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ORIGINAL ARTICLE

X-linked mental retardation-hypotonic facies syndrome: Exome sequencing identifies novel clinical characteristics associated with c.5182G>C mutation in the *ATRX* gene

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Abstract

Background: X-linked mental retardation-hypotonic facies syndrome-1 (MRXFH1), caused by a mutation in the *ATRX* gene, is a rare syndromic form of X-linked mental retardation (XLMR) that is mainly characterized by severe intellectual disability, dysmorphic facies, and skewed X-inactivation pattern in carrier women.

Method: In this study, due to the genetic heterogeneity of the disease, we performed exome sequencing (ES) on a 15-year-old boy with primary microcephaly and intellectual disability. Also, Sanger sequencing, cosegregation analysis, and structural modeling were done to identify and verify the causative variant in the proband and other affected individuals in the family. In addition, we collected data from previously reported cases to compare with our patients' phenotypes.

Results: ES revealed a previously reported missense variant in the *ATRX* gene (c.5182G > C, p.Ala1728Pro), segregating with the new clinical characteristic including primary microcephaly in the pedigree. This variant meets the criteria of being likely pathogenic based on the ACMG variant interpretation guideline.

Conclusions: The findings of this study extend the spectrum of phenotypes associated with the identified variant and provide further details on its clinical features.

KEYWORDS

ATRX, Exome sequencing, Iranian population, primary microcephaly, X-linked mental retardation, XLMR

1 | INTRODUCTION

Intellectual disability (ID) is an important genetic disorder that impairs an individual's ability to understand and comprehend and causes the affected individuals to struggle with daily living activities (Shea, 2012). Patients have signs of high levels of genetic and phenotypic heterogeneity. In addition, some patients may show very low IQ, as

low as 20, in contrast, other patients may show IQ levels somehow close to a normal person (Shevell, 2008). Some patients may manifest structural abnormalities such as short stature, microcephaly, hypotonia, and development delay (McDermott et al., 2007). It is noteworthy that the global incidence of this disease is reported to be 2–3% of the general population, and its incidence can be higher in consanguine populations (McDermott et al., 2007).

The list of identified genes associated with ID is constantly growing, and the impairment of a wide range of genes and cellular pathways may lead to ID. Some patients manifest syndromic ID, whereas others may show only one or two symptoms. XLMR shows a global prevalence of 1/600 males, and it comprises several genetic syndromes like fragile-X syndrome, Holmes-Gang syndrome, Juberg-Marsidi syndrome, Carpenter-Waziri syndrome, Cabezas syndrome, Chudley-Lowry syndrome, Menkes syndrome, Smith-Fineman-Myers syndrome, alpha-thalassemia with mental retardation syndrome (Abidi et al., 2005; Al-Owain et al., 2011; Basehore et al., 2015), and X-linked mental retardation-hypotonic facies (MRXHF1) (Bouazzi et al., 2016; Gibbons et al., 1995). Diagnosis is achieved via precise family history, physical evaluations, and meticulous genetic evaluations.

The *ATRX* gene (OMIM #300032) is located on the Xq21.1; from base pair 77,504,878 to 77,786,216 on chromosome X and has 35 exons (NG_008838.3; https://www.ncbi.nlm.nih.gov/). *ATRX* gene encodes a protein which is a chromatin remodeler with an ATPase/helicase domain and belongs to the SWI/SNF2 superfamily of helicases/ATPases. This protein consists of several domains involved in essential cellular activities such as transcription regulation, DNA recombination and repair, and meiosis and mitosis via chromatin remodeling (Chiurazzi et al., 2004). Domains are composed of a cysteine-rich region at the N-terminal of the gene called, ATRX-DNMT3-DNMT3L (ADD) domain and an SWI/SNF2 ATPase/helicase-like domain at the C-terminal of the gene. The ADD domain contains an N-terminal GATA-like zinc finger, a PHD finger, and a C-terminal α-helix. Spontaneous mutations in the PHD-like region in humans are associated with alpha-thalassemia with intellectual disability and sometimes with seizure and gonadal dysgenesis (Gibbons et al., 1997). The ATRX protein is phosphorylated throughout meiotic maturation in a calcium-dependent way and alters chromatin structure via deacetylation of histones (De La Fuente et al., 2004). Mutations in the *ATRX* gene have been shown to cause x-linked alpha-thalassemia/mental retardation (OMIM #301040) and mental retardation with hypotonic facies (OMIM #309580) (Stevenson et al., 2000). In this study, we report a 15-year-old boy with ID and primary microcephaly with a hemizygous missense mutation in the *ATRX* gene using exome sequencing (ES).

2 | METHODS

2.1 | Subjects

A 15-year-old boy with ID and primary microcephaly from an Iranian consanguineous family (first cousin once removed) was ascertained for analysis (pedigree is shown in Figure 1). His brother and cousin were also diagnosed with primary microcephaly. Comprehensive family history was obtained during genetic counseling. Informed written consent was taken from parents as guardians in cases under legal age according to the Ethics Committees and Review Boards of Isfahan University of Medical Sciences. A blood sample was collected in EDTA-containing tubes from all family members, including parents and their affected and healthy children.

2.2 | DNA extraction and molecular study

Genomic DNA was extracted using Prime Prep GenomicDNA Extraction kit from blood (GeNet Bio, Korea) according to the manufacturer's instructions. The DNA purity and concentration were directly determined using Nanodrop 2000 Spectrophotometer (Nanodrop 2000 Thermo Scientific, USA), and its quality was checked on 1% agarose gel.

2.3 | ES and bioinformatics analysis

ES was used to enrich all exons of protein-coding genes as well as some other important genomic regions. NGS was performed to sequence close to 100 million reads on the Illumina platform (Macrogen Company, Novaseq 6000 platform) with 150-bp paired-end reads. DNA was fragmented and prepared for Illumina library, and fragments were subjected to exome capturing as well as splicing sites and flanking intronic sequences of all genes. All fragments were sequenced and amplified at the same time. The average depth of coverage was 100× for >95% of the sequences. After sequencing, raw data were transformed into the FASTQ file. The bioinformatic analysis included Genome Analysis Toolkit (GATK) (https://gatk.broadinstitute.org/) for variant calling, Burrows-Wheeler Aligner (BWA) (http://bio-bwa.sourceforge.net/) for genome alignments and variant detection (hg19, NCBI Build 38), and Picard to mark duplicate reads were used. Variant filtering was performed based on homozygous missense, start codon change, splice site, nonsense, stop loss, and indel variants with minor allele frequency <1% in databases, such as dbSNP version 147, 1000 genomes

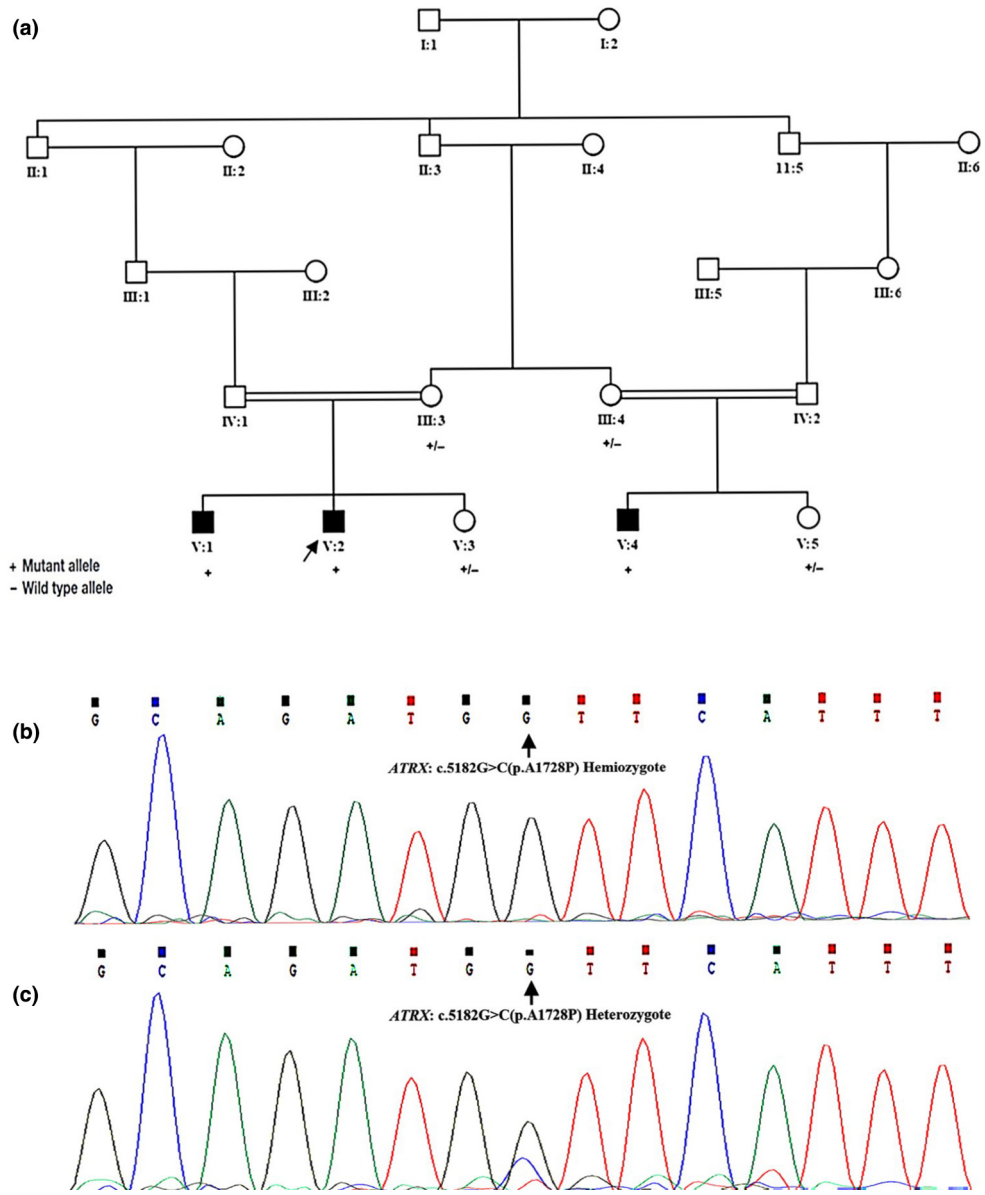

FIGURE 1 (a) Pedigree of a consanguineous family with three members affected. The electropherogram analysis shows hemizygote (b), and heterozygote (c) variants (c.5182G > C [p.Ala1728Pro]) in pedigree.

project phase 3 databases (https://www.internationalgenome.org/), NHLBI GO exome sequencing project (ESP) (https://evs.gs.washington.edu/), exome aggregation consortium (ExAC) (http://exac.broadinstitute.org), and Iranome (http://www.iranome.ir/). After the filtration, the reported variant was evaluated by different in silico software tools such as PANTHER (http://www.pantherdb.org/), MutationTaster (http://www.mutationtaster.org/), SIFT (https://sift.bii.a-star.edu.sg/), PolyPhen 2.0 (http://genetics.bwh.harvard.edu/pph2/), PhD-SNP (https://snps.biofold.org/phd-snp/phd-snp.html), and MutPred2 (http://mutpred.mutdb.org/) to predict its damaging effect on protein in terms of function. Conservation prediction of the ATRX protein sequence was analyzed with ConSurf (https://consurf.tau.ac.il/), a web server used for identifying functional regions in proteins by analyzing the evolutionary dynamics of amino acid substitutions among homologous sequences. The MEGA6 software was also used to check the conservation of the mutant variant in a number of species.

2.4 | Structural modeling of ATRX protein

The three-dimensional (3D) structure of the wild-type and mutated protein was constructed by protein structure prediction, Phyre-2 server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). These 3D structures were visualized by Jmol molecular viewer.

2.5 | Protein stability evaluation

The stability of the protein was evaluated by the MUpro (http://mupro.proteomics.ics.uci.edu/) and I. Mutant (http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi). Based on energy change value, these bioinformatics tools predict if a mutation decreases or increases protein stability.

2.6 | Variant confirmation and cosegregation analysis

The candidate variant was confirmed using Sanger sequencing. Then, cosegregation analysis was performed, using exon-specific custom primers to examine the segregation of genotype and observed phenotype among the family members. PCR amplification and sequencing of this variant were performed using the forward primer: 5′-CTCAGAGGACCCTGACTACCA-3′ and the reverse primer: 5′-TTTCACAGCAGACTAAGATGAACC-3′ in exon 20. Electropherogram was compared with the reference sequence (NM_000489.6), encoding a 2492 amino acids protein (NP_000480.3), using the SeqMan software version 5.00© (DNASTAR, Madison, WI, USA). Variant nomenclature was based on Human Genome Variation Society (HGVS), and variant interpretation was done according to the American College of Medical Genetics and Genomics (ACMG) guideline (Richards et al., 2015).

3 | RESULTS

3.1 | Clinical evaluations

The proband was a 15-year-old boy who manifested ID and primary microcephaly. He also showed signs of craniosynostosis. Facial features observed were hypertelorism, depressed nasal bridge, and small and low-set ears. His palate was normal except for the hypertrophy of the palatal ridges. Ophthalmological examination was normal. He had speech impairment, but his hearing ability was normal. His gait was impaired and he could not walk alone. He had an elder brother who also suffered from the same symptoms and had been subjected to skull surgery for microcephaly. Another affected individual was present in the family, a peer cousin, who also manifested the same symptoms as the proband and his brother. The three affected individuals were delivered at term following an uneventful pregnancy. The standard karyotype was normal for all of them. Fragile-X screening, performed on all the three affected members, pointed out the existence of two alleles with values in the normal range.

TABLE 1 In silico analysis of the identified variant in the *ATRX* gene

Gene and Transcript	cDNA/amino acid change	OMIM ID	Zygosity	CADD score	SIFT	PolyPhen2-HVAR	PhD-SNP	Mutation Taster	MutPred2	PANTHER
ATRX NM-000489.6	c.5182G>C (p.Ala1728Pro)	309580	Hem	24	Damaging	Probably Damaging	Disease causing	Disease causing	Pathogenic	Probably damaging

Abbreviation: Hem, Hemizygous.

3.2 | Molecular findings and *in silico* analysis

ES of the proband was identified as a hemizygous missense variant in the *ATRX* gene on Xq21.1 with the change of guanine to cytosine on position 5182 and alanine substitution to proline (c.5182G > C, p.Ala1728Pro). The researchers could identify the variant via Sanger sequencing. The candidate variant was shown to be cosegregating with the phenotype (Figure 1) and absent from dbSNP version 147, 1000 genomes project phase 3, NHLBI GO ESP, ExAC, Iranome, HGMD, and Clinvar databases. This mutation was assessed as being deleterious by Mutation Taster as well as several other prediction tools such as SIFT, PolyPhen 2.0, PANTHER, PhD-SNP, MutationTaster, and MutPred2 (Table 1). Moreover, different types of mutations in the *ATRX* gene were shown based on nonprofessional HGMD (Figure 2). The ConSurf server shows an average conservation score of 6 out of 9 for alanine residues, also suggesting alanine as a buried residue in the protein structure. By implementing the MEGA6, an evolutionary analysis software, alanine residue was shown to be conserved among the designated organisms (Figure 3). Based on 2015 ACMG guidelines for interpreting genetic variants, the variant was categorized as pathogenic (Stevenson et al., 2000). In summary, *ATRX*: c.5182G > C variant met the PM1, PM2, PP1, PP2, and PP3 criteria.

3.3 | Structural prediction of the ATRX protein

Secondary structure prediction was achieved based on template/homology modeling by an online ephyra-2 server. The 3D model of the ATRX protein was constructed with template structure c1z3iX. The sequence identity between the query and modeled residues was 36% which was higher than the average 25%. The comparison of van der Waals forces in the wild-type and mutant proteins indicated that it was modified in the mutant protein (Figure 4).

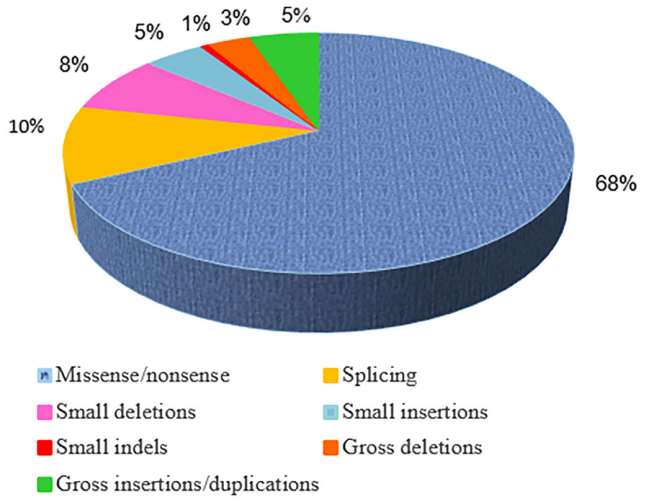

FIGURE 2 Proportion of different types of mutations in *ATRX* gene based on nonprofessional HGMD.

3.4 | Effect on the protein stability

MUpro and I- Mutant were used to predict changes in the protein stability. The result of IMutant showed that the identified amino acid substitution was recorded as decreasing the stability of the ATRX protein ($\Delta\Delta G < 0$). The result of protein stability changes by MUpro showed that the identified variant decreased the stability of the ATRX protein (Table 2).

4 | DISCUSSION

In this study, we reported a missense variant in the *ATRX* gene (c.5182G > C, p.Ala1728Pro), cosegregating with the new clinical characteristic phenotype in a consanguineous family. The proband was a male with an *ATRX* hereditary mutation manifested with primary microcephaly, developmental delay, hypotonia, facial dysmorphism, psychomotor impairment, and no signs of hematologic abnormality.

The *ATRX* gene encodes a large protein, consisting of 2492 residues (283 kDa), which belongs to the family of SWI/SNF DNA helicases. It influences the expression of many downstream genes during embryonic development, thereby taking part in the regulation of many essential cellular signaling pathways such as chromatin remodeling and transcription regulation (Wada et al., 2000). Findings prove the critical role of the ATRX protein in cerebral development and the survival of nerve cells in the developing cortex and hippocampus (Bérubé et al., 2005). Given that the *ATRX* gene is extremely conserved across species and the identified variant has been shown to be conserved among different species, the resultant mutant residue may cause incorrect regulation of the pertinent genes. The mutated transcript "NM_000489.6" is translated to protein "NP_000480.3," also known as transcriptional regulator ATRX isoform 1. This isoform is the longest isoform of the gene and consists of ATRX-DNMT3-DNMT3L (ADD) domain and SNF2_N, SNF2 family, containing a GATA-like zinc finger at the N-terminus, and an extended C-terminal that compact together to form a single globular domain containing a helicase/ATP domain in which the identified

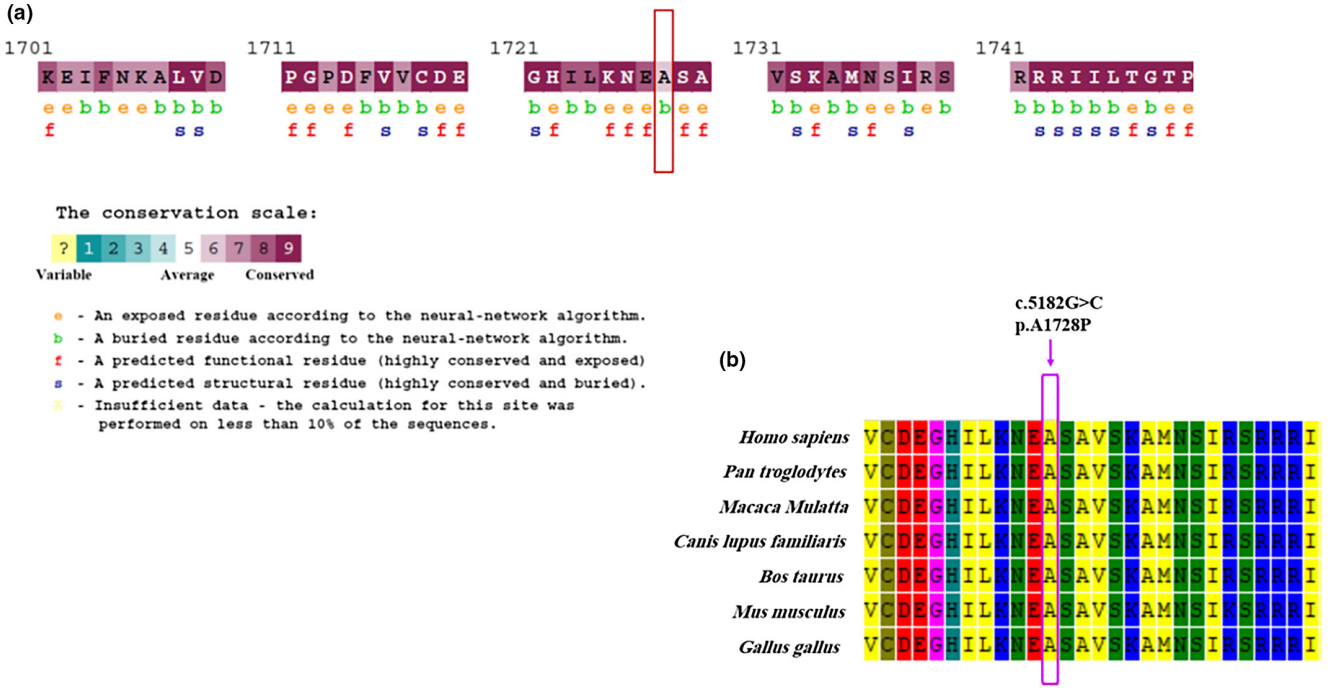

FIGURE 3 Evolutionary conservation of the mutated residue (a) ConSurf data analysis of alanine residues. (b) MEGA6 evolutionary analysis software was used to check the conservation of the mutated residue in several species.

variant is located (Barresi et al., 2010). These are extremely conserved domains, suggesting their functional importance in the aforementioned essential cellular activities and neurological development. Notably, *ATRX* mutations cluster mainly in the following domain: ADD (50%) and helicase domains (30%) (Gibbons et al., 2008). Most patients with *ATRX* mutations often present profound ID (IQ ~ 20). These patients show a broad range of phenotypes, ranging from patients who will never obtain autonomous walk to those who can walk and say a few words or a few sentences. Variations are also observed in urogenital abnormalities and other symptoms (Bérubé et al., 2005; Gibbons et al., 2008).

ATRX gene-related disorders were first described in 1980 by Smith et al. who described two brothers with ID, microcephaly, short stature, and unusual facial appearance. The muscle tone of these brothers was hypotonic, and one patient showed hyperreflexia (Smith et al., 1980). Later, Stephenson and Johnson reported a case that was clinically very similar to that described by Smith. Therefore, it was renamed as Smith-Fineman-Myers syndrome (Stevenson et al., 2000). Mattei et al. also reported a family with Juberg-Marsidi syndrome in which seven males in five sibships were affected. They showed deafness, severe ID, facial dysmorphism, and genital abnormalities, including the small penis, hypospadias, and cryptorchidism (Mattei et al., 1983). Alpha-thalassemia is also observed in many individuals with mutations in the *ATRX* gene, which is due to a decrease in the expression of alpha-globin genes while the gene has a normal structure. This supports the hypothesis that ATRX is involved in regulating gene expression (Gibbons et al., 2003).

A comparison of clinical symptoms in patients with any of the aforementioned syndromes associated with mutations in the *ATRX* gene suggests that severe intellectual disability, microcephaly, and facial abnormalities are among the major clinical manifestations in these patients. Based on previous studies, it seems that mutations in the ADD domain are usually more likely associated with microcephaly, severe psychomotor impairment, and constitute more severe urogenital abnormalities than mutations in the helicase domain (Barresi et al., 2010; Bérubé et al., 2005; Gibbons et al., 2008). Also, in the literature review of the clinical characteristics of previously reported cases with a mutation in helicase/ATP-binding domain, summarized in Table 2, especially the reported case in the Hu et al. study and its comparison with our patients' phenotypes, it seems that mutations in this domain are rarely associated with microcephaly, while all three patients in our study had severe primary microcephaly (Hu et al., 2019). Alpha-thalassemia is also described as one of the most important clinical signs associated with *ATRX* gene mutations, and according to our review (Table 3), unlike previous findings with helicase/ATP-binding domain mutations, HbH was not present in our patient (Chudley & Lowry, 1992).

Given the range of tissues in which the *ATRX* gene is expressed, it appears that mutations in this gene have

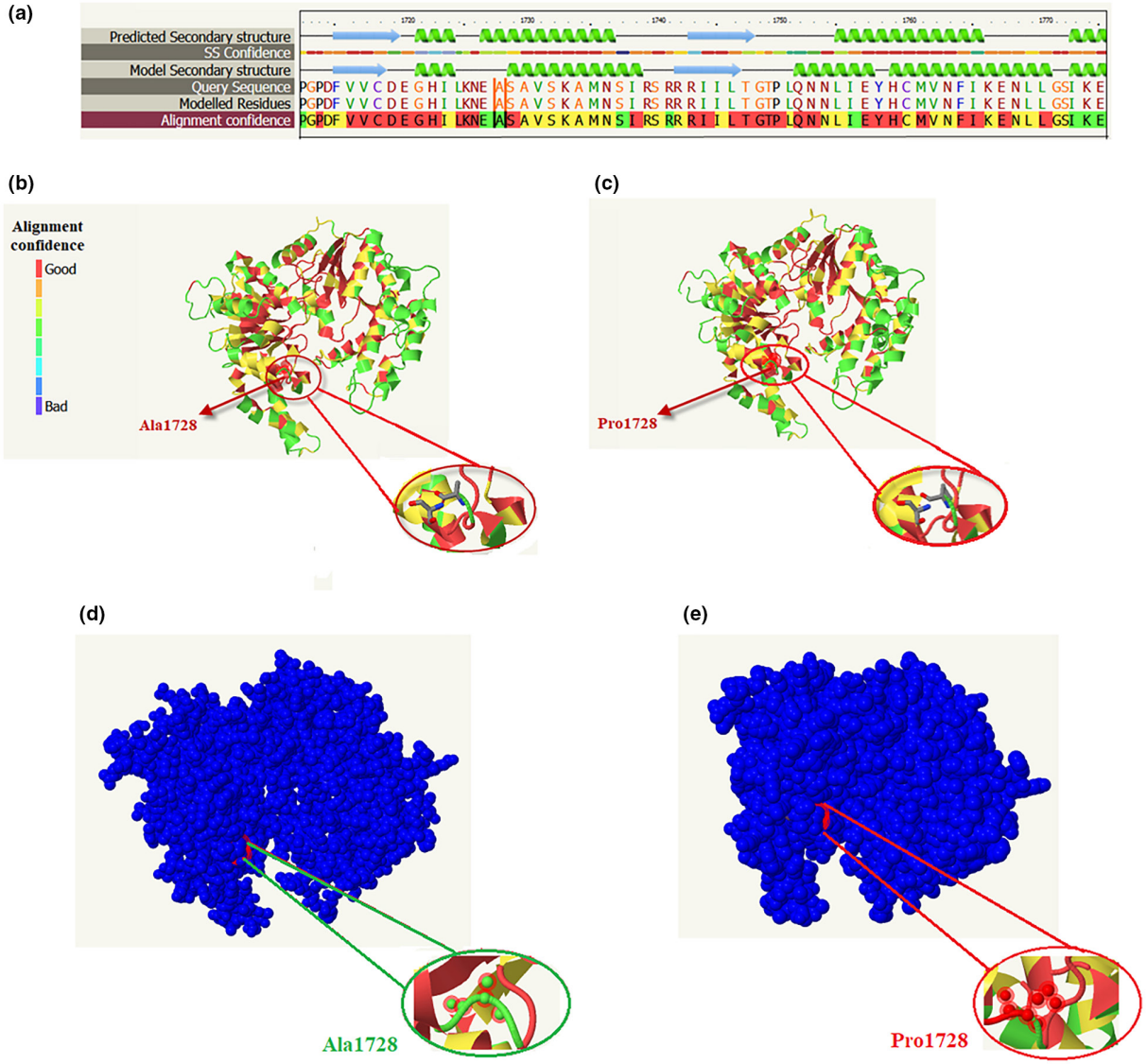

FIGURE 4 Structural analysis of the ATRX protein (NP_000480.3). (a) Prediction of the secondary structure of the ATRX protein based on template/homology modeling by Phyre-2 server. The first row displays the predicted secondary structure of the ATRX protein, in which Ala1728 is located in the α-helix structure. The second row represents SS confidence, blue is low confidence, whereas red is high confidence. The third and fourth rows indicate the amino acid sequence of ATRX and modeled residues, respectively. The last row illustrates the alignment confidence. (b) 3D structure prediction of the ATRX protein based on Phyre-2 server. Green highlighted the Ala1728 residue indicates that the alignment confidence has an average score (score = 4 of 8). (c) The van der Waals forces in the wild-type and mutated ATRX protein. The green and red balls show van der Waals forces that are rendered by Ala and Pro in wild-type and mutated ATRX protein, respectively. The detailed structure shows that van der Waals forces in the mutated type have been changed in comparison with the wild type.

TABLE 2 Prediction of the effect of identified variant on ATRX protein stability using (a) MUpro (b) I. Mutant

			(a) MUpro				(b) I. Mutant	
			Method 1: SVM		Method 2: Neural network			
Variant	ΔΔG	Prediction	Confidence score	Effect	Confidence score	Effect	ΔΔG	Stability
A1728P	−1.227	Decrease	−0.622	Decrease	−0.993	Decrease	−0.44	Decrease

Abbreviations: SVM, support vector machine; ΔΔG < 0, Decrease stability; ΔΔG > 0, Increase stability.

TABLE 3 Clinical findings of affected individuals and genotypes of the present case and previously reported cases due to helicase/ATP-binding domain mutations

cDNA/amino acid change	Type of syndrome	MR	Microcephaly	HbH inclusion bodies	urogenital abnormality	Psychomotor impairment	Faceabnormality	Ref
c.4817G > A p.S1606N	Alpha-thalassemia-X-linked intellectual disability syndrome	+	−	+	NR	NR	NR	Niranjan et al. (2015)
c.5041A > G p.H1609R	ATRX syndrome	+	−	+	+	NR	+	Gibbons et al. (1995)
c.5055T > C p.C1614R	ATRX syndrome	+	NR	+	+	NR	NR	Gibbons et al. (1995)
c.5069C > T P.T1621M	Mental retardation without alpha-thalassaemia	+	−	+	−	−	−	Yntema et al. (2002)
c.5079G > A p.A1622T	Intellectual disability/ developmental delay	+	−	+	+	NR	NR	Gibbons et al. (2000)
c.4934T > C p.L1645S	ATRX syndrome	+	+	+	−	+	+	Wada et al. (2000)
c.5027G > C p.G1676A	ATRX syndrome	+	−	NR	−	−	+	Badens et al. (2006)
c.5254T > C p.I1680T	ATRX syndrome	+	NR	+	+	NR	NR	Gibbons et al. (2000)
c.5352C > T p.P1713S	Mental retardation without alpha thalassaemia	+	−	−	−	−	+	Villard et al. (1996)
c.5440G > A p.R1742K	Mental retardation and spastic paraplegia	+	−	+	+	+	+	Lossi et al. (1999)
c.5282T > p.1761M > T	Intellectual disability, X-linked	+	NR	+	NR	NR	+	Jensen et al. (2011)
c:5488-5663del p.Y1758X	Intellectual disability, X-linked	+	NR	+	+	+	+	Villard et al. (1996)
c.5182G > C, p.Ala1728Pro	Intellectual disability, X-linked	+	−	NR	NR	NR	NR	Hu et al. (2019)
c.5182G > C, p.Ala1728Pro	X-linked mental retardation-hypotonic facies syndrome	+	+	−	+	+	+	This study

Abbreviations: NR, not reported; +, percent; −, absent.

pleiotropic effects and can lead to phenotypes with common features but with some differences. In addition, the presence of important domains in this large protein may additionally explain different phenotypes in patients with the *ATRX* gene defects. This phenotypic variation exists even among individuals with similar mutations (Villard, Toutain et al., 1996). It appears that modifying genes and genes encoding other heterochromatin protein components may also be involved in this phenotypic variation. The identified variant in this study is in the helicase/ATP-binding domain, where 33% of all sequence alterations are clustered. The latter domain is involved in ATP-dependent RNA or DNA unwinding and its alterations are expected to alter the protein function. Mutations impairing the ATP-binding ability of the ATRX protein can impair its ability in chromatin remodeling during the cell cycle, thereby altering the expression of many downstream genes (Badens et al., 2006; Dyer et al., 2017).

In conclusion, in this study, we described the new clinical characteristic phenotype in an Iranian pedigree. It seems that a wide phenotypic spectrum is associated with different *ATRX* mutations, and even with the same mutations in this gene. Additional surveys on the variations of the protein domains and their interaction with other proteins can help better recognize the mechanism of numerous genetic disorders associated with this gene and may help design better therapeutic methods in the forthcoming years.

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CONFLICT OF INTEREST

The authors, hereby, declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

MAT designed and supervised the study; FS recruited patients and performed sample collection; FS and MJ acquired the clinical data; FS Performed the experiments; FS, ZN analyzed data; FS and ZN mainly contributed to the preparation of the manuscript. All the coauthors critically reviewed and approved the last version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

ETHICS STATEMENT

This study was approved by the ethics committee of Isfahan University of Medical Sciences (ethics number: IR.MUI.MED.REC.1399.203).

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