Phenotype and genotype spectrum of variants in guanine nucleotide exchange factor genes in a broad cohort of Iranian patients

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Abstract
Background: Guanine nucleotide exchange factors (GEFs) play pivotal roles in neuronal cell functions by exchanging GDP to GTP nucleotide and activation of GTPases. We aimed to determine the genotype and phenotype spectrum of GEF mutations by collecting data from a large Iranian cohort with intellectual disability (ID) and/or developmental delay (DD).

Methods: We collected data from nine families with 20 patients extracted from Iranian cohort of 640 families with ID and/or DD. Next-generation sequencing (NGS) was used to identify the causing variants in recruited families. We also compared our clinical and molecular findings with previously reported patients carrying mutations in these GEF genes in the literature published until mid-2021.

Results: We identified disease-causing variants in eight GEF genes including ALS2, IQSEC2, MADD, RAB3GAP1, RAB3GAP2, TRIO, ITSN1, and DENND2A. The major clinical manifestations in 203 previously reported cases along with our 20 patients with disease causing variants in eight GEF genes were as follow; speech disorder (85.2%), ID (81.6%), DD (81.1%), inability to walk (71.3%), facial dysmorphisms features (52.4%), abnormalities in skull morphology (55.6%), hypotonia and muscle weakness (47%), and brain MRI abnormalities (43.4%).

Conclusion: Our study provides new insights into the genotype and phenotype spectrum of mutations in GEF genes.

Keywords
developmental delay, guanine nucleotide exchange factors, intellectual disability, Iranian families
1 | INTRODUCTION

Small GTPases are monomeric G proteins which act as cellular switches catalyzing the hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP). These proteins are involved in a broad variety of cellular functions including cell growth, differentiation, polarity, adhesion, migration, and membrane trafficking (Cromm, Spiegel, Grossmann, & Waldmann, 2015; Vetter & Wittinghofer, 2001; Williams & Rottner, 2010). These molecules are divided into five main families including Ras, Rho, Rab, Arf, and Ran GTPases based on their structure and functions. They are activated and inactivated when bound to GTP and GDP, respectively. Release of GDP from inactive GTPases is an extremely slow process (Bourne, Sanders, & McCormick, 1990; Cherfils & Zeghouf, 2013). However, the binding of guanine nucleotide exchange factors (GEFs) to their GTPases promotes the dissociation of GDP leading to rebinding of GTP to the GTPase (Bos, Rehmann, & Wittinghofer, 2007; Cherfils & Zeghouf, 2013). Based on Gene Ontology (GO) terms (http://amigo.geneontology.org/amigo/term/GO:0005085), there are about 207 human genes with GEF function which act on different GTPase molecules. Genes with GEF functions have some specific highly conserved domains such as CDC25, DHR2, Sec7, DH, and PH domains which are different between GEF genes based on their targets (Jackson & Casanova, 2000; Lemmon, 2008; Snyder et al., 2002; Zheng, 2001). Due to the importance of GTPases and GEF proteins in neurologic processes including neuronal survival, growth and migration, synaptic transmission, and plasticity, many studies have been conducted aiming to unravel their role in development of neurological disease. These studies have demonstrated that mutations of GEF genes are linked to diverse phenotypes, such as neurodegenerative disorders, neurodevelopmental disorders (NDD), and especially intellectual disability (ID). It is believed that the deteriorative effect of these mutations are caused mostly by interruptions in different cellular processes (Droppelmann, Campos-Melo, Volkening, & Strong, 2014). The first report on the pathogenicity of mutations in genes encoding GEF proteins was published by Kutsche et al. in 2000, which demonstrated that mutation in ARHGEF6 (OMIM: 300267), a gene acting as Rho GEF, is associated with ID (Kutsche et al., 2000). Following this report, numerous studies uncovered the link between mutations in these genes with different inheritance patterns and the occurrence of ID and other neurologic phenotypes. In the current study, we present the phenotypic and molecular spectrum associated with mutations in GEF genes on 20 affected subjects with ID and/or developmental delay (DD) extracted from a large Iranian cohort of families with these disorders. Our study expands the genetic and phenotypic heterogeneity of ID and/or DD associated with mutations in GEF genes which were previously reported in the literature.

2 | MATERIALS AND METHODS

Our study subjects included 20 patients from nine families with confirmed disease-causing variants in GEF genes. These patients were recruited from a large Iranian cohort consisting 540 mostly consanguineous ID families with ≥2-affected individuals, and 100 families with only one patient in each family referred to the Genetics Research Center (GRC), at the University of Social Welfare and Rehabilitation Sciences between 2007 and 2018. The blood samples were collected from the patients and participating members of each family for next processing. Genomic DNA was extracted from 2cc of EDTA anticoagulated venous blood samples by salting-out method (MWer, Dykes, & Polesky, 1988). The quality of extracted DNA was then controlled by measuring the optical density in Nanodrop2000 (Thermo Scientific) device, and approximately 2 μg of gDNA was subsequently used for next-generation sequencing (NGS). Except for the sample from family 3, which subjected to whole genome sequencing (WGS), other samples underwent whole exome sequencing (WES). Agilent SureSelect 50 Mb (V5 for families 1, 2, 5, 7, 8, and 9 and V4 for families 4 and 6) was used for exome target enrichment. Furthermore, WGS for family 3 was performed on the Illumina HiSeq X Ten sequencer (Macrogen). The pathogenicity of identified variants were evaluated using the American College of Medical Genetics/Association of Molecular Pathology (ACMG/AMP) guidelines (Richards et al., 2015). Identified variants by WES were subsequently confirmed by Sanger sequencing of affected pedigrees. Our detailed methodology for molecular diagnosis were as previously described (Hu et al., 2019; Kahrizi et al., 2019).

Finally, we reported molecular and phenotypic spectrum of ID and/or DD patients associated with mutations in GEF genes. The clinical information and phenotypes have been recorded using the human phenotype ontology (HPO). We also compared our clinical and molecular findings with previously reported patients with these core phenotypes (ID and/or DD) in the literature published until mid-2021.

3 | RESULTS

3.1 | Variant spectrum

We identified 10 different disease-causing variants in eight GEF genes from our study subjects. Most families
recruited in this study (8 out of 9 families) had consanguineous marriages; only 1 family with mutations in MADD (OMIM: 603584) was nonconsanguineous. The disease-causing variants in ALS2 (OMIM: 606352), ITSN1 (OMIM: 602442), RAB3GAP1 (OMIM: 602536), RAB3GAP2 (OMIM: 609275), and DENND2A (HGNC: 22212) were inherited in homozygous pattern while inheritance of disease-causing variants in IQSEC2 (OMIM: 300522), TRIO (OMIM: 601893), and MADD was hemizygous, heterozygous, and compound heterozygous, respectively. In two families of our cohort which included seven patients, two disease-causing variants in ALS2 including a missense variant, p.(Y1607H) located in VPS9 domain (family 1) and a canonical splice site variant, c.1640+1G>A located in RCC1 domain (family 2) were identified. Helal et al. (2018) reported that c.1640+1G>A variant is located on a common haplotype indicating a founder mutation. Although, patients in this study did not show any cognition problems, we linked the ALS2 variants to ID phenotype for the first time in our previous study (Hu et al., 2019). Two males with moderate ID in family 3 had a novel hemizygous missense variant, p.(R1122C), in IQSEC2 gene. Mutations in IQSEC2 have been previously associated with ID, autism spectrum disorder (ASD), and epilepsy (Barrie et al., 2020; Levy et al., 2019; Shoubridge et al., 2010; Zerem et al., 2016). We also identified compound heterozygosity for the MADD gene with a frameshift, p.(M1187X), and a missense variant, p.(P354L), in two affected females in a family with non-related parents. Interestingly, compound heterozygous variants in MADD have been reported in several cases (Table S1) (Anazi et al., 2017; Schneeberger et al., 2020). In family 5, we indicated a splice site variant in ITSN1 as a novel ARID gene in a patient with mild ID. However, Feliciano et al. (2019), had reported ITSN1 variants in six patients with ASD.

Furthermore, we describe two families with symptoms characterizing Warburg micro syndrome 1 (WARBM1). Our analysis revealed a missense variant in RAB3GAP1 and a splice site variant in RAB3GAP2 in these families. Several previous studies demonstrated numerous pathogenic variants in these genes associated with WARBM1 and Marfson syndromes, with ID as the major phenotype of these disorders (Table S1) (Abdel-Hamid et al., 2020; Aligianis et al., 2005; Koparir et al., 2019). We also report two variants in two sporadic families with consanguineous marriages including a de novo frameshift variant, p.(Val-1698LeufsTer61) in TRIO in an affected male (family 8) and an inherited missense variant, p.(Arg930Trp), located at dDENN domain of a novel ARID gene, DENND2A, in a female with nonsyndromic ID. Several WES studies have identified numerous pathogenic de novo variants in TRIO in different cohorts of patients with neurodevelopmental disorders, such as ID and/or ASD (Ba et al., 2016; Sadybekov, Tian, Arnesano, Katritch, & Herring, 2017). The spectrum of identified variants and characteristics of families with disease-causing variants in eight GEF genes observed in our cohort are collected in details in Table 1.

### 3.2 | Phenotype description

From all 20 affected subjects, 12 patients (60%) were females and eight subjects (40%) were males. All patients had ID and/or DD with four patients showing severe ID, seven patients with moderate and eight patients having mild ID. Fourteen affected subjects (70%) showed inability to walk and 12 patients (60%) manifested speech disorder. Seven individuals had abnormal skull morphology including microcephaly, macrocephaly, and acrocephaly and 10 affected (50%) had upper motor neuron dysfunction including spasticity and spastic tetraplegia. The other clinical manifestations and pedigrees of affected subjects in the present study are listed in Table 2 and Figure S1 (Fig A through I).

Patients with disease-causing variants in eight GEF genes in our cohort, show some similarities in clinical manifestations compared to cases in the literatures (Tables S1 and S2). In Table 3, we summarized all molecular frequencies and clinical characteristics of 219 ID/DD cases with disease-causing variants in six GEF genes. Because of the low amount of studies on ITSN1 and DENND2A genes, their data are not shown in Table 3. In addition to ID and/or DD, speech disorder and inability to walk were among other frequently seen neurological features. Developmental regression was only seen in most patients with ALS2 or IQSEC2 variants. Overall, upper motor neuron dysfunction (e.g. spasticity, spastic tetraplegia, and spastic tetraparesis) was seen more frequently in patients with ALS2, RAB3GAP1, or RAB3GAP2 disease-causing variants. Besides, seizure was common in patients with variants in IQSEC2, MADD, or RAB3GAP1. Stereotypic behavior was common in cases with variants in IQSEC2 or TRIO and optic nerve atrophy/hypoplasia was more frequently seen in patients with RAB3GAP1 or RAB3GAP2 variants. In relation to growth abnormality category, short stature was obviously higher in cases with variants in RAB3GAP1 or RAB3GAP2, while failure to thrive was more common in patients with MADD variants. Microcephaly was more common in patients with variants in RAB3GAP1, RAB3GAP2, or TRIO and was a rare feature in patients with ALS2 variant. Eye abnormalities such as congenital cataract, microphthalmia, and microcornea were only observed in patients with RAB3GAP1 or RAB3GAP2 variants while nystagmus and/or strabismus was more common in patients with ALS2 or IQSEC2 variants. Dysmorphic
| Family N | Gene name | Affected/sex | Ethnicity/parental relationship | Zygosity/inheritance | Variation | Mutation type | ACMG classification | Protein domain |
|----------|-----------|--------------|-------------------------------|---------------------|-----------|--------------|-------------------|-----------------|
| 1        | ALS2      | 3/3 F        | Persian/Second cousin         | Homo/AR             | Chr 2: 202569196A>G; c.4819T>C (p.Y1607H) (NM_020919.4) | Missense       | Likely Pathogenic (PM1, PM2, PP1, PP3, BP1) (Hu et al., 2019) | VPS9 |
| 2        | ALS2      | 4/2 M, 2 F   | Arab/First cousin             | Homo/AR             | Chr 2: 202612252C>T; c.1640+1G>A (NM_020919.4) | Splice site    | Pathogenic (PVS1, PM2, PP3) (Hu et al., 2019) | RCC1 |
| 3        | IQSEC2    | 2/2 M        | Persian/First cousin          | Hemi/XLR            | Chr X: 53265591G>A; c.3364C>T (p.R1122C) (NM_001111125.3) | Missense       | Likely pathogenic (PM1, PM2, PP1, PP3) (Hu et al., 2019) | - |
| 4        | MADD      | 2/2 F        | Persian/non related           | Compound Hetero/AR  | Chr 11: 47317089delA; c.3559del A (p.M1187X) (NM_003682.4) | Frameshift and missense | Pathogenic (PVS1, PM2, PP3, PP5)/Likely pathogenic (PM1, PM2, PP3, PP5) (Hu et al., 2019) | -/DENN |
| 5        | ITSN1     | 3/2 F, 1 M   | Zaboli/First cousin           | Homo/AR             | Chr 21: 35107347A>G; c.186-2A>G (NM_003024.3) | Splice site    | Pathogenic (PVS1, PM2, PP3) (Hu et al., 2019) | EH domain |
| 6        | RAB3GAP1  | 3/2 F,1 M    | Persian/First cousin          | Homo/AR             | Chr 2: 135892885T>C; c.1550T>C (p.L517P) (NM_001172435.2) | Missense       | Uncertain Significance (PM2, PP1, PP3, BP1) (Hu et al., 2019) | - |
| 7        | RAB3GAP2  | 1/1 M        | Persian/First cousin          | Homo/AR             | Chr 1: 220368212T>C; c.961-2A>G (NM_012414.4) | Splice site    | Pathogenic (PVS1, PM2, PP3) | RAB3GAP2_N |
| 8        | TRIO      | 1/1 M        | Persian/Consanguine           | Hetero/De novo      | Chr 5: 14420018_14420028delGGTACGCGAGCAinsTCTGGTGCGGACC; c.5091_5101delinsTCTGGTGCGGACC (p.Val1698LeufsTer61) (NM_003682.4) | Frameshift     | Pathogenic (PVS1, PM2, PP3) | - |
| 9        | DENND2A   | 1/1 F        | Lur/First cousin              | Homo/AR             | Chr 7: 140221778G>A; c.2788C>T (p.Arg930Trp) (NM_015689.4) | Missense       | Uncertain Significance (PM1) (Kahrizi et al., 2019) | dDENN |

*aGenBank reference sequence: ALS2: NG_008775.1; IQSEC2: NG_021296.2; MADD: NG_029462.1; ITSN1: NG_029504.1; RAB3GAP1: NG_016972.1; RAB3GAP2: NG_015837.2; TRIO: NG_052962.1; DENND2A: NC_000007.14.*
Facial features were common in all cases, however, it was not reported in patients with ALS2 variants. Genital anomalies such as cryptorchidism and hypogenitalism were only recorded in cases with variants in MADD, RAB3GAP1, or RAB3GAP2.

Hypotonia and muscle weakness were more frequently documented in all patients, except for cases with TRIO variants (11.7%). Other muscular abnormalities such as muscle atrophy and decreased muscle mass, were only reported in patients with ALS2 variants (56%). In total, behavioral abnormalities were most common in patients with IQSEC2 and TRIO disease-causing variants.

Brain MRI abnormalities were more frequently documented than other brain MRI abnormalities.

### TABLE 2 Classification and frequency of clinical and radiological data of 20 patients with disease-causing variants in eight GEF genes in our cohort

| Family N | Gene name | Diagnosis | Clinical phenotype |
|----------|-----------|-----------|--------------------|
| 1        | ALS2      | HSP with ID | Mild ID (HP:0001256), Slight psychomotor delay (HP:0001263), Inability to walk (HP:0002540), Stuttering (HP:0025268), Spasticity (HP:0001257), Spastic tetraplegia (HP:0002510) |
| 2        | ALS2      | HSP with ID | Mild ID (HP:0001256), Seizure (HP:0001250), Inability to walk (HP:0002540), Spasticity (HP:0001257), Stuttering (HP:0025268), Strabismus (HP:0000486) |
| 3        | IQSEC2    | Nonsyndromic ID | Moderate ID (HP:0002342), Macrocephaly (HP:0000256); +4SD and +5SD, Long face (HP:0000276) |
| 4        | MADD      | Nonsyndromic ID | Moderate ID (HP:0002342), Macrocephaly (HP:0000256); +4SD and +5SD, Long face (HP:0000276) |
| 5        | ITSN1     | Nonsyndromic ID | Severe ID (HP:0010864), History of psychomotor delay (HP:0001263), Microcephaly (–2SD) (HP:0000252); Their OFCs were 51 (–2SD), 50 (–5SD), and 49 cm (–5SD), Cranial MRI in one affected individual showed agenesis of corpus callosum (HP:0001274) with Lateral and temporal ventriculomegaly (HP:0002119), spasticity (HP:0001257), Increased deep tendon reflexes (HP:0001347), Inability to walk (HP:0002540), Absent speech (HP:0001344), Poor eye contact (HP:0000817), No social interaction (HP:0008763) |
| 6        | RAB3GAP1  | Warburg micro syndrome 1 | Moderate ID (HP:0002342), Congenital cataract (HP:0000519), Optic atrophy (HP:0000648), Ataxia (HP:0001251), Inability to walk (HP:0002540) |
| 7        | RAB3GAP2  | Warburg micro syndrome 1 | Developmental delay (HP:0001263), Delayed speech and language development (HP:0000750), Inability to walk (HP:0002540), Large forehead (HP:0002003), Long face (HP:0000276), Acrocephaly (HP:0000263), Congenital cataract (HP:0000519), Hypotonia (HP:0001252), Nystagmus (HP:0000639), Cryptorchidism (HP:0000028), Anemia (HP:0001903), Muscle weakness (HP:0001324), Cortical atrophy (HP:0002120) |
| 8        | TRIO      | Nonsyndromic ID | Mild ID (HP:0001256), Microcephaly (–2SD) (HP:0000252); The OFCs was 50 cm (–2.9 SD), Hyperactivity (HP:000752), Aggression (HP:0000718), Attention deficit hyperactivity disorder (ADHD) (HP:0007018), Kyphosis (HP:0002808) |
| 9        | DENND2A   | Nonsyndromic ID | Severe ID (HP:0010864), Seizure (HP:0001250), Absent speech (HP:0001344), Restlessness (HP:0000711), Triangular face (HP:0000325), Hypertelorism (HP:0000316) |

*GenBank reference sequence: ALS2: NG_008775.1; IQSEC2: NG_021296.2; MADD: NG_029462.1; ITSN1: NG_024504.1; RAB3GAP1: NG_016972.1; RAB3GAP2: NG_015837.2; TRIO: C_000005.10; DENND2A: NC_000007.14.

### DISCUSSION

Considering the importance of small GTPases and therefore GEFs in different cellular processes from proliferation to cell migration, these proteins have highly conserved domains which are crucial for their functions (Bos et al., 2007; Ozturk & Kinzy, 2008). As the expression profiles of these proteins are expanded to different types of cells and tissues, alterations in their functions can lead to various clinical phenotypes. For instances, many studies have indicated that mutations of GEF genes are linked...
| Molecular and clinical characteristics | ALS2* (25 cases) | IQSEC2* (54 cases) | MADD* (27 cases) | RAB3GAP1* (49 cases) | RAB3GAP2* (30 cases) | TRIO* (34 cases) |
|--------------------------------------|------------------|--------------------|------------------|---------------------|---------------------|------------------|
| **Variant frequency**                |                  |                    |                  |                     |                     |                  |
| Missense                            | 3 (12%)          | 21 (38.8%)         | 18 (66.6%)       | 8 (16.3%)           | 5 (16.6%)           | 21 (61.7%)       |
| Nonsense                             | 1 (4%)           | 6 (11.1%)          | 13 (48.1%)       | 14 (28.5%)          | 14 (46.6%)          | 3 (8.8%)         |
| Frameshift                           | 6 (24%)          | 18 (33.3%)         | 5 (18.5%)        | 18 (36.7%)          | 5 (16.6%)           | 10 (29.4%)       |
| Splice site                          | 15 (60%)         | 1 (1.8%)           | 6 (22.2%)        | 8 (16%)             | 6 (19.3%)           | 0 (0%)           |
| Del/ins                              | 0 (0%)           | 2 (3.7%)           | 0 (0%)           | 5 (10%)             | 1 (3.2%)            | 0 (0%)           |

**Growth**

|                                | ALS2* (25 cases) | IQSEC2* (54 cases) | MADD* (27 cases) | RAB3GAP1* (49 cases) | RAB3GAP2* (30 cases) | TRIO* (34 cases) |
|--------------------------------|------------------|--------------------|------------------|---------------------|---------------------|------------------|
| Short stature (≤ -2SD);         | 0P/14N/11NA (0%) | 1P/12N/42NA (1.8%) | 0P/2N/25NA (0%)  | 22P/11N/16NA (44.8%)| 6P/11N/13NA (20%)  | 2P/21N/11NA       |
| Failure to thrive (HP:0001508)  | 0P/0N/25NA (0%)  | 3P/0N/51N (5.5%)   | 18P/7N/2NA (66.6%)| 8P/1N/40NA (16.3%) | 1P/0N/29NA (3.3%)  | 3P/0N/31NA (8.8%) |

**Head and neck**

|                                | ALS2* (25 cases) | IQSEC2* (54 cases) | MADD* (27 cases) | RAB3GAP1* (49 cases) | RAB3GAP2* (30 cases) | TRIO* (34 cases) |
|--------------------------------|------------------|--------------------|------------------|---------------------|---------------------|------------------|
| Microcephaly (HP:0000252)      | 1P/18N/6NA (4%)  | 10P/16N/28NA (18.5%)| 4P/13N/10NA (14.8%)| 46P/0N/3NA (93.8%) | 23P/5N/2NA (76.6%) | 14P/16N/4NA       |
| Other abnormal skull morphology (HP:0000929) | 0P/19N/6NA (0%) | 8P/22N/24NA (14.8%) | 2P/15N/10NA (7.4%) | 0P/45N/4NA (0%) | 5P/23N/2NA (16.6%) | 8P/22N/4NA (23.5%) |
| Nystagmus (HP:0000639)/Strabismus (HP:0000486) | 5P/3N/17NA (20%) | 15P/8N/31NA (27.7%) | 1P/2N/24NA (3.7%) | 0P/2N/47NA (0%) | 2P/2N/28NA (6.6%) | 2P/8N/24NA (5.8%) |
| Congenital cataract (HP:0000519) | 0P/0N/25NA (0%) | 0P/0N/54NA (0%) | 0P/0N/27NA (0%) | 49P/0N/0NA (100%) | 28P/0N/2NA (93.3%) | 0P/0N/34NA (0%) |
| Microphthalmia (HP:0000568)     | 0P/0N/25NA (0%) | 0P/0N/54NA (0%) | 0P/0N/27NA (0%) | 35P/8N/6NA (71.4%) | 23P/4N/3NA (76.6%) | 0P/0N/34NA (0%) |
| Microcornea (HP:0000482)        | 0P/0N/25NA (0%) | 0P/0N/54NA (0%) | 0P/0N/27NA (0%) | 29P/11N/8NA (59.1%) | 16P/6N/8NA (53.3%) | 0P/0N/34NA (0%) |
| Dysmorphic facial features (HP:0001999) | 0P/0N/24NA (0%) | 23P/11N/20NA (42.5%) | 23P/1N/3NA (85.1%) | 33P/4N/12NA (67.3%) | 14P/0N/16N (46.6%) | 23P/3N/8NA (67.6%) |

**Genital**

|                                | ALS2* (25 cases) | IQSEC2* (54 cases) | MADD* (27 cases) | RAB3GAP1* (49 cases) | RAB3GAP2* (30 cases) | TRIO* (34 cases) |
|--------------------------------|------------------|--------------------|------------------|---------------------|---------------------|------------------|
| Cryptorchidism (HP:000028)     | 0P/0N/25NA (0%) | 0P/0N/54NA (0%) | 11P/12N/4NA (40.7%) | 18P/18N/13NA (36.7%) | 9P/8N/13NA (30%) | 0P/0N/34NA (0%) |
| Hypogenitalism (HP:0003241)    | 0P/0N/25NA (0%) | 0P/0N/54NA (0%) | 7P/16N/4NA (25.9%) | 30P/6N/13NA (61.2%) | 14P/3N/13NA (46.6%) | 0P/0N/34NA (0%) |

**Skeletal**

|                                | ALS2* (25 cases) | IQSEC2* (54 cases) | MADD* (27 cases) | RAB3GAP1* (49 cases) | RAB3GAP2* (30 cases) | TRIO* (34 cases) |
|--------------------------------|------------------|--------------------|------------------|---------------------|---------------------|------------------|
| Scoliosis (HP:0002650)/Kyphoscoliosis (HP:0002751) | 6P/18N/1NA (24%) | 11P/25N/18NA (20.3%) | 4P/2N/21NA (14.8%) | 7P/17N/25NA (14.2%) | 1P/1N/28NA (3.3%) | 11P/15N/8NA (32.3%) |
| Molecular and clinical characteristics | ALS2 (25 cases) | IQSEC2 (54 cases) | MADD (27 cases) | RAB3GAP1 (49 cases) | RAB3GAP2 (30 cases) | TRIO (34 cases) |
|---------------------------------------|----------------|------------------|----------------|---------------------|---------------------|----------------|
| Abnormality of limbs (HP:0040064) and Abnormality of joint mobility (HP:0011729) | 0P/0N/25NA (0%) | 1P/7N/46NA (1.8%) | 0P/0N/27NA (0%) | 6P/0N/43NA (12.2%) | 4P/0N/26NA (13.3%) | 9P/0N/29NA (26.4%) |
| Neurologic | | | | | | |
| Intellectual disability (Total) HP:0001249 | 13P/0N/12NA (52%) | 49P/0N/5NA (90.7%) | 12P/0N/15NA (44.4%) | 48P/0N/1NA (97.9%) | 25P/0N/5NA (83.3%) | 31P/2N/1NA (91.1%) |
| Global developmental delay HP:0001263 | 15P/6N/4NA (60%) | 36P/1N/17NA (66.6%) | 24P/2N/1NA (88.8%) | 40P/0N/9NA (81.6%) | 30P/0N/0NA (100%) | 33P/0N/1NA (97%) |
| Developmental regression HP:0002376 | 11P/14N/0NA (44%) | 29P/13N/12NA (53.7%) | 0P/23N/4NA (0%) | 0P/23N/26NA (0%) | 0P/1N/29NA (0%) | 0P/0N/34NA (0%) |
| Seizures HP:0001250 | 4P/4N/17NA (16%) | 44P/6N/4NA (81.4%) | 16P/9N/2NA (59.2%) | 11P/29N/9NA (22.4%) | 3P/19N/8NA (10%) | 6P/21N/7NA (17.6%) |
| Speech disorder HP:0002167 | 25P/0N/0NA (100%) | 46P/1N/7NA (85.1%) | 22P/1N/4NA (81.4%) | 34P/0N/15NA (69.3%) | 28P/2N/0NA (93.3%) | 31P/0N/3NA (91.1%) |
| Inability to walk HP:0002540 | 25P/0N/0NA (100%) | 27P/5N/22NA (50%) | 22P/3N/2NA (81.4%) | 43P/0N/6NA (87.7%) | 28P/0N/2NA (93.3%) | 11P/0N/23NA (32.3%) |
| Upper motor neuron dysfunction HP:0002493 | 25P/0N/0NA (100%) | 5P/22N/27NA (9.2%) | 0P/3N/25NA (0%) | 35P/2N/12NA (71.4%) | 26P/3N/1NA (86.6%) | 4P/0N/30NA (11.7%) |
| Optic nerve atrophy HP:0000648/Optic Nerve hypoplasia HP:0000609 | 0P/0N/25NA (0%) | 1P/0N/53NA (1.8%) | 0P/0N/27NA (0%) | 31P/9N/9NA (63.2%) | 16P/8N/6NA (53.3%) | 2P/0N/32NA (5.8%) |
| Stereotypy HP:0000733 | 0P/7N/18NA (0%) | 23P/5N/26NA (42.5%) | 1P/0N/26NA (3.7%) | 0P/0N/49NA (0%) | 0P/0N/30NA (0%) | 6P/15N/13NA (17.6%) |
| Musculature | | | | | | |
| Hypotonia HP:0001252/Muscle weakness HP:0001324 | 18P/0N/7NA (72%) | 25P/11N/18NA (46.2%) | 20P/3N/4NA (74%) | 22P/23N/4NA (44.8%) | 16P/0N/14NA (53.3%) | 4P/2N/28NA (11.7%) |
| Abnormality of the musculature HP:0003011 | 14P/4N/7NA (56%) | 0P/2N/52NA (0%) | 0P/0N/27NA (0%) | 0P/0N/49NA (0%) | 0P/0N/30NA (0%) | 0P/0N/34NA (0%) |

(Continues)
| Molecular and clinical characteristics | ALS2* (25 cases) | IQSEC2* (54 cases) | MADD* (27 cases) | RAB3GAP1* (49 cases) | RAB3GAP2* (30 cases) | TRIO* (34 cases) |
|--------------------------------------|-----------------|--------------------|------------------|----------------------|----------------------|------------------|
| Behavioral                           |                 |                    |                  |                      |                      |                  |
| Hyperactivity (HP:0000752)/Attention  | 0P/0N/25NA (0%) | 6P/4N/44NA (11.1%) | 2P/0N/25NA (7.4%)| 0P/0N/49NA (0%)     | 0P/0N/30NA (0%)     | 5P/1N/29NA (14.7%)|
| deficit hyperactivity disorder (ADHD)|                 |                    |                  |                      |                      |                  |
| (HP:0007018)                         |                 |                    |                  |                      |                      |                  |
| Autistic behavior (HP:0000729)       | 0P/0N/25NA (0%) | 24P/11N/19NA (44.4%)| 2P/0N/25NA (7.4%)| 0P/0N/49NA (0%)     | 0P/0N/30NA (0%)     | 6P/10N/18NA (17.6%)|
| Aggressive behavior (HP:000718)/Self-| 0P/14N/11NA (0%)| 14P/17N/23NA (25.9%)| 8P/17N/2NA (29.6%)| 0P/0N/49NA (0%)     | 0P/0N/30NA (0%)     | 12P/13N/9NA (35.2%)|
| injurious behavior (HP:0100716)/Self-|                 |                    |                  |                      |                      |                  |
| mutilation (HP:000742)               |                 |                    |                  |                      |                      |                  |
| Drooling (HP:0002307)                | 5P/6N/14NA (20%)| 9P/5N/40NA (16.6%) | 0P/0N/27NA (0%)  | 0P/0N/49NA (0%)     | 0P/0N/30NA (0%)     | 2P/15N/13NA (5.8%)|
| Brain MRI                            |                 |                    |                  |                      |                      |                  |
| White matter abnormalities (HP:0002500)| 1P/7N/17NA (4%) | 3P/33N/18NA (5.5%) | 0P/0N/27NA (0%)  | 27P/13N/9NA (55.1%) | 14P/10N/6NA (46.6%)| 1P/3N/30NA (3%)   |
| Abnormal corpus callosum f            | 0P/7N/18NA (0%) | 1P/35N/18NA (1.8%) | 0P/0N/27NA (0%)  | 39P/2N/8NA (79.5%)  | 20P/7N/3NA (66.6%) | 2P/2N/30NA (5.8%)|
| Abnormal cerebral morphology (HP:0002060) | 1P/6N/17NA (4%) | 13P/23N/18NA (24%)| 0P/0N/27NA (0%)  | 24P/17N/8NA (48.9%) | 12P/14N/4NA (40%)  | 0P/4N/30NA (0%)   |
| Other MRI finding h                   | 0P/7N/18NA (0%) | 14P/21N/19NA (25.9%)| 0P/0N/27NA (0%)  | 35P/6N/8NA (71.4%)  | 18P/7N/3NA (60%)   | 3P/1N/30NA (8.8%) |

Abbreviations: N, negative; NA,: not available; P, positive.

*Including: Macrocephaly (HP:0000929), Acrocephaly (HP:0000256), Brachycephaly (HP:0000248), and Plagiocephaly (HP:0001357).

*Including: Micrognathia (HP:0000347), Large ears (HP:0000400), Long palpebral fissure (HP:0000637), Abnormal facial shape (HP:0001999) such as long and narrow face, broad or narrow forehead and ...

*Also including assisted walk, walk with support & difficulty walking.

*fUpper motor neuron dysfunction including: spasticity (HP:0001257), spastic tetraplegia (HP:0002510)/spastic tetraparesis (HP:0001285)/spastic diplegia (HP:0001264)/spastic paraparesis (HP:0002313).

*Including: Myopathy (HP:0003198), Muscle atrophy (HP:0003202), and Decreased muscle mass (HP:0003199).

**Hydropsia of the corpus callosum (HP:0002079)/Abnormal corpus callosum morphology (HP:0001273)/Thin corpus callosum (HP:0033725).

**Cerebral degeneration (HP:0007313)/Cerebral atrophy (HP:0002059)/Cerebral cortical atrophy (HP:0002120).

**Polymicrogyria (HP:0002126), Delayed myelination (HP:0012448), Ventriculomegaly (HP:0002119).

*GenBank reference sequence: ALS2: NG_008775.1; IQSEC2: NG_021962.2; MADD: NG_024462.1; ITS1: NG_029504.1; RAB3GAP1: NG_016972.1; RAB3GAP2: NG_015837.2; TRIO: C_000005.10; DENND2A: NC_000007.14.
to a broad range of disorders, such as neurodegenerative disorders, neurodevelopmental disorders (NDD), and especially intellectual disability (ID). It is thought that the pathogenic effects of these mutations are mostly caused as a result of interruptions and/or malfunctions in different cellular processes (Droppelmann et al., 2014).

In the current study, we present the molecular and phenotypic spectrum associated with mutations in GEF genes on patients from a large Iranian cohort of families with ID and/or DD. We described 20 patients from nine families with identified disease-causing variants in eight GEF genes. In Table S1, we presented data from 157 families affected by ID and/or DD, harboring variants in eight GEF genes. Sixty-five out of the 157 families presented in this study were consanguineous. The major clinical findings in all patients were speech disorder (85.2%), ID (81.6%), DD (81.1%), inability to walk (71.3%), abnormalities in skull morphology (55.6%; microcephaly: 45.2% and other abnormalities: 10.3%), facial dysmorphisms (52.4%), hypotonia and muscle weakness (47%), brain MRI abnormalities (43.4%), seizures (38.1%), and spasticity (35.4%). The distribution of ID severity was as follow; 46.6% of cases were classified as having severe to profound ID, followed by 15.2% of cases with moderate, and 14.3% with mild ID. Furthermore, the most common brain imaging findings were abnormalities in corpus callosum morphology (65% of cases with brain abnormalities, Table S2). The spectrum of clinical manifestations in all cases is presented in Table S2. Here, we discuss the molecular and phenotypic spectrum of 223 ID and/or DD patients with disease-causing variants in eight GEF genes (203 cases from the literatures and 20 cases from our cohort).

### 4.1 | ALS2

All affected subject with ALS2 variants showed mild ID (Table S2). Until now, more than 50 different pathogenic missense, nonsense, frameshift, and splice site variants in ALS2 have been reported in ALS2-related disorders (Flor-de-Lima, Sampaio, Nahavandi, Fernandes, & Leão, 2014). ALS2 encodes the alsin protein which acts as a GEF protein for Rab5, a GTPase which in turn regulates endosomal trafficking, postsynaptic development, and neuronal survival (Otomo et al., 2003). Pathogenic variants in ALS2 can lead to neurological conditions resulting from regressive degeneration of the upper motor neurons in the pyramidal tracts. ALS2-related disorders include infantile ascending hereditary spastic paraplegia (IAHSP), juvenile primary lateral sclerosis (JPLS), and juvenile amyotrophic lateral sclerosis (JALS) (Flor-de-Lima et al., 2014; Orban, Devon, Hayden, & Leavitt, 2007; Silani et al., 2020). Most patients with pathogenic variants in ALS2 have normal development and cognitive function. However, some cases with ID and/or DD were reported in patients described here. Interestingly, from all 25 affected subject with variants in ALS2, 15 cases from four consanguineous Iranian families had a specific splice site variant (c.1640+1G>A) which represents a founder mutation with specific phenotype (Helal et al., 2018; Hu et al., 2019). Three cases showed a missense variant in VPS9 domain of alsin protein. This domain catalyzes GEF on Rab5 protein and also plays a vital role in endosomal localization of the alsin protein (Carney, Davies, & Horazdovsky, 2006; Otomo et al., 2003).

### 4.2 | IQSEC2

Variants in IQSEC2, mostly missense and frameshifts, have been reported in 54 ID patients (Table 3 and Table S1). Of these patients, 57.4%, 24%, and 3.7% had severe to profound, moderate, and mild ID, respectively (Table S2). Despite the presence of epileptic encephalopathy (EE) in only 13% of patients described here, Zerem et al., (2016) indicated that EE is a main clinical feature occurring in more than 60% of sporadic cases. In addition, Radley et al. (2019) reported that 64.2% of patients with IQSEC2 variants had drooling, however, this phenotype was absent in other reported patients (Table S1). Autistic behavior with the frequency of 44.4%, followed by aggressive and self-injurious behavior reported in 25.9% of affected cases were the other common phenotypes, although some studies indicate that the latter phenotype occurs in most cases (Table S1) (Ewans et al., 2017; Shoubridge et al., 2010).

Furthermore, short stature was reported in only one patient with IQSEC2 variant (Barrie et al., 2020). Failure to thrive, was also observed in three patients, reported in two different studies (Barrie et al., 2020; Gandomi et al., 2014). About 44.4% of all cases showed structural brain abnormalities, which was in line with previous studies (Alexander-Bloch, McDougle, Ullman, & Sweetser, 2016; Gandomi et al., 2014; Radley et al., 2019). Moreover, abnormalities of the cerebral morphology were more frequently reported (24%) than other brain abnormalities in these patients (Table 3). The incidence of facial dysmorphic phenotypes was reported more variably from high rates in some studies to lower frequency in other studies (Gilissen et al., 2014; Olson et al., 2015; Zerem et al., 2016). Dysmorphic facial features including long palpebral fissure, synophrys, high forehead, short philtrum, hypoplastic midface, micrognathia, and large ears, were present in the average frequency of 42.5% of patients.

IQSEC2 plays an important role in membrane trafficking and synaptic transmission via GEF activity on specific
Arfs (ADP ribosylation factors) such as ARF1 and ARF6. Several Studies have reported that most pathogenic mutations of IQSEC2 are located at functional domains of this gene such as Sec7 domain with GEF activity and IQ domain which accelerates GEF activity. It has been suggested that patients with mutations disrupting these domains usually manifest more severe phenotype (Ewans et al., 2017; Mignot et al., 2019; Radley et al., 2019; Shoubridge et al., 2010; Zerem et al., 2016).

4.3 | MADD

Variants in MADD gene were first shown to be associated with autosomal recessive ID in our previous study (Hu et al., 2019). Previous investigations revealed that MADD is able to act as a Rab3-GEF, Rab27-GEF, and Rab3 effector, thus it is involved in formation and trafficking of synaptic vesicles (Bae et al., 2016; Imai, Ishida, Fukuda, Nashida, & Shimomura, 2013; Yoshimura, Gerondopoulos, Linford, Rigden, & Barr, 2010). MADD is also involved in neuronal survival through antiapoptotic and proapoptotic functions of its different isoforms which is triggered by its interaction with type 1 tumor necrosis factor receptor (TNFR1) (Al-Zoubi et al., 2001; Del Villar & Miller, 2004). To date, a number of 27 patients with variants in MADD has been reported, with 15 cases (55%) of them showing compound heterozygous variants. Most patients with MADD variants reported by Schneeberger et al. (2020) manifested a wide spectrum of clinical phenotypes from DD to even multisystem disorders. In this study, craniofacial dysmorphism was reported in almost all of the cases. In total, 24 rare changes in MADD have been reported including 11 missense, 10 truncating, and three splice site variants, as well as one multi exon deletion. Of these, eight variants are clustered in the central DENN domain, three variants are located at Ser-rich domain, and one variant lies in death domain, while the remaining variants are located at regions of the protein, without affecting any known domain (Anazi et al., 2017; Hu et al., 2019; Schneeberger et al., 2020).

4.4 | RAB3GAP1 and RAB3GAP2

RAB3GAP is a heterodimeric complex containing a catalytic (RAB3GAP1) and a non-catalytic subunit (RAB3GAP2) acting as a RAB3GEF which is involved in exocytosis of hormones and neurotransmitters (Li & Chin, 2003; Takai, Sasaki, Shiratani, & Nakanishi, 1996). Numerous studies have demonstrated that mutations in RAB3GAP1 and RAB3GAP2 genes may lead to clinical phenotypes like WARBM1 and Martsolf syndromes (Abdel-Hamid et al., 2020; Derbert et al., 2004; Koparir et al., 2019; Tenawi, Al Khudari, & Alasmr, 2020). These rare autosomal recessive disorders are mostly characterized by abnormalities of eye and central nervous system, although Martsolf syndrome shows a milder phenotype (Martsolf, Hunter, Haworth, & Herrmann, 1978; Warburg, Sjö, Fledelius, & Pedersen, 1993). In our cohort, we identified two patients with WARBM1 syndrome carrying variants in these genes (Tables 1 and 2). We also presented data from other studies, which included 46 cases with RAB3GAP1 and 29 cases with RAB3GAP2 variants (Table 3; Tables S1 and S2).

The frequency of different ID types in these patients was as follow: 89.7% and 8.1% of patients with RAB3GAP1 variants had severe to profound and moderate ID, respectively while 43.3%, 23.3%, and 16.6% of cases with RAB3GAP2 variants showed severe to profound, moderate, and mild ID, respectively (Table S2). It is noteworthy that cases with WARBM1 syndrome mostly showed severe to profound ID while patients with Martsolf syndrome manifested variable phenotype mostly from mild to moderate ID, especially in cases with mutations in RAB3GAP2 (Abdel-Hamid et al., 2020; Aligianis et al., 2005; Handley et al., 2013; Xu et al., 2020).

Some clinical features occur at different frequencies between cases with variants in these genes. Some examples include; short stature (44.8% vs. 20%), failure to thrive (16.3% vs. 3.3%), microcephaly (93.8% vs. 76.6%), dysmorphic facial features (67.3% vs. 46.6%), scoliosis/kyphoscoliosis (14.2% vs. 3.3%), and seizures (22.4% vs. 10%). In addition to microcephaly, five patients with other abnormalities in skull morphology (one case with acrocephaly and four cases with brachycephaly) were reported in cases with RABGAP2 variants (Borck et al., 2011; Gumus, 2018; Hu et al., 2019). It is of note that no behavioral abnormalities were reported in all 79 patients with RAB3GAP variants. However, abnormal corpus callosum was observed at more prevalence in brain MRI imaging compared to other brain abnormalities (79.5% and 66.6% in cases with RAB3GAP1 and RAB3GAP2 variants, respectively). Polymicrogyria was also common in these cases (59.1% for RAB3GAP1 and 60% for RAB3GAP2 cases) while ventriculomegaly was reported in only six patients with RAB3GAP1 variants (Abdel-Hamid et al., 2020; Koparir et al., 2019), and three cases with RAB3GAP2 variants (Table S1) (Abdel-Hamid et al., 2020).

4.5 | TRIO

TRIO encodes a large protein which acts as a GEF protein for RHOA, RHOG, and RAC1 GTPases. Therefore, it is thought to serve a critical role in neurodevelopmental functions such
as neuronal migration, cortical axon growth, dendritic arborization, and synaptic motility or maintenance (Briançon-Marjollet et al., 2008; Debant et al., 1996; Hall & Lalli, 2010; Schmidt & Debant, 2014). Heterozygous mutations in TRIO have been associated with intellectual disability accompanied by microcephaly or macrocephaly (Ba et al., 2016; Barbosa et al., 2020; Pengelly et al., 2016). TRIO was first introduced as a candidate gene for ID by De Ligt et al. (2012), when two pathogenic de novo variants in this gene were identified in two unrelated cases with severe ID. In this study, we have collected 34 ID cases with variants in TRIO from seven different studies. Of these, 29.4% of patient were diagnosed with mild ID, 17.6% showed moderate ID, and 29.4% were reported to have severe ID (Table S2). Other abnormalities in skull morphology were reported only in two studies; Pengelly et al. reported a case with plagiocephaly and Barbosa et al. identified seven cases with macrocephaly (Table S1) (Barbosa et al., 2020; Pengelly et al., 2016). In these two recent studies, various dysmorphic facial features were also identified in patients with TRIO variants. These dysmorphic features included short or straight nose, broad nasal root, slightly bulbous nasal tip, micrognathia, low anterior hairline, facial asymmetry, large fleshy ears, protruding ears, synophrys, ptosis, and Angelman-like facies (Barbosa et al., 2020; Pengelly et al., 2016). In 4 out of 34 patients, at least one brain abnormality was observed in MRI imaging. These abnormalities comprised ventriculomegaly in three cases, abnormal corpus callosum morphology in two cases, and white matter abnormalities documented in one patient (Table S1) (Barbosa et al., 2020; De Ligt et al., 2012). TRIO contains two major catalytic domains, including GEF1 (activator of RHOG and RAC1) and GEF2 (activator of RHOA), and several accessory motifs such as Sec14 and spectrin-like repeats (spectrin repeats domain) at its N terminus region and a serine/threonine kinase domain at its C terminus region (Bellanger et al., 1998; Schmidt & Debant, 2014). In two studies by Pengelly and Barbosa et al, all cases with plagiocephaly (p.Asn1080Ile) and macrocephaly (p.Arg1078Trp), had missense variants located at spectrin repeats domain leading to increased RAC1 activation of TRIO protein (Barbosa et al., 2020; Pengelly et al., 2016). Moreover, most cases with missense heterozygous variants in spectrin repeats domain revealed moderate to severe ID. Besides, studies have shown that missense variants in GEF1 domain, decreases binding and activation of RAC1 and most cases with these variants are diagnosed with mild to moderate ID along with microcephaly (Ba et al., 2016; Barbosa et al., 2020; Katrancha et al., 2017; Pengelly et al., 2016; Schultz-Rogers, Muthusamy, Pinto e Vairo, Klee, & Lanpher, 2020). We noticed that 6 out of 34 cases (17.6%) with TRIO variants discussed here had seizures. Strikingly, seizures were only described in patients with variants in the spectrin domain (p.Arg1078Trp and p.Asn1080Ile) or with truncating mutations (p.Val2351Cysfs*62 and p.Phe2473Serfs*54) (Barbosa et al., 2020; Pengelly et al., 2016). However, the other clinical manifestations were observed across all mutational spectrum.

### 4.6 | ITSN1 and DENND2A

ITSN1 encodes Intersectin-1 protein which acts as a GEF for CDC42 and is involved in the assembly and maturation of clathrin-coated vesicles. It plays an importance role in synaptic vesicle endocytosis in brain neurons and regulates neuronal migration and synaptic plasticity in the hippocampus (Hunter, Russo, & O’Bryan, 2013; Jakob et al., 2017). We identified a homozygous variant in ITSN1 as a novel ARID gene in our cohort (Hu et al., 2019). Feliciano et al. (2019), reported variants in ITSN1 gene in patients with ASD and introduced this gene as an autism-related candidate gene. In their study, two cases with Asperger disorder and four cases with autism were reported with only one patient showing mild ID and the other cases having normal intelligence. Although, the clinical phenotypes of patients in our cohort were more severe than cases described by Feliciano et al., some shared features including autistic behaviors, poor eye contact, and no social interaction were also observed.

Similar to ITSN1, DENND2A is a GEF gene which activates RAB9A and RAB9B acting in trafficking among the trans-Golgi network (TGN) and late endosome (Yoshimura et al., 2010). However, the exact function of this gene especially in neuronal cells is yet to be identified. In our cohort, we presented an inherited missense variant in the main domain of DENND2A (dDENN) in a sporadic case with severe ID and suggested this gene as a novel ARID gene.

### 5 | CONCLUSION

Because of the importance of GEF proteins in the function of small G proteins and GTPases, they serve critical roles in neuronal functions such as neuronal survival, migration, axon growth, and guidance, as well as in synaptic formations including the regulation of the actin cytoskeleton, spine remodeling, and synaptic plasticity. Therefore, it is predictable that alterations in GEF genes may lead to neurodevelopmental disorders. Our study adds more evidence on phenotypic spectrum resulting from mutations in specific GEF genes and expands the genetic and phenotypic heterogeneity of GEF-related disorders.
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CONFLICT OF INTEREST
All authors declare no conflict of interest.

ETHICAL COMPLIANCE
All participants singed an informed consent form and the study was approved by the Ethics Committee of the University of Social Welfare and Rehabilitation Sciences (USWR).

DATA AVAILABILITY STATEMENT
The data supporting our findings in Iranian patients are openly available at doi: 10.1038/s41380-017-0012-2 and doi.org/10.1523/ENEURO.0031-16.2016. The data supporting our findings in Iranian patients are openly available at doi: 10.1038/s41380-017-0012-2 and doi.org/10.1523/ENEURO.0031-16.2016.

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