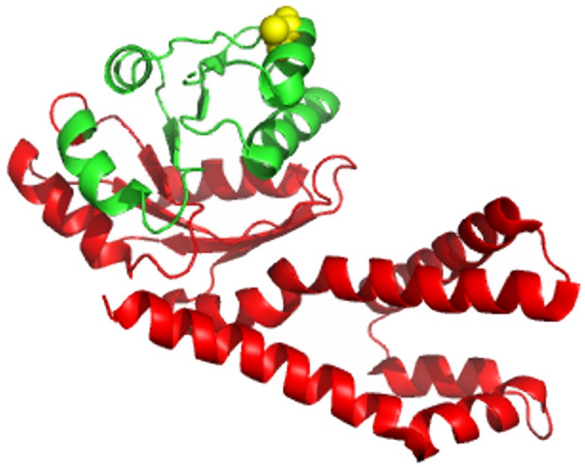

Fig. 3 Schematic description of the location of the novel pathogenic c.135dup variant in the hypomyelinating leukodystrophy type 10 (HLD10) patient

similarities. However, their genetic roles in human disorders appear to be largely different [3–9]. *PYCR1* mutations cause Cutis Laxa. In contrast, *PYCR2* mutations are associated with hypomyelinating leukodystrophy type 10 (HLD10) and intellectual deficits. Importantly, these phenotypes are more severe in *PYCR2*/HLD10 patients. This may be, in part, given the fact that higher levels of PYCR2 protein exist in the brain than PYCR1. The PYCR2 protein consists of two common domains, known as the dimerization domains, and the NAD(P) binding part, encompassing 268 residues that is equal to 84% of the PYCR2 protein [3]. The NAD(P) binding domain is important for the function of the pyrroline-5-carboxylate reductase (PYCR) enzyme. The pathogenicity of the Arg-119Cys variant in the NAD(P)-binding domain has been recently validated and it was represented that the Arg-119Cys strongly impairs the catalytic efficiency of PYCR2 protein up to 366 times lower than that of the non-mutant enzyme, confirming its pathologic role in HLD10 [22]. This evidence proposes that the molecular alterations in the NAD(P)-binding domain could take part with the *PYCR2* function, resulting in the HLD10 phenotypes.

The p.R46Q*36 variant reported in our study is located in the P5CR dimerization domain and also is considerably close to another homozygous missense variant, p.Val86Gly, that previously was reported in a *PYCR2*-deficient patient (Fig. 5). In vitro functional studies of such variations in PYCR2 protein has exhibited that they disrupt protein dimerization, putatively leading to a loss-of-function [11]. By applying whole-exome or targeted sequencing, Zaki et al. found homozygous mutations of *PYCR2* in 14 patients from 11 consanguineous families and showed that *PYCR2*-related syndrome can cause protein dysfunction with distinguishable clinical phenotypes in patients. In terms of genotype–phenotype correlation in *PYCR2*, reduced levels of protein were seen due to truncating mutations. Additionally, missense mutations were mentioned to have no effects on the levels of protein, however they impaired the protein multimerization [11]. Depletion in *PYCR2* has been shown to cause neurodegeneration

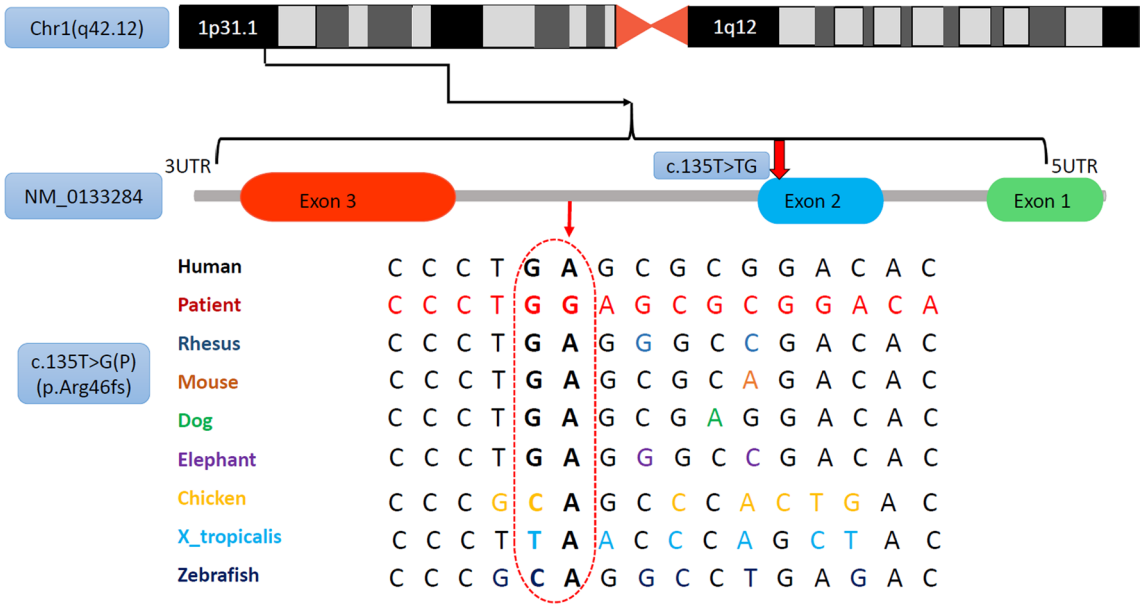

Fig. 4 Schematic description of the location of our novel variant in *PYCR2* gene and multispecies alignment for the candidate variant throughout different genomes

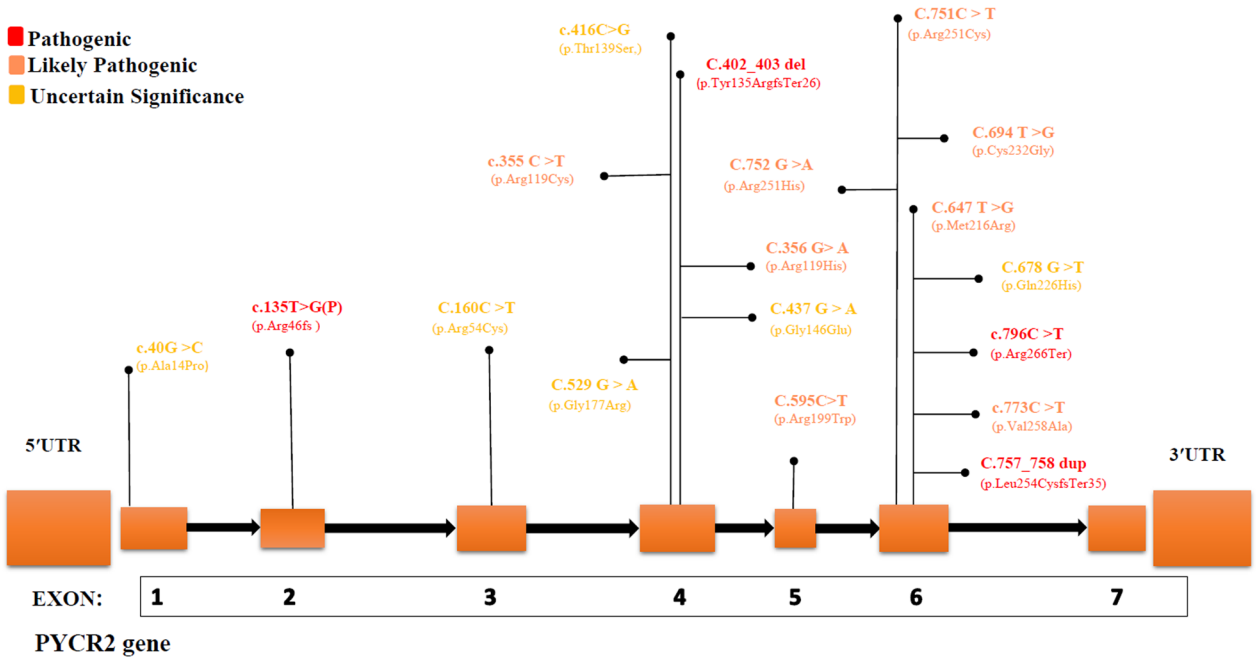

Fig. 5 Schematic representation of the identified variants in *PYCR2* gene

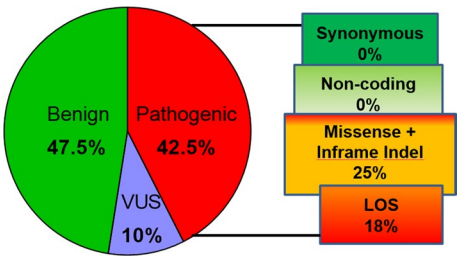

Fig. 6 Distribution of various types of mutations in *PYCR2* gene

via raising the level of cerebral glycine through SHMT2 upregulation [10]. Afroze et al. confirmed the disease-causing impacts of *PYCR2* deficiency by detecting a known homozygous mutation in their patients [7]. As well, Srivastava et al. discovered a compound heterozygous variant in *PYCR2* gene via clinical WES in a patient suffering from failure to thrive, global developmental, and dysmorphic facies [23]. Manaspon et al. found the novel *PYCR2* mutations, in both compound heterozygous and homozygous forms, in the first Thai families with developmental delay and microcephaly. They, also, suggested that most of the HDL10 patients have homozygous variants and belong to a consanguineous pedigree, which is in-line with Zaki et al. results where all cases were from a consanguineous family. Moreover, they noted that loss of *PYCR2* in an *in vitro* design resulted in incremented susceptibility to apoptosis under oxidative stress [16].

On the other hand, in a study by Meng et al., non-consanguineous family history was reported in three out of five patients with homozygous variants in *PYCR2*. These observations are consistent with our data and underscore the deleterious impacts of *PYCR2* mutations (Fig. 6), as well as the potential application of WES in characterization of novel rare and unknown variants. Noteworthy, more in-depth investigations could be utilized to heighten the knowledge about this specific area of science. The limitation of this study was the unavailability of MRI images for the patient. Finally, as a forward step, we suggest the transgenic animal models and other supplementary examinations for our novel mutation in *PYCR2* gene to further strongly confirm these results in multiple biological settings.

Conclusion

In this study, we examined a 9-year-old girl with clinical presentation related to HLD10. WES was performed and assisted us to detect a novel and rare homozygous frameshift variant in the *PYCR2* gene, known as c.135dup. The homozygous status of the variant in the patient and its heterozygous status in her parents were confirmed through Sanger sequencing. The pathogenicity of the discovered variant was interpreted via ACMG guidelines and led us to classifying this variant as a pathogenic mutation. These data could be of paramount significance for genetic counselling of patients with HLD and for understanding the underlying mechanisms in this disorder.

Abbreviations

PYCR2	Pyrroline-5-carboxylate reductase 2
HLD10	Hypomyelinating leukodystrophy type 10
WES	Whole-exome sequencing
CNS	Central nervous system
NGS	Next-generation sequencing
QC	Quality control
SAM	Sequence alignment mapping
GATKv4	Genome analysis toolkit version 4
VCF	Variant call format
VQSR	Variant quality score recalibration
GnomAD	Genome aggregation database
ExAC	Exome aggregation consortium
MAF	Minor allele frequency
ACMG	American College of Medical Genetics
MRI	Magnetic resonance imaging

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None.

Author contributions
MA, ZET, and MZ contributed to selecting idea of study and gathering the samples and data from the participants. They performed the whole-exome sequencing analysis, and the HR and MG were in charge of step-by-step supervision of the research. HR conducted the protein evaluations, and the Sanger results were assessed by MG. The manuscript was written by MA, ZET, and MZ. MG and HR helped in methodology writing, editing of manuscript, and final revision. All authors have read and approved the manuscript.

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Availability of data and materials
The data that support the findings of this study are available on request from the corresponding author.

Declarations

Ethical approval and consent to participate
This study was performed in-line with the principles of the 1964 Helsinki Declaration, and the approval was granted by the Ethics Committee of Pasteur Institute of Iran. Written informed consents were obtained from the parents of the affected child (under 16 years old).

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

Competing interests
The authors declare that they have no competing interest.

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