A novel homozygous variant in an Iranian pedigree with cerebellar ataxia, mental retardation, and dysequilibrium syndrome type 4

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Abstract

Background: Cerebellar ataxia, mental retardation, and dysequilibrium (CAMRQ) syndrome is a rare and early-onset neurodevelopmental disorder. Four subtypes of this syndrome have been identified, which are clinically and genetically different. To date, altogether 32 patients have been described with ATP8A2 mutations and phenotypic features assigned to CAMRQ type 4. Herein, three additional patients in an Iranian consanguineous family with non-progressive cerebellar ataxia, severe hypotonia, intellectual disability, dysarthria, and cerebellar atrophy have been identified.

Methods: Following the thorough clinical examination, consecutive detections including chromosome karyotyping, chromosomal microarray analysis, and whole exome sequencing (WES) were performed on the proband. The sequence variants derived from WES interpreted by a standard bioinformatics pipeline. Pathogenicity assessment of candidate variant was done by in silico analysis. The familial cosegregation of the WES finding was carried out by PCR-based Sanger sequencing.

Results: A novel homozygous missense variant (c.1339G > A, p.Gly447Arg) in the ATP8A2 gene was identified and completely segregated with the phenotype in the family. In silico analysis and structural modeling revealed that the p.G477R substitution is deleterious and induced undesired effects on the protein stability and residue distribution in the ligand-binding pocket. The novel sequence variant occurred within an extremely conserved subregion of the ATP-binding domain.

Conclusion: Our findings expand the spectrum of ATP8A2 mutations and confirm the reported genotype-phenotype correlation. These results could improve genetic counseling and prenatal diagnosis in families with clinical presentations related to CAMRQ4 syndrome.

Keywords

ATP8A2, Cerebellar ataxia, dysequilibrium syndrome 4, mental retardation, rare disease, whole exome sequencing
1 | INTRODUCTION

Cerebellar ataxia, mental retardation, and dysequilibrium (CAMRQ) syndrome is a heterogeneous genetic condition characterized by non-progressive cerebellar ataxia, various severities of intellectual disability, and cerebellar atrophy. The other possible clinical features are severe hypotonia, retarded motor development, dysarthria, seizures, optic atrophy, and short stature. Besides, quadrupedal locomotion has been seen in a few patients with this syndrome. Indeed, CAMRQ is an extremely rare condition, and about 50 affected individuals have been described in the world literature. The pattern of inheritance in all of the possible phenotypes is autosomal recessive. Four subtypes of CAMRQ syndrome have been identified (CAMRQ1-CAMRQ4), which differ in both the clinical and genotypical spectrum. Mutations in four genes have been identified as the causes of these heterogeneous conditions: very low-density lipoprotein receptor (VLDLR) gene (CAMRQ1), WD repeat domain 81 (WDR81) gene (CAMRQ2), carbonic anhydrase 8 (CA8) gene (CAMRQ4), and ATPase phospholipid transporting BA2 (ATP8A2) gene (CAMRQ4). Cerebellar ataxia, mental retardation, and dysequilibrium syndrome type 4 (CAMRQ4, OMIM 615 268) is suspected in patients with ataxia, intellectual disability, hypotonia, dysarthria, and global developmental delay, with or without brain abnormalities. The ATP8A2 (OMIM 605 870) gene located at 13q12.1 that spans 653 878 bp and contains 37 exons. ATP8A2 encodes a member of the P4 ATPase subfamily that effectively facilitates the phospholipid flipping across cellular membranes. While 14 P4 ATPases genes have been determined in the human genome, only some of them, including ATP8B1, ATP10A, ATP11A, and ATP8A2, have been reported in human diseases. Although the ATP8A2 gene is not expressed ubiquitously, it is strongly expressed in the brain, retina, and testis. The maximum tissue concentration of ATP8A2 is in the cerebellum. There is evidence about the significant role of ATP8A2 in the evolution of the central nervous system. In the first study, a de novo balanced translocation t(10;13) interrupting the ATP8A2 coding sequence was detected in a patient with major axial hypotonia and moderate intellectual disability. The previous literature has described 32 patients from different ethnicities with ATP8A2 mutations and phenotypic features assigned to CAMRQ4. Almost all of these mutations have been located in the cytoplasmic region that includes phosphorylation (P), nucleotide-binding (N), and actuator (A) domains.

Whole exome sequencing (WES) is thought to be a fast and cost-effective technique to identify probable disease-causing variations. The application of WES in a Turkish family with four members affected by intellectual disability, mild cerebral and cerebellar atrophy, dysarthria, and truncal ataxia revealed a missense variant p.I376M in the ATP8A2 gene.

In the current study, we reported a novel homozygous missense variant of ATP8A2 in an Iranian consanguineous family, of which three siblings showed non-progressive cerebellar ataxia, hypotonia, intellectual disability, dysarthria, and cerebellar atrophy.

2 | MATERIALS AND METHODS

2.1 | Subjects

We enrolled a consanguineous Iranian family with 3 affected and 5 unaffected members in our study. The patients involved in this study were clinically examined and followed up by a pediatric neurologist. (Figure 1A). This study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki. Informed consent was taken from all participants before the entrance to the study. Ethical approval code was acquired from Birjand University of Medical Sciences (Irbums.REC.1397.253).

2.2 | Clinical findings

The proband (IV.4) was a 9-year-old girl from healthy and first-cousin parents with Arab descent (Figure 1A). Following an uncomplicated pregnancy, she was born with normal weight, height, and head circumference. The first noticeable clinical sign was severe neonatal hypotonia. Afterward, she demonstrated a lag in all psychomotor and developmental milestones. In the aspect of motor performance assessment, she was wheelchair-bound and could not walk, sit, or hold her head. She has been suffering from some feeding difficulties since infancy. Index case has five siblings: two healthy sisters (IV. 5, IV. 6), one healthy brother (IV. 3), one affected sister (IV. 2), and one affected brother (IV. 1). Patients IV. 1 and IV. 2 were 17 and 15 years old with phenotypical characteristics similar to those present in the proband. (Table 1). All 3 patients had limited intellectual functioning with IQ levels ranging from 20 to 40. Also, they had delayed speech and language as well as signs of dysarthria. Nevertheless, the hearing examination of patients was not shown any significant impairment. In these patients, deep tendon reflexes were absent. There was no record of similar diseases in the family. Also, metabolic investigations consist of plasma thyroid hormones, uric acid, lactic acid, plasma and urine amino acids, liver function test, ammonia, and urinary organic acid profiles were in standard limits. The brain magnetic resonance imaging (MRI) significantly indicated cerebellar atrophy in all the patients.

2.3 | Chromosome karyotyping

Evaluation of possible chromosomal anomalies in the proband was performed by the G-banding chromosome karyotyping according to the routine laboratory procedure.

2.4 | DNA extraction

Peripheral blood samples were collected from all family members, and genomic DNA (gDNA) was isolated using a DNA extraction kit (Sinaclon, Iran) according to the manufacturer’s procedure. The
integrity of isolated gDNA was verified on agarose gel electrophoresis. Quantity and purity of gDNA samples were considered by a nanodrop spectrophotometer (Nanodrop Technologies).

2.5 | Chromosomal microarray analysis

CMA was performed on the proband’s DNA by Affymetrix CytoScan HD array platform following the manufacturer’s protocol. Raw data were interpreted using the Chromosome Analysis Suite (ChAS) software.

2.6 | Whole exome sequencing

The gDNA sample of the proband (IV.0.4) was exposed to WES analysis (Macrogen Company, South Korea). Following the library preparation and adaptor ligation, exome capture was performed by Agilent SureSelect Human All Exome Kit V6 (Agilent Technologies, Santa Clara, CA, USA). Next, the Illumina HiSeq 4000 instrument was used to paired-end sequencing based on the manufacturer’s instructions. For secondary data analysis, FASTQ files imported to CLC Genomics Workbench 12.0 (https://www.qiagenbioinformatics.com). Briefly, raw reads were trimmed for length, adaptor sequence, and quality. High-quality reads were subsequently aligned to the human reference genome (hg19/GRCh37). After removing the duplicate reads, unique mapped reads were introduced to the basic variant caller in CLC Workbench software. For functional annotation and frequency analysis of variants, wANNOVAR online software (http://wannovar.wgbli.org) was used. In primary filtering, non-functional variants consist of all intronic variations were removed. Then, the only variants located in the exons and splicing sites were filtered against the accessible databases (dbSNP, 1000 Genomes Project, Exome Sequencing Project (ESP), gnomAD, and ExAC). Alterations with a minor allele frequency (MAF) lower than 0.01 were predicted to affect protein function. Classified variants, as benign and likely benign in the ClinVar (https://www.ncbi.nlm.nih.gov/clinvar), were also excluded. After that, variants were prioritized by functional, phenotypical, and expression data which previously reported in OMIM, HPO, Orphanet, Genecards, and PubMed databases. Then, homozygous and compound heterozygous variants, compatible with autosomal recessive inheritance mode, were considered.

2.7 | Pathogenicity prediction of candidate variant

Pathogenicity of candidate variants was estimated by in silico predictor programs: PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and PROVEAN (http://provean.jcvi.org/index.php) tools were used to predict the potential impact of amino acid substitution on protein
|                  | Current study | Cacciagli et al, 2010 | Onat et al, 2013 | Martin-Hernandez et al, 2016 | Quintas et al, 2017 | Alshali et al, 2018 | McMillan et al, 2018 | Guissart et al, 2020 |
|------------------|---------------|-----------------------|------------------|-----------------------------|---------------------|--------------------|--------------------|---------------------|
| **Age of onset** | Birth         | 1 mo                  | ND               | Birth ~1 mo                 | 6 mo                | 1-4 mo             | Birth ~6 mo        | Birth ~4 y          |
| **Developmental delay** | Yes          | Yes                   | Yes              | Yes                         | Yes                 | Yes                | Yes                | Yes                 |
| **Intellectual disability** | Yes; IQ:20-40 | Yes                   | Yes              | Yes                         | Yes                 | Yes                | Yes                | Yes                 |
| **Seizures**     | No            | ND                    | ND               | ND                          | ND                  | NA                 | 2/11               | 3/6                 |
| **Lower leg reflexes** | Absent       | ND                    | ND               | ND                          | ND                  | 2/5: Absent, 1/5: Reduced | ND                 | 2/6: Reduced        |
| **Upper extremities reflexes** | Absent      | ND                    | ND               | ND                          | ND                  | 2/5: Absent, 1/5: Reduced | ND                 | 4/6: Brisk          |
| **Speech disabilities** | Dysarthria | Few words at 3 y      | Dysarthria      | Monosyllabic and disyllabic words, or with the aid of pictograms | Dysarthria, words or short sentences | Dysarthria or no speech | 9/11: Non-verbal, 2/11: mono or di-syllabic words, or with the aid of pictograms | 4/6: Dysarthria | 1/6: Anarthria | 1/6: None verbal |
| **Encephalopathy** | Yes           | Yes                   | Yes              | Yes                         | Yes                 | Yes                | Yes                | 3/6                 |
| **Feeding difficulties** | Yes          | ND                    | ND               | ND                          | ND                  | 10/11              | 3/6                |                     |
| **Optic atrophy** | No            | No                    | ND               | Yes                         | Yes                 | 3/5                | 7/9                | 2/6                 |
| **Hearing impairment** | No           | No                    | ND               | 1/2                         | ND                  | 1/5                | 2/11               | 2/5                 |
| **Anomalies on brain MRI** | Cerebellar atrophy | Normal               | Mild atrophy of cerebral cortex, corpus callosum and inferior cerebellum | Delayed myelination, thin corpus, callosum cerebral atrophy | Normal | 1/3: Ventriculomegaly, Mild atrophy of the corpus callosum, Cerebral cortex, and inferior cerebellum. | 5/10: Mild cerebral Atrophy, Delayed myelination, Subcortical white matter volume loss, Thin corpus callosum | 2/6: Mild cerebellar Atrophy; 1/6: No cerebellar atrophy (white matter hypersignal); 1/6: Heterotopias; 1/6: Reduced number of cerebralgyri, callosal hypoplasia; 1/6: Bilateral frontal atrophy |

(Continues)
function based on the evolutionary considerations and physical characteristics of amino acids. Besides, Meta-SNP (http://snps.biofold.org/meta-snp/index.html) analysis, a highly reliable predictor integrating the outputs of PhD-SNP, SIFT, PANTHER, and SNAP was applied. MutationTaster (http://www.mutationtaster.org) web-based application was employed to estimate the impact of the candidate variants on the regulatory and splice sites, as well as gene product features. Finally, the IRANOME database (http://www.irano.me.ir), a source of WES data belonging to 800 healthy Iranian individuals from different ethnicities, was used to ensure that the candidate variant is not present in an ethnically matched normal population.

2.8 | Polymerase chain reaction and segregation analysis

The nucleotide sequence of the ATP8A2 gene (NG_042855.1) was taken from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov). Specific primers flanking the expected variation were designed using primer3 (http://primer3.ut.ee/). PCR was performed in a total volume of 25 μL, and agarose gel electrophoresis was run to verify the presence of an amplified sequence with the correct size. The accuracy of WES analysis was confirmed by conventional automated Sanger sequencing on ABI-3130 Genetic Analyzer. In the same way, the presence of the candidate variant in other family members was also assessed.

2.9 | Homology modeling and structural analysis

As no PDB model of the human ATP8A2 has been reported so far, the amino acid sequence was downloaded from the UniProt database with accession number Q9NTI2. Three-dimensional structures were predicted for the wild type and mutant protein (based on the WES results) through a homology modeling-based online tool, Phyre2 (Protein Homology/analogy Recognition Engine V 2.0). The refinement of top-ranking predicted structures was performed by energy minimization of structures and elimination of residues located in disallowed regions through ModRefiner (https://zhanglab.ccmb.med.umich.edu/ModRefiner/) algorithm. For quality evaluation of modeled protein structures through Ramachandran plot analysis, the refined models (wild + Mutated) were submitted to RAMPAGE (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) server. Next, structural analysis was used to determine the pathogenicity mechanism. We have calculated the stability of the mutated protein compared to the normal type by measuring ΔΔG through the structure-based mode of I-Mutant 3.0 webserver (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi). Moreover, SNPeffect 4.0 (http://snpeffect.switchlab.org/) database was applied to variant phenotyping analysis by studying the effect of variant on aggregation propensity (TANGO), amyloid propensity (WALTZ), chaperone binding (LIMBO), and structural stability of the protein (FoldX). To investigate the variant’s effect on potential ligand-binding sites,
the verified structures (wild type and mutant) were introduced to 3DLigandSite (http://www.sbg.bio.ic.ac.uk/3dligandsite).

3 | RESULTS

3.1 | Genetic analysis

Chromosomal analysis demonstrated a 46,XY karyotype without any chromosomal abnormalities. Also, the CMA testing detected no pathogenic copy number variant (CNV) in the proband. A primary variant calling on WES data of the proband unveiled 80,863 variants, of which 30,027 variants located in coding regions. As the disease phenotype revealed autosomal recessive segregation; candidate variants in either homozygous or compound heterozygous form were considered. After the stepwise variant-filtering strategy mentioned above, 81 homozygous variants and 2 compound heterozygous variants left (Supporting Information Table S1). By removing all tolerated or benign entries through the in silico prediction programs, only six variants left (Supporting Information Table S2). Among them, only one homozygous missense variant "NM_016529.6:c.1339G > A, NP_057613.4:(p.Gly447Arg)" in exon 14 of the ATP8A2 gene on 13q12.1 was predicted as pathogenic (Table 2) and well-matched with the phenotype and inheritance mode. Based on the MutationTaster results for this variation, the phastCons conservation score equal to 1 reflected a high probability of evolutionary conservation among 46 different species. Moreover, phyloP presented a score of 5.623 (where the score of 6 is the highest probability of conservation). The multiple protein sequence alignment is shown in Figure 1B. This variant has not been reported in any public variant database. The Human Gene Mutation Database (http://www.biobase-international.com/product/hgmd) was checked to support the novelty of the variant. The existence of the p.G447R variant was then confirmed through PCR-Sanger sequencing. According to segregation analysis, the sequence variant was homozygous in the affected individuals (IV.0.1, IV.0.2, IV.0.4) (Figure 1C) and heterozygous in the parents (III.1, III.2), one healthy brother (IV.3), and one healthy sister (IV.5) (Figure 1D). The only healthy sibling IV.0.6 was wild type (Figure 1E). The identified novel variant was not found in 100 healthy and ethnically matched controls.

According to the American College of Medical Genetics (ACMG) guideline, the sequence variant c.1339G > A in ATP8A2 classified as "likely pathogenic" due to belonging to PM2 (pathogenic moderate: absent from controls in Exome Sequencing Project, 1000 Genomes or ExAC), PP1 (pathogenic, supporting: cosegregation with the disease in multiple affected family members in a gene definitively known to cause the disease), PP2 (pathogenic, supporting: missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease), PP3 (pathogenic, supporting: multiple lines of computational evidence support a deleterious effect on the gene or gene product), and PP4 (pathogenic, supporting: patient’s phenotype or family history is highly specific for a disease with a single genetic etiology).

3.2 | Structural analysis of the protein upon the mutation

In 3D structures made by the Phyre2 server, 91% of the sequence have been modeled with 100.0% confidence. After high-resolution refinement, the reliability of refined structures was evaluated using Ramachandran plots. Depending on the percentage of residues located in favored (95.5%), allowed (3.5%), and outlier (1%) conformations, the predicted models had high accuracy. The predicted structures were visualized by PyMol software (Figure 2A, and B). The structural stability of human ATP8A2 protein is largely decreased upon the p.G477R variant. I-Mutant Suite 3.0 computed free energy change (DDG) value equal to −1.04 Kcal/mol with a reliability index (RI) of 8 for this variation. According to the SNPeffect 4.0 output, dTANGO equals −8.50, indicating that this mutation does not influence the aggregation tendency of our protein. For the p.G447R substitution, dWALTZ equals −51.23, which means that this substitution decreases the amyloid propensity of our protein. Besides, dLIMBO equals 0.00, which refers to that the mutation has no significant effect on the chaperone binding tendency. About the FoldX algorithm prediction, the substitution of Glycine to Arginine at position 447 results in a severely reduced protein stability with ddG of 7.94 kcal/mol. According to 3DLigandSite output, the amino acid residue 447 is adjacent to the active site, and mutation p.G447R affected the ligand-binding pocket. Due to the mutation-induced protein restructuring, three extra residues consist of Phe 569, Phe 1161, and Gln 1163 fall into the active sites (Figure 2C, and D). It may dramatically decrease the ligand-binding affinity, ligand specificity, and the catalytic activity of ATP8A2.

| Tools        | PolyPhen-2 | PROVEAN | MutationTaster | Meta-SNP                | CADD |
|-------------|------------|---------|----------------|-------------------------|------|
| Mutation    |            |         |                |                         |      |
| c.1339G > A | p.G447R    |         |                | 0.842: RI: 7; Disease-causing | 34   |
| ATP8A2      |            |         |                | SIFT 0.010 | SNAP 0.755 | PANTHER 0.895 | PhD-SNP 0.905 |

Abbreviation: RI, Reliability Index.

TABLE 2 The interpreted values derived from in silico pathogenicity assessment tools
4 | DISCUSSION

In this study, we identified a novel homozygous missense variant c.1339G > A in ATP8A2 gene related to cerebellar ataxia, mental retardation, and dysequilibrium 4 (CAMRQ4) syndrome in one Iranian family. The phenotypical features of our patients were comparable with previously reported cases with this syndrome. All available published articles that describe the association of ATP8A2 mutations and CAMRQ4 are cited in Table 1. Taking into account of our patients, a total of 35 patients have been identified with CAMRQ4 syndrome thus far. The phenotypical spectrum of ATP8A2 mutations is broad. In most reports of CAMRQ4, encephalopathy, developmental delay, intellectual disability, hypotonia, and dysarthric speech have been described. Besides, more severe phenotypes with optic atrophy, absence of ambulation, unhealthy brain MRI, non-verbal or absent speech, and feeding difficulties have also been reported.19-21

Based on multiple in silico tools, the mutation p.G447R in ATP8A2 is deleterious. In the protein structure of ATP8A2, a replacement of a hydrophobic, small, and buried residue (GLY) with a charged, bigger, and exposed residue (ARG) may alter the protein folding pathway and subsequently the protein stability. Folding free energy is a critical biophysical feature of proteins that determines the global stability of the 3D structure.22

The mutation p.G447R in ATP8A2 could make changes in folding, expression, and flippase activity of ATP8A2 by disrupting its interaction with CDC50A. Moreover, since the membrane ATP8A2:TMEM30A complex enhances neurite outgrowth,13 p.G447R mutation can be involved in the pathogenesis of neurodegenerative disorders by interrupting the neuronal differentiation.

In conclusion, WES-based approaches could be diagnostic procedures in rare diseases with heterogeneous clinical presentations, where an accurate diagnosis is difficult.26,27 Our findings expand the spectrum of known ATP8A2 mutations and may provide new approaches to preimplantation or prenatal genetic diagnosis in families with ATP8A2-related diseases. Carrier screening is crucial to...
prevent transmission risk of disease to the next generations and help the families to informed decision-making for future pregnancies. It is well known that disabling syndromes like CAMRQ4 enforced a heavy load of financial and psychological problems on the patients and their families, as well as on healthcare society. Therefore, early diagnosis of these patients is indispensable to apply the psychological and medical interventions and subsequently modify the quality of life.

ACKNOWLEDGMENTS

The authors thank the patients and their family members for their participation in this study. We wish to thank Dr Ata Ghadiri for his valuable comments during this project. This work was supported by Birjand University of Medical Sciences (No. 455747).

AUTHOR CONTRIBUTIONS

Study conception and design were conducted by Pegah Ghandil and Mohsen Naseri. Material preparation, data collection, and analysis were performed by Ali Akbar Momen, Pegah Ghandil, and Malihe Mohamadian. Drafting and revising of the study were performed by Malihe Mohamadian, Pegah Ghandil, Mohsen Naseri, and Afsane Bahrami. All authors read and approved the final study.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Mohamadian M, Ghandil P, Naseri M, Bahrami A, Momen AA. A novel homozygous variant in an Iranian pedigree with cerebellar ataxia, mental retardation, and dysequilibrium syndrome type 4. J Clin Lab Anal. 2020;34:e23484. https://doi.org/10.1002/jcla.23484