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ORIGINAL ARTICLE

Genotype–phenotype correlation in seven motor neuron disease families with novel *ALS2* mutations

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Abstract

Autosomal-recessive mutations in the *Alsin Rho guanine nucleotide exchange factor (ALS2)* gene may cause specific subtypes of childhood-onset progressive neurodegenerative motor neuron diseases (MND). These diseases can manifest with a clinical continuum from infantile ascending hereditary spastic paraplegia (IAHSP) to juvenile-onset forms with or without lower motor neuron involvement, the juvenile primary lateral sclerosis (JPLS) and the juvenile amyotrophic lateral sclerosis (JALS). We report 11 patients from seven unrelated Turkish and Yemeni families with clinical signs of IAHSP or JPLS. We performed haplotype analysis or next-generation panel sequencing followed by Sanger Sequencing to unravel the genetic disease cause. We described their clinical phenotype and analyzed the pathogenicity of the detected variants with bioinformatics tools. We further reviewed all previously reported cases with *ALS2*-related MND. We identified five novel homozygous pathogenic variants in *ALS2* at various positions: c.275_276delAT (p.Tyr92CysfsTer11), c.1044C>G (p.Tyr348Ter), c.1718C>A (p.Ala573Glu), c.3161T>C (p.Leu1054Pro), and c.1471

Abbreviations: ACMG, American College of Medical Genetics and Genomics; *ALS2*, Alsin Rho guanine nucleotide exchange factor ALS2 gene; DH/PH, Dbl homology and pleckstrin homology; GEF, guanine nucleotide exchange factor; IAHSP, infantile ascending hereditary spastic paraplegia; JALS, juvenile amyotrophic lateral sclerosis; JPLS, juvenile primary lateral sclerosis; MND, motor neuron diseases; MORN, membrane occupation and recognition nexus; NGS, next generation sequencing; NMD, nonsense-mediated decay; PD, Parkinson's disease; RCC, regulator of chromatin condensation; ROH, regions of homozygosity; VPS9, vacuolar protein sorting 9.

+1G>A (NM_020919.3, NP_065970.2). In our cohort, disease onset was in infancy or early childhood with rapid onset of motor neuron signs. Muscle weakness, spasticity, severe dysarthria, dysphagia, and facial weakness were common features in the first decade of life. Frameshift and nonsense mutations clustered in the N-terminal Alsin domains are most prevalent. We enriched the mutational spectrum of *ALS2*-related disorders with five novel pathogenic variants. Our study indicates a high detection rate of *ALS2* mutations in patients with a clinically well-characterized early onset MND. Intrafamilial and even interfamilial diversity in patients with identical pathogenic variants suggest yet unknown modifiers for phenotypic expression.

KEYWORDS

amyotrophic lateral sclerosis (ALS), familial ALS, infantile ascending hereditary spastic paraplegia (IAHSP), juvenile amyotrophic lateral sclerosis (JALS), linkage analysis, whole-exome sequencing

1 | INTRODUCTION

Autosomal recessive mutations in the Alsin Rho guanine nucleotide exchange factor gene (*ALS2*) cause an early onset progressive degeneration predominantly of the upper motor neurons and have been implicated as disease-causing in around 1.5% of patients with amyotrophic lateral sclerosis (ALS) (Hadano et al., 2001; Kenna et al., 2013). The resulting motor neuron diseases (MND) can clinically manifest as one of three different *ALS2*-related disorders with overlapping phenotypes: infantile ascending hereditary spastic paraplegia (IAHSP, OMIM #607225) (Eymard-Pierre et al., 2002; Lesca et al., 2003) and two juvenile-onset forms with or without lower motor neuron involvement, juvenile primary lateral sclerosis (JPLS, OMIM #606353) and juvenile amyotrophic lateral sclerosis (JALS, OMIM #205100) (Gros-Louis et al., 2003; Yang et al., 2001).

ALS2 is located on chromosome 2 at cytoband 2q33 (Hentati et al., 1994) and transcribed into two isoforms, a long one with 34 exons encoding Alsin, a polypeptide of 1,657 amino acids (NP_065970.2), and a short one comprising only exons 1–4 with an open reading frame for 396 amino acids (NP_001129217.1). There is no experimental evidence that the short isoform is indeed translated into the predicted protein, however, it may be rapidly degraded (Devon et al., 2006; Soares, Barlow, Porteous, & Devon, 2009).

ALS2 is ubiquitously and abundantly expressed in various tissues including motor neurons. The encoded protein Alsin is a member of the guanine nucleotide exchange factors (GEFs) and has been shown to activate the small GTPase Rab5 (Otomo et al., 2003). Its structure contains three GEF domains that regulate the activity of Rho, Rac1, and Rab5 GTPases. Beyond that Alsin shows a complex domain architecture with three to seven (various description in the literature) identified regulator of chromatin condensation (RCC)-like domains located near the N-terminus, a centrally located Dbl homology and pleckstrin homology (DH/PH) domain, eight membrane occupation and recognition nexus (MORN) domains related to membrane binding, and a vacuolar protein sorting 9 (VPS9) domain at the C-terminus (Eker, Unlu, Al-Salmi, & Crosby, 2014; Soares et al., 2009) (Figure 1a). The presence of these functional domains and findings of recent functional studies indicate multiple important subcellular functions for Alsin, including modulation of endosome and mitochondrial trafficking as well as endocytosis (Hadano et al., 2006; Hadano, Kunita, Otomo, Suzuki-Utsunomiya, & Ikeda, 2007; Hsu, Spannl, Ferguson, Hyman, & Parton, 2018; Otomo et al., 2003).

We identified five novel pathogenic mutations in *ALS2* in 11 patients from seven families by haplotype analysis, next-generation sequencing (NGS) or based on clinical features with subsequent Sanger sequencing. We describe their neurological phenotype and evaluate the mutations' impact on protein structure and function. To further elucidate genotype–phenotype correlations, we reviewed all cases reported in literature regarding their clinical phenotype and genetic background.

2 | MATERIALS AND METHODS

2.1 | Cohort of patients

The cohort we recruited consists of eight patients from six Turkish families with IAHSP [family 1 (case 1), family 2 (case 2), family 3 (cases 3 and 4), family 4 (case 5), family 5 (case 6), and family 7 (cases 10 and 11)] and three patients from a consanguineous Yemeni family (family 6, cases 7, 8, and 9), who were diagnosed with JPLS. Informed consent was obtained from all patients or their legal representatives. The study was approved by the local ethics committee of the Medical Faculty, University Hospital Cologne, University of Cologne (17-096).

2.2 | Molecular genetic analysis

To identify the pathogenic variants that lead to upper MND in our patients, we performed haplotype analysis in four families and next-

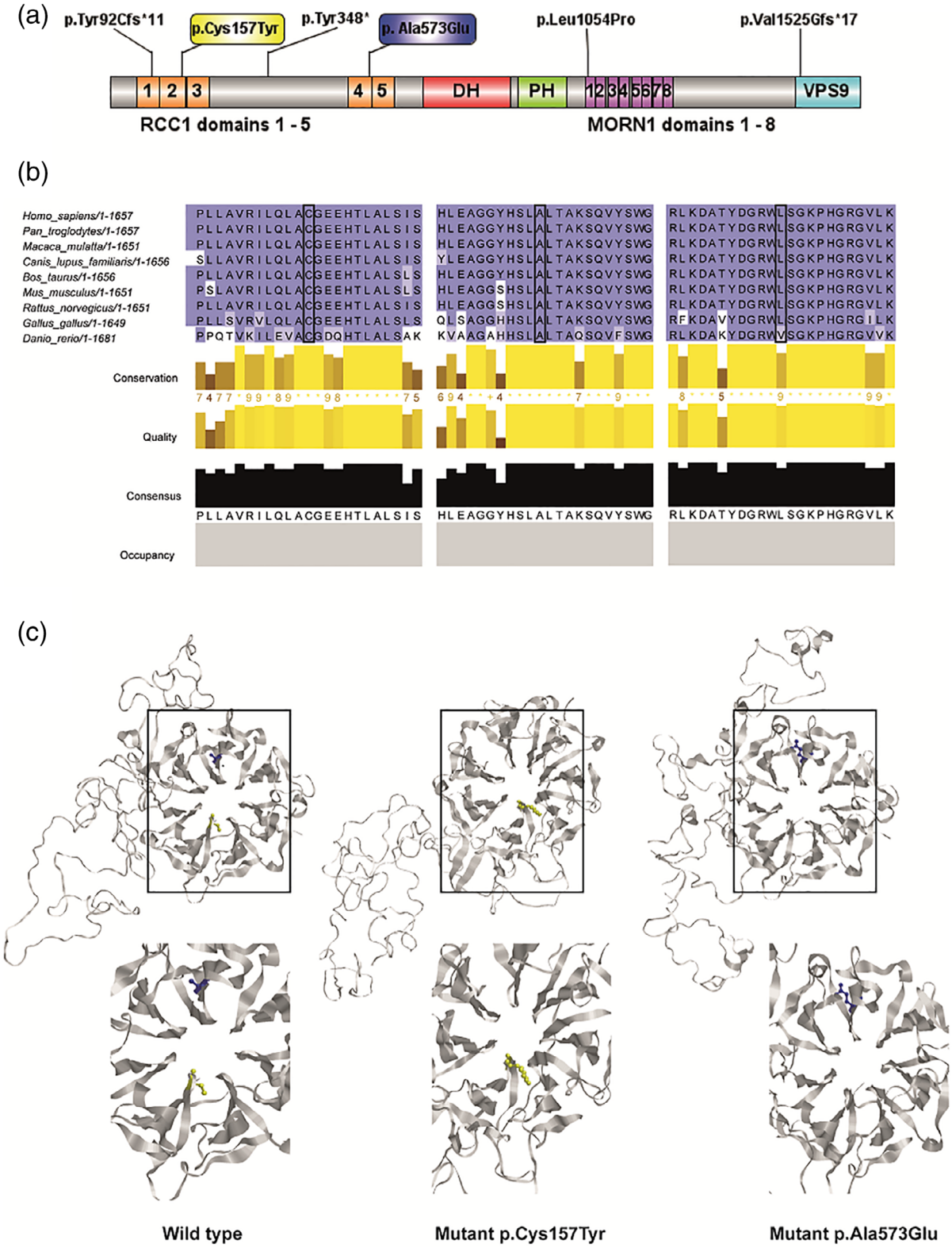

FIGURE 1 (a) Linear model of the structure of the human Alsin, displaying the localization of variants described in this report. (b) Multiple sequence alignment analysis performed for the missense variants p.Ala573Glu, p.Cys157Tyr, and p.Leu1054Pro indicates that these mutations are located in highly conserved areas. (c) The 3D structure model of Alsin wild type protein (UniProtKB Q96Q42) and both variants located in the RCC domain (yellow: p.Cys157Tyr and blue: p.Ala573Glu). The crystal structure of human RCC2 (PDB 5gwn.1) was used as a template. Both mutations are located in the propeller structure and are likely to disrupt its architecture. RCC domains promote the exchange of Ran-bound GDP by GTP, a loss of function leads to severe disruption of nucleo-cytoplasmic transport and nuclear-envelope assembly, which might lead to neuron damage. Legend: RCC, regulator of chromosome condensation; DH, Dbl homology; PH, Pleckstrin homology domain; MORN, membrane occupation and recognition nexus; VSP9, voltage-sensitive phosphatase 9 [Color figure can be viewed at wileyonlinelibrary.com]

generation sequencing in one family with subsequent Sanger sequencing. Haplotype analysis was performed for the families 1–3 and 5 to confirm homozygosity at the *ALS2* locus. Genomic DNA was extracted from whole-blood samples according to standard protocols. Based on their phenotypic characteristics, the four polymorphic microsatellite markers D2S116, D2S309, D2S2309, and D2S2214 were used for genotyping. The order of the markers and their genetic distances are taken from the deCode map. PCRs were performed with fluorescently labeled primers and semiautomated genotyping was performed on an ABI 310 using the genescan v.3.1 software (Applied Biosystems, Foster City, California) (Olmez et al., 2006). Primer pairs were designed using the Primer 3 program (Rozen & Skaletsky, 2000). For sequencing, all coding exons including exon–intron boundaries were amplified from genomic DNA and sequenced using the BigDye Terminator 3.1 Sequencing Kit (Applied Biosystems). We performed sequence comparisons using the DNASTAR package (Lasergene). Haplotypes were constructed manually and phases were determined assuming a minimum number of recombinants. Dideoxy sequencing was performed for coding exons and flanking introns of *ALS2* after linkage to its locus was demonstrated (Appendix S1).

In the Yemeni family 6, NGS was used to uncover the genetic cause of the syndrome. The genomic DNA of the index patient (case 7) was extracted using standard methods and enriched with a gene panel for mendelian diseases (TruSight One Panel, Illumina, The United States) according to the manufacturer's best-practice protocol. Sequencing was performed on an Illumina HiSeq 4000 Sequencing System (Illumina, California). The mean coverage was 84×. About 10× coverage was attained for 97.7% of target sequences and 20× coverage was achieved for 94.5% of target sequences.

In the families 4 and 7, diagnosis was suspected based on highly evocative clinical features consisting of early bulbar manifestation—early dysarthria after initially unremarkable speech development—and confirmed via dideoxy sequencing. For all families, dideoxy sequencing was additionally performed in available family members to confirm co-segregation (Appendix S1).

Data analysis was performed with the Varbank V2.12 exome pipeline of the Cologne Center for Genomics (CCG) (https://varbank.ccg.uni-koeln.de/varbank2/). Sequencing data were filtered for rare and homozygous variants (minor allele frequency <0.1%, allele read frequency of 75–100%) following the expected autosomal recessive mode of inheritance. Variants were considered if they were located in runs of homozygosity (ROH) corresponding to a total of 420 Mb in the patient. Variants affecting protein-coding sequences or splice-sites were considered. We performed dideoxy sequencing for validation of the variants and confirmation of co-segregation for available relatives (Appendix S1). All positions in *ALS2* were annotated to NCBI RefSeq NM_020919.3. Amino acid positions were assigned to NCBI RefSeq NP_065970.2. The pathogenic variants have been submitted to ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) and can be accessed under SCV000998812 to SCV000998818.

2.3 | Bioinformatic in silico prediction of mutational effects

To further evaluate the variants, we used the best-practice filtering scheme based on the American College of Medical Genetics and Genomics (ACMG) guidelines (Richards et al., 2015) and common online prediction tools for variant interpretation (Table S2). The Exome Aggregation Consortium (http://exac.broadinstitute.org/) and the Greater Middle East Variome Project (http://igm.ucsd.edu/gme/) were checked for the variants (Table S2).

A modified exon-based missense tolerant ratio (MTR) gene viewer result for *ALS2* (ENST00000264276, NCBI Ref Seq NM_020919) with window size 21 (http://mtr-viewer.mdhs.unimelb.edu.au/mtr-viewer/) was performed to assess putative pathogenic mutations in regions with high intolerance toward missense variations. We also performed a modified analysis for minor allele frequencies from the gnomAD population database (frameshift, missense, essential splice site, and splice region) and CADD scores (http://shiva.rockefeller.edu/PopViz/) *ALS2* (ENSG00000003393, NCBI Gene ID 57679) to assess the mutational impact based on genetic variation.

2.3.1 | Multiple sequence alignment

Multiple sequence alignment was generated for the missense mutations p.Cys157Tyr, p.Ala573Glu, and p.Leu1054Pro between selected orthologues by using the NCBI HomoloGene Protein Multiple Alignment platform. The alignment was analyzed with the MUSCLE algorithm (Edgar, 2004), provided by the Jalview web services and visualized with the Jalview online tool (version 2.11.0). Amino acid color labels were selected for the block substitution matrix 62 (Figure 1b).

2.3.2 | Protein modeling

The linear protein model was visualized with the IBS illustrator tool (http://ibs.biocuckoo.org/online.php). The 3D protein structures of Alsin wild type and both missense mutations affecting the RCC-like domains (p.Cys157Tyr and p.Ala573Glu) were modeled with the User Template Mode of SWISS-MODEL online server (https://swissmodel.expasy.org/). The crystal structure of the human RCC2 domain (PDB 5GWN) was selected as the template for homology modeling. The PDB files were visualized and evaluated using RasMol Molecular Graphics Visualization Tool (http://www.openrasmol.org/).

2.4 | Literature review

A literature search in Medline and Web of Science was conducted using the search terms *ALS2*, Alsin, JALS, JPLS, and/or IAHSP. References of all matching results were additionally scanned. We collected

the clinical and genetic characteristics for all 82 patients described in the literature up to July 2020 (Tables S3 and S4) to compare their phenotypic and genotypic features with our cohort (Daud et al., 2016; Devon et al., 2003; Eymard-Pierre et al., 2002; Eymard-Pierre et al., 2006; Flor-de-Lima, Sampaio, Nahavandi, Fernandes, & Leao, 2014; Gros-Louis et al., 2003; Hadano et al., 2001; Helal et al., 2018; Herzfeld et al., 2009; Kim et al., 2016; Kress et al., 2005; Lesca et al., 2003; Luigetti et al., 2013; Mintchev, Zamba-Papanicolaou, Kleopa, & Christodoulou, 2009; Nishiyama et al., 2017; Panzeri et al., 2006; Racis et al., 2014; Sheerin et al., 2014; Shirakawa et al., 2009; Siddiqi et al., 2014; Sztriha et al., 2008; Tariq, Mukhtar, & Naz, 2017; Verschuuren-Bemelmans et al., 2008; Wakil et al., 2014; Xie, Cen, Xiao, & Luo, 2015).

3 | RESULTS AND DISCUSSION

3.1 | Clinical findings and genotype–phenotype correlations

Our cohort of patients with *ALS2*-related diseases consists of 11 patients from seven unrelated families. Three patients are from a Yemeni family (cases 7, 8, and 9), the other families are Turkish. The cases 1–6, 10, and 11 were diagnosed with IAHSP, while the three Yemeni patients (cases 7, 8, and 9) were diagnosed with JPLS.

The age range at the first clinical assessment was between one and 20 years. Disease onset was in infancy or early childhood, usually with a rapid onset of motor neuron signs and particular dysarthria. All of our patients developed upper limb involvement during the disease course, although weakness and spasticity were more prominent in the lower limbs with clearly associated pyramidal signs. Severe dysarthria or even anarthria were common features in the first decade of life, accompanied by facial weakness and pronounced dysphagia. Early bulbar manifestation appears to be a highly evocative sign for *ALS2* mutations. Remarkably, the dysarthria rapidly progresses to anarthria. Cerebral MRI and EEG were normal in all but one patient (case 1), who suffered from seizures. The intellectual development was unremarkable in all our patients except for one, who showed mild mental retardation (case 2). For detailed clinical information, see Table 1, Table S1, case presentations, and patients' photos provided in the Appendix S1.

To this date, 82 patients with early-onset MND due to variants in *ALS2* have been described in the literature (Tables S3 and S4). Of these patients, 53 cases (64.6%) were diagnosed with IAHSP. Four cases had JPLS (4.9%), while 25 cases were reported with JALS (30.5%). However, the phenotypes of the *ALS2*-associated disorders overlap markedly. Clinically, patients present with a continuum of disease severity: some patients may never achieve the ability to walk independently, while in others ambulation is preserved until early adulthood (Orrell, 1993). Calculated from literature, the average age of disease onset of IAHSP is 15.5 months (n = 40; see Table S3). Amongst our patients, the age of onset of IAHSP was at 14.8 months (n = 8). For JPLS, cases from the literature have an average age of onset of 24 months (n = 4), while the average age of onset in our JPLS patients (n = 3) is at 17.3 months. For JALS, the average age of onset calculated from 21 cases is at 4.9 years.

We report five novel pathogenic mutations in *ALS2*. Amongst our patients, we report three missense mutations (p.Cys157Tyr, p.Ala573Glu, and p.Leu1054Pro), a nonsense mutation (p.Tyr348Ter), two frameshift mutations (p.Tyr92CysfsTer11, p.Val1525GlyfsTer17), and a splice site mutation (c.1471+1G>A), all in homozygous state. Two pathogenic variants found in our cohort have previously been published:

Firstly, Sheerin et al. (2014) reported a patient harboring the mutation c.4573dupG (RefSNP rs730882256) that we also detected in our case 5, leading to frameshift at p.Val1525GlyfsTer17. Both patients originate from Turkey but are, to the best of our knowledge, not related. They show several similar symptoms in different degrees of severity and were classified into different *ALS2*-related diseases (JALS in Sheerin et al., IAHSP in our case). Our patient first presented symptoms at 16 months, while Sheerin et al. reported their patient's symptom onset at approximately 2–3 years. However, Sheerin's patient reached major milestones in early childhood at an appropriate age and became wheelchair-bound at the age of 8 years, while our patient has never achieved independent ambulation.

Furthermore, Sheerin et al. reported generalized dystonia in their patient that was poorly responsive to deep brain stimulation. In line with this, our patient also showed dystonic posturing of the hands, rendering him unable to open his hands and to hold a pen at the latest examination at 28 years. To date, the novel phenotype of *ALS2* variants associated with dystonia has only been reported in four unrelated consanguineous families: two families of Bangladeshi and Turkish descent, with JALS and homozygous loss-of-function variants in *ALS2* affecting the DH/PH respectively the VPS9 domains (Sheerin et al., 2014); a large consanguineous Pakistani family with JALS and a homozygous splice site variant affecting the RCC-like domain (Siddiqi et al., 2014); and a consanguineous Iranian family with IAHSP, generalized dystonia and a splice-site variant affecting the RCC-like domain (Helal et al., 2018). Our patient barely exhibits hand dystonia at the age of 28 years, in contrast to previously published cases with generalized dystonia at earlier ages. Of note, volume reduction in basal ganglia measured by cranial MRI examinations was previously reported in ALS patients, even more extensively in those carrying a *C9orf72* hexanucleotide repeat expansion (Bede et al., 2013). However, there is no comprehensive report on *ALS2*-associated basal ganglia damage to this date.

Secondly, we identified another previously described mutation in our cohort: Case 6 carries a homozygous c.470G>A, p.Cys157Tyr mutation in *ALS2*. Eymard-Pierre et al. (Eymard-Pierre et al., 2006) found this variant in two of their patients from a large consanguineous family from Turkey (RefSNP rs121908138). Both siblings started supported walking at the age of 3 and 7 years and were wheelchair-bound at 12 and 10 years, respectively. They were classified to have IAHSP. The younger sister displayed subtle cerebellar atrophy in cMRI. Eymard-Pierre et al. reported that the younger sister had more severe motor development impairment and faster disease progression

TABLE 1 Clinical and genetic aspects of our patients

Family	Case	Age/ gender/ origin	Clinical phenotype	Pathogenic variant	Pre- and perinatal history	Motor development	Onset of disease	Spasticity/ pathological reflexes	Dysarthria/ anarthria	Current status	Additional findings	Consanguinity/ familial history
1	1	8 yr/F/ Turkey	IAHSP	c.1718C>A, p.Ala573Glu	Uneventful	Walking at 18 mo	19 mo	Spasticity + Babinski + Clonus +	Close to anarthria	Walks w/o support	Epilepsy; genu recurvatum	+
2	2	11 yr/F/ Turkey	IAHSP	c.1044C>G, p.Tyr348Ter	Uneventful	Walking delayed	15 mo	Spasticity + Babinski + Clonus +	Close to anarthria	Walks w/o support	Foot drop	+ affected brother, 25 yr, never able to walk, dysarthria
3	3	22 yr/F/ Turkey	IAHSP	c.3161T>C, p.Leu1054Pro	Uneventful	Tip toe walking at 15 mo	15 mo	Spasticity + Babinski + Clonus +	Anarthria	Walks with support	Achilles tendon release at 3 yr	+
	4	19 yr/F/ Turkey	IAHSP	c.3161T>C, p.Leu1054Pro	Uneventful	Walking at 8 mo	13 mo	+	Anarthria	Loss of ambulation at 14 yr	A prominent weakness of upper extremities	
4	5	28 yr/M/ Turkey	IAHSP	c.4573dupG, p. Val1525GfsTer17 (rs730882256)	Uneventful	Never attained walking	16 mo	Spasticity + Babinski + Clonus +	Anarthria	Loss of ambulation at 4 yr	Scoliosis; dystonia with predominance in hands	+ affected cousin
5	6	13 yr/F/ Turkey	IAHSP	c.470G>A, p.Cys157Tyr (rs121908138)	Uneventful	Tip toe walking at 14 mo	14 mo	+	Dysarthria	Walks w/o support	Achilles tendon release at 3 yr; pes planus	+
6	7	15 yr/F/ Yemen	JPLS	c.275_276delAT, p.Tyr92CfsTer11	Uneventful	Walking at 14 mo	24 mo	+	Anarthria at 9 yr	Loss of ambulation at 10 yr	Achilles tendon release at 3 yr	+ 8 affected relatives
	8	18 yr/F/ Yemen	JPLS	c.275_276delAT, p.Tyr92CfsTer11	Uneventful	Walking at 14 mo	16 mo	+	Anarthria	Loss of ambulation at 3 yr	Unilateral foot polydactyly	
	9	19 yr/F/ Yemen	JPLS	c.275_276delAT, p.Tyr92CfsTer11	Uneventful	Walking with abnormal gait at 12 mo	12 mo	+	Anarthria	Loss of ambulation	Teeth malocclusion	
7	10	19 yr/M/ Turkey	IAHSP	c.1471+1G>A	Uneventful	Never attained walking	12 mo	+	Anarthria	Wheelchair-bound	–	NA
	11	17 yr/M/ Turkey	IAHSP	c.1471+1G>A	Uneventful	Never attained walking	12 mo	+	Anarthria	Wheelchair-bound	–	

Note: All positions in *ALS2* were annotated to NCBI RefSeq NM_020919.3. Amino acid positions were assigned to NCBI RefSeq NP_065970.2.
Abbreviations: yr, years; mo, months; w/o, without; F, female; M, male; +, present; –, absent; TA, achilles tendon.

than her older sister. On the contrary, our patient had a less progressive disease course: he achieved the ability to walk with 14 months and was still ambulatory at the latest clinical examination at 11 years. We did not detect any abnormal MRI findings.

Daud et al. (2016) observed a high degree of intra-familial clinical homogeneity associated with specific *ALS2* mutations in two families. In contrast, despite having identical pathogenic *ALS2* variants, families in our cohort demonstrate both intra- and interfamilial phenotypic variability in disease severity, symptoms, and imaging findings. It remains elusive, whether environmental factors, modifier genes and/or epigenetic alterations are important for the clinical presentation of *ALS2*-related diseases.

3.2 | Genetic results and analysis

3.2.1 | Determination of the molecular impact of *ALS2* variants

For a better understanding of the functional consequences of disease-causing variants, it is important to review the current functional knowledge on the protein. Hsu et al. have implicated Alsin as an essential key player in a cytoprotective mechanism of damaged mitochondria (Hsu et al., 2018). Laser-damaged mitochondria responded to oxidative stress with the mitochondria outer membrane permeabilization releasing cytochrome c. In turn, this triggered the assembly of the GTPase Rab5, which led to the internalization of damaged mitochondria into early endosomes. Alsin significantly co-localized with Rab5 around mitochondria following immunocytochemical analysis. After damaging mitochondria, iPSC spinal motor neurons showed enrichment of Rab5 on mitochondria, whereas Alsin-deficient neurons showed no Rab5 enrichment (Hsu et al., 2018). In an Alsin-KO mouse model, there were observations of disintegrated and fused mitochondria with defective inner membranes, expanded core and broken cristae as well as major Golgi complex defects in electron microscopy (Gautam et al., 2016). In line with the implication of Alsin in Rab5 translocation as a priming step of mitochondria-related stress response, we conclude that the previously only rarely reported phenotype patients with *ALS2*-related dystonia conjoins with deficient mitochondria degradation into early endosomes.

We uncovered a homozygous single-base exchange in exon 7 of *ALS2* (c.1718C>A) in case 1, leading to an amino acid exchange of alanine to glutamic acid at position p.Ala573Glu. The mutation is located in the RCC1-like domain 4 of Alsin. In case 2, genetic analysis revealed a homozygous variant in exon 4 (c.1044C>G) resulting in a nonsense mutation at position p.Tyr348Ter. The transcript is predicted to be subject to degradation by NMD pathway, according to NMDEscPredictor. Both sisters of the third family (cases 3 and 4) are affected by the same homozygous single-base exchange c.3161T>C in exon 18, leading to an amino acid alteration from leucine to proline at position p.Leu1054Pro in the MORN domain 1. In cases 7, 8, and 9, a deletion of two nucleotides was discovered at c.275_276delAT by NGS. It results in a frameshift with a predicted premature stop-codon at p.Tyr92CysfsTer11 in the RCC1-like domain 1. In the patients 10 and 11, a splice-site mutation in intron 5 at c.1471+1G>A was found. As discussed above, the pathogenic variants detected in our cases 5 and 6 were previously reported by Sheerin et al. (c.4573dupG, p.Val1525GlyfsTer17) (Sheerin et al., 2014) and by Eymard-Pierre et al. (c.470G>A, p.Cys157Tyr) (Eymard-Pierre et al., 2006). These mutations affect the C-terminal VSP9 domain and the RCC1-like domain 2. Eymard-Pierre et al. detected for their pathogenic variant a decreased protein stability by analysis of protein levels both by immunoblotting of extracts from lymphoblasts and by expression of the mutant complementary DNA in human embryonic kidney (HEK293) cells.

None of the novel variants were found in the Genome Aggregation (gnomAD) Database or the Greater Middle East Variome Project (Table S2). To evaluate the pathogenic potential of the variants, we used MutationTaster2 for in-silico prediction based on protein structure/function and evolutionary conservation. All mutations were predicted to be "disease-causing." Both missense mutations were further predicted as "deleterious" based on sequence homology with the PROVEAN prediction tool. Both mutations leading to a stop mutation were considered to be subject to degradation by nonsense-mediated decay (NMD) by MutationTaster2 and NMDEscPredictor. The splice-site mutation was predicted as an "alteration of the WT donor site, most probably affecting splicing" by Human Splicing finder (weblinks in Appendix S1).

Missense tolerant ratio (MTR) calculations for our patients' variants revealed putative pathogenic missense mutations in regions with high intolerance toward missense variations (Figure 2a). In comparison to the *ALS2* variants from gnomAD population database (frameshift, missense, essential splice site, and splice region), we observed high CADD scores in our patients' novel, unreported variants (Figure 2b). Ultimately, variants were scored based on the standards and guidelines of the ACMG for the interpretation of variants (Richards et al., 2015) as "likely pathogenic" for both missense mutations and the splice-site mutation and "pathogenic" for both nonsense mutations (Table S2).

The three missense mutations p.Cys157Tyr, p.Ala573Glu, and p.Leu1054Pro were further analyzed by multiple sequence alignment of human Alsin with selected orthologues, indicating that these mutations are located in strictly conserved areas (Figure 1b). Moreover, we built a structural model of the RCC1-like domains of Alsin to clarify the structural and functional implications of the missense mutations in this region (p.Cys157Tyr, p.Ala573Glu; Figure 1c). Human RCC2 domain served as a template (PDB 5GWN). It revealed that the N-terminal RCC-like domains encode a seven-bladed propeller with an interruption in blade 5 for an intercalated disordered sequence. Both mutations are located in the propeller structure (blade 3, respectively, blade 6) and not in the intercalated disordered sequence. Both mutated residues are predicted to be exposed on the inter-blade interface of the protein surface. Taken together, the modeling results imply that these pathogenic missense mutations are likely to disrupt the architecture of the propeller structure and cause a dysfunction of

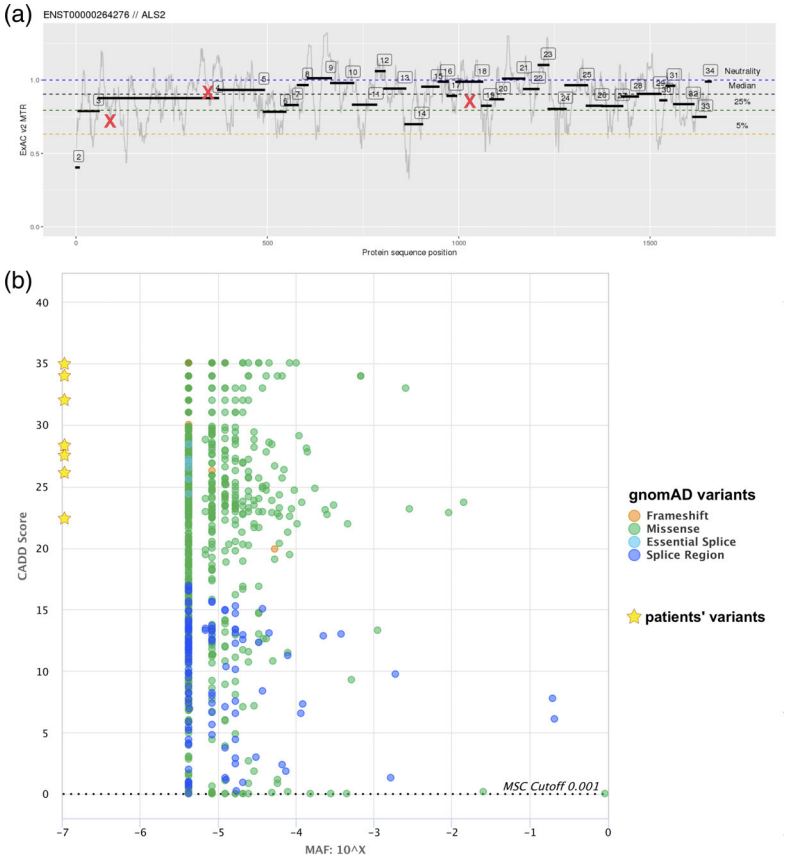

FIGURE 2 Bioinformatic analyses reveal pathogenicity and intolerance toward missense variants of our patients' variants. (a) Modified exon-based missense tolerant ratio (MTR) gene viewer result for *ALS2* (ENST00000264276, NCBI Ref Seq NM_020919) with window size 21; patients' missense variants are marked with red crosses. MTR calculations revealed putative pathogenic mutations in regions with high intolerance toward missense variations. (b) Modified PopViz results for *ALS2* (ENSG00000003393, NCBI Gene ID 57679) with minor allele frequencies and CADD scores for our patients' variants (yellow stars) and variants from gnomAD population database (frameshift orange, missense green, essential splice site pink, and splice region blue). Mutation Significance Cutoff (MSC) was 0.001. Our patients' variants are absent from gnomAD and are predicted as likely pathogenic with CADD score values above 20, while the mostly heterozygous gnomAD variants from healthy subjects show a broad variability of CADD scores [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Literature review of domain location and distribution of reported pathogenic variants in Alsin-related MND

	IAHSP		JPLS		JALS		Total *ALS2*-related MND	
Domain	Number of variants	Percentage	Number of variants	Percentage	Number of variants	Percentage	Number of variants	Percentage
RCC-like	15	53.6	2	66.7	4	50	21	53.8
DH/PH	8	28.6	1	33.3	2	25	11	28.2
MORN	4	14.3	–	–	1	12.5	5	12.8
VPS9	1	3.6	–	–	1	12.5	2	5.1
Total	28	100	3	100	8	100	39	100

the RCC-like domain in Alsin. Our modeling results are consistent with previously reported computational models, built upon human RCC1 sequence (PDB 1A12) or third RCC1-like domain of human HERC2 (PDB 3KCI) (Sato et al., 2018; Soares et al., 2009).

We further investigated if there is a potential correlation between disease phenotype and the affected protein domains (Table 2) or the mutation type (Table 3). Pathogenic variants are distributed throughout the whole protein; pathogenic variants are reported in every domain. However, mutations were mostly located in the RCC1-like domains (53.6%), and affect the C-terminal domains in descending order. These findings apply to all three *ALS2*-related diseases similarly. Nonsense and frameshift mutations were in general the most frequent mutation types to cause *ALS2*-related disorders (25 and 30.6%, respectively).

In line with previous studies, we confirm that there is no clear genotype–phenotype correlation for the three disease entities (Daud et al., 2016; Helal et al., 2018; Racis et al., 2014). Interestingly, we observed hardly any diversity in the phenotypes in advanced disease stages: although in different disease severities, patients with all different mutation types in *ALS2* will eventually develop similar symptoms leading to a loss of ambulation, affection of the upper extremities and bulbar symptoms. We, therefore, suggest to scrutinize the common diagnostic criteria for IAHSP, JPLS, and JALS and swap them for a genotype-centered and personalized approach.

TABLE 3 Literature review of the mutation type of pathogenic variants reported in Alsin-related MND

	IAHSP		JPLS		JALS		Total *ALS2*-related MND	
Type of mutation	Number of variants	Percentage	Number of variants	Percentage	Number of variants	Percentage	Number of variants	Percentage
Missense	4	16.0	1	33.3	2	25.0	7	19.4
Nonsense	8	32.0	–	–	1	12.5	9	25.0
Frameshift	7	28.0	–	–	4	50.0	11	30.6
Splice site	3	12.0	2	66.7	1	12.5	6	16.7
Com. het.	3	12.0	–	–	–	–	3	8.3
Total	25	100	3	100	8	100	36	100

4 | CONCLUSION

In conclusion, we identified five novel pathogenic variants in *ALS2* that expand the phenotypic spectrum of IAHSP and JPLS. Moreover, the systematic review of all patients with *ALS2*-related MND reported in the literature and its genotype–phenotype correlations led us to believe that there is a high detection rate of Alsin mutations in clinically well-characterized infantile-onset MND. We observed a significant clinical heterogeneity, so further research is required to understand the correlation between genotype and phenotype. Inter- and even intrafamilial diversity of symptoms, the severity of the disease, and clinical course can be detected. We observed no correlation between disease severity and affected domain or type of mutation.

There is a need for further analysis of genetic modulators and mechanisms in Alsin-related pathways and environmental modification of monogenic diseases.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Rosanne Sprute collected data, analyzed and interpreted the results, summarized the findings, prepared the figures and tables, and wrote and submitted the manuscript. Hannah Jergas and Hülya-Sevcan Daimagüler contributed to the manuscript writing, genetic analyses and the literature review. Hormos Salimi Dafsari contributed to the interpretation of clinical findings and bioinformatics analysis of the patients and the manuscript writing. Salem Alawbathani participated in the collection of clinical data. Kerstin Becker contributed to exome analysis. Akgün Ölmez participated in the recruitment of the patients and haplotype analysis. Hatice Karasoy participated in the recruitment of the patients and collected clinical data. Peter Nürnberg contributed to exome analysis. Francesco Muntoni contributed to the interpretation of results. Haluk Topaloglu collected clinical data. Gökhan Uyanik contributed to haplotype analysis and sequencing. Sebahattin Cirak designed the study concept, coordinated and supervised the work, critically revised the manuscript, and acquired funding.

DATA AVAILABILITY STATEMENT

The datasets supporting the conclusions of this article are included within the article and its additional file.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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