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Two Novel Biallelic Variants in TECPR2 and FA2H Genes Causing Complicated Hereditary Spastic Paraplegia in Iranian families from lur Ethnicity: Case Series.

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Abstract

We herein report first Iranian families with spastic paraplegia 35 and 49 and claim that TECPR2 gene causes complicated spastic paraplegia 49 with or without sensory autonomic neuropathy. In addition, we show how coexistence of SPG49 and griscelli syndrome can lead to misdiagnosis.

1 Introduction

Hereditary spastic paraplegia (HSPs) refers to a heterogeneous group of rare inherited neurodegenerative disorders. They are generally characterized by progressive and length-dependent degeneration of distal retrograde axons of the corticospinal tracts (CST) and posterior columns of the spinal cord.[1-3]Clinically, these conditions share the primary symptoms of progressive spasticity, hyperreflexia and mild weakness of the lower limbs in the "Pure" form. In the "complicated/complex" form, additional symptoms such as peripheral nerve involvement, extrapyramidal disturbances, cerebellar ataxia, polyneuropathy, cognitive impairment, optic atrophy and seizures might be added.[2, 4, 5]

So far, at least 76 clinical types of HSPs and around 80 corresponding genes with different patterns of inheritance have been reported.[1, 2]Almost, 21 HSP-associated genes involved in the autosomal recessive (AR) form of these disorders. One of the complicated HSPs with AR inheritance pattern is spastic paraplegia 35 (SPG35, MIM 612319), also known as fatty acid hydroxylase-associated neurodegeneration (FAHN). The condition is caused by pathogenic alterations in *FA2H* , located on chromosome 16q23.1. This gene encodes endoplasmic reticulum (ER) enzyme fatty acid 2-hydroxylase. The enzyme is a membrane-bound protein with NADPH-dependent mono oxygenase activity, converting free fatty acids to 2-hydroxy fatty acids (hFAs), which, subsequently are incorporated into membrane sphingolipids as essential components of myelin. These compounds show particular temporal expression pattern and are unessential in early development, but are required as the individual matures.[6-9]Considering the substantial role of the enzyme in the maintenance of the myelin sheath around neuronal axons, deficiency of its coding gene can manifest diverse demyelinating phenotypes such as dysmyelinating, leukodystrophy associated cognitive decline, dysarthria, spastic paraparesis with or without dystonia and neurodegeneration with brain iron accumulation (NBIA).[10-14]Although the frequency of *FA2H* mutations in patients with HSPs has been obscure at least in

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Asia, it was considered as the second most common subtype of AR-HSP.[5]SPG35 has early onset and shows heterogeneous neuroimaging patterns such as a variable degree of white matter lesions (WMLs), thin corpus callosum, cortical and cerebellar atrophy and iron accumulation in the globus pallidus.[4, 7, 10, 15]In addition, genetic alterations in *TECPR2* , tectonin beta-propeller repeat containing 2 (TECPR), have been reported with another complicated form of autosomal recessive HSP called Spastic Paraplegia 49 (SPG49).[16] This gene encodes a protein which contains two main domains, tryptophan-aspartic acid repeat (WD repeat) and TECPR, and plays a significant role in autophagy process.[17-19] The disorder characterized by developmental delay, generalized hypotonia, microcephaly, short stature and dysmorphic faces. Affected individuals evolved progressive spasticity in the lower body muscles. However, in 2016 three more affected individuals with additional autonomic-sensory neuropathy features have been introduced.[20] Therefore, Heimer et al reclassified disorder as a new subtype of hereditary sensory-autonomic neuropathy, adding controversy to the exact class of this disorder. So far, only three pathogenic variations have been reported in *TECPR2* , implying that SPG49 is a rare genetic disorder with unknown frequency and heterogenous phenotype.[16, 20]

We herein report two Iranian Lur families with typical features of HSPs. Subsequent genomics and bioinformatics analyses have suggested novel pathogenic variants in TECPR2 and FA2H genes.

2 CASE PRESENTATION

2.1 Patient A

Two affected siblings (shown in Figure 1) from first cousin Lur parents were referred to Madar Medical Genetics center. The proband was 3.7-year-old girl who was hospitalized immediately after delivery due to respiratory distress. Then, until age 1.5 she was repeatedly hospitalized because of recurrent respiratory infections and decreased consciousness, diagnosed as pneumonia. She had generalized hypotonia and moderate developmental delay since she has not acquired ambulation until the time of study, however, she was able to creep and crawl. She can stand up only by physical aid. The proband achieved limited level of communication, using few words to call her parents. Physical examination illustrated short stature, microcephaly, brachycephaly and synostotic trigonocephaly. The other clinical features include failure to thrive, short neck, dysmorphic face, triangular face, severe strabismus, myopia and amblyopia. The proband experienced three episodes of seizures following temperatures up to 40, phenobarbital administered in order to control seizures. She showed aggressive behavior and easy mood changes, injuring other infants by physically attacking them. Detailed clinical examination revealed specific facial dysmorphic features such as protruding ears, absent antihelical fold in left ear, sparce eyebrows, bulbus nose, wide nasal bridge, flat nasal tip as well as chubby cheeks. The auditory brain-stem responses (ABR) examination was normal at age 2. Furthermore, her parents complained about her increased appetite in addition to gastroesophageal refluxes. Neurological examination showed the extension of hallux during Babinski test, indicating upper motor neuropathy. However, unlike previously reported patients, no sign of sensory-autonomic neuropathy including sleep disturbances, apnea, decreased pain sensitivity, blood pressure, arrythmia was identified at the time of study. Laboratory findings indicated elevated levels of platelets and SGOT. The proband's 2-year-old sister represented with similar clinical features of the proband (Figure 1). Furthermore, the affected sisters had silver-gray hairs and partial white patches on their skin. The younger sister's hair was completely silvery or gray in majority of parts, while the proband's hair was considerably darker. Similarly, white eyelashes and light iris were observed in both sisters. The proband had family history of griscIli appearance since her father and two of aunts and uncles represented with similar appearance. Since both siblings had griscelli phenotype in addition to developmental delay and neurological regression, the disorder was mistakenly diagnosed as phenylketonuria (PKU).

2.2 Patient B

An 11-year-old male who exhibited a history of gait difficulties, frequent falls and clumsiness was requited to this study. The proband (Shown in Figure 2) was the first child born to healthy consanguineous parents from Lorestan province, Iran. The proband was born naturally and delivery was uneventful. However, the mother had a miscarriage history, and she was pregnant for a male fetus at the time of the study. The process of the

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childhood development was remarkable, since the proband was able to hold his neck, sit, communicate and walk at the estimated time. By the age of 4 years, the first sign of the disorder appeared as he represented with lower limb spasticity and gait difficulties. The proband's toes became spastic which led to fixed plantar flexion of the foot, indicating pes caus. Subsequently, he acquired motor difficulties including hyperreflexia, tremor and ataxia. Strabismus and poor vision were also observed in eye examination. The rapid progression of the disorder resulted in loss of previously acquired developmental milestones, leading to mild-sever cognitive decline, intellectual disability and progressive loss of ambulation. The family also complained about the proband's urinary urgency. Electromyography (EMG) and nerve conduction velocity (NCV) evaluations of skeletal muscles at the age of five, presented no evidence of myopathy or peripheral neuropathy. The magnetic resonance imaging (MRI) of brain and spinal cord disclosed very mild abnormal signal intensity in centrum semiovale, suggesting leukoencephalopathy or periventricular leukomalacia (PVL) (shown in Figure 2). With this regard, clinical findings proposed mild spastic paraplegia.

2.3 Molecular analysis

Whole-exome sequencing (WES) was implemented in Madar Medical Genetics Center, Khorramabad, Lorestan, Iran to identify the subtype of HSPs and the corresponding gene, due to heterogenous nature of the disorder. To this end, the genomic DNA of the probands and their relatives were extracted from peripheral blood based on an established salting out protocol.[21] Only the probands' samples were subjected to WES. For Patient B, the SureSelect Human All Exon V6 Kit (Agilent Technologies Inc., Santa Clara, CA, USA) was used to capture exonic region and paired-end sequencing was carried out on illumine NextSeq (Illumina Inc., San Diego, CA, USA). For patient A, Human Core Exome Kit (Twist Bioscience) was used for this purpose. The Burrows-Wheeler Aligner[22]was implemented to align the sequence reads to GRC38 human reference genome. GATK HaplotypeCaller[23, 24] tool was utilized to call all variants within the target regain and annotation was performed using ANNOVAR. At the next step, all variants with more than 0.01 alle frequency in 1000genome, genomAD exome and GenomAD genome were removed and the remaining variants were prioritized according to bioinformatics predictions, inheritance pattern and clinical information. Once WES data was analyzed, the co-segregation analysis of the disease-associated variant was done for understanding the inheritance pattern of the disorder. For this purpose, specific primers Table 1 were designed using online tools such as primer3 (https://primer3.ut.ee), oligoanalyzer (https://eu.idtdna.com/calc/analyzer) and ensembl databases (https://ensembl.org). After PCR amplification, Sanger sequencing was performed using Applied Biosystems 3500 Genetic Analyzer and the sequences were aligned to the reference genome by Codon Code Aligner (https://www.codoncode.com/aligner).

2.4 Genetic findings and co-segregation study

2.4.1 Patient A

In patient A, following WES, a frameshift variant defined as c.1568delC (NM_014844, 14:102434385 GCR38, p.S523Ffs*12) in exon 9 of*TECPR2* was identified. This variant was not found neither in GenomeAD, 1000genome, Iranome databases nor in our local database for 300 Lur individuals, being extremely rare. The c.1568delC variant was a single nucleotide deletion of cytosine, leading to a premature stop codon only 11 amino acids after deletion site (shown in Figure 1).*TECPR2* encodes 1411 amino acid length protein in canonical isoform (NM_014844), which contains three WD and ten TECPR domains. As a consequence of this frameshift mutation, approximately 63% of critical region of original protein is eliminated after translation, remaining only 37%. In addition, another homozygous deletion defined as c.1135_1136del (NM_024101, 2:237540378 GRC38, p.D379Cfs*19) in exon 10 of *MLPH* was detected in the proband. This mutation describes the griscIli appearance of affected siblings, since mutations in this gene have been reported with Griscelli syndrome, type 3. Genotyping family members including mother and siblings reveled that affected sister harbored c.1568delC variant in homozygote manner, while mother and the healthy elder daughter of the family were carriers (shown in Figure 1). However, father of the family was unavailable to participate in the study. As expected, two affected siblings were homozygote for c.1135_1136 deletion in *MLPH* , confirming the Griscelli syndrome, type 3 in the family.

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2.4.2 Patient B

As a consequence of WES, a homozygous three-nucleotide deletion in exon 5 of *FA2H* was identified. The c.685_687delATC mutation was a non-frameshift deletion of nucleotides 685 to 687 (NM_024306) which leads to deletion of a non-polar, uncharged amino acid Isoleucine at 229th residue. For understanding the pattern of the inheritance, the proband's relatives underwent co-segregation analysis. The results revealed heterozygotes status of parents and male fetus, suggesting an AR pattern of inheritance (shown in Figure 2). Since the pathogenicity of the mutation has not been reported in gene variant public databases, we applied several variant effect predictor websites to identify its potential pathogenicity. The predictors predicted the mutation according to ACMG parameters as a probably pathogenic variant (http://www.varsome.com/). Our analysis also showed that the nucleotides and their corresponding amino acid are highly conserved among different species (up to Chrysochloris Asiatica and considering 4 species) (shown in Figure 2).

3 Discussion

In the current study, we identified two novel pathogenic homozygous deletions in *TECPR2* and *FA2H* in two Iranian families, who were diagnosed with complicated spastic paraplegia. Patient A and her sibling were homozygous for two frameshift deletions in *TECPR2*and *MLPH* genes, while patient B was homozygous for non-frameshift mutation in *FA2H* . Regarding *TECPR2* , in 2012, it was the first time when a frameshift single nucleotide deletion (c.3416delT) in exon 16 of *TECPR2* was linked to a complicated form of HSP, named SPG49.[16] All five affected individuals in the study were from the same ethnic group and shared similar clinical features of generalized hypotonia, developmental delay, progressive spasticity in lower body, dysmorphic features, and recurrent respiratory infections. Since the fact that all individuals were from unrelated Jewish Bukharin families and harbored similar genetic variation, the c.3416delT mutation was considered as a founder mutation in that specific ethnic group. There had not been any TECPR2 related phenotype until 2016, when three affected individuals with c.C566T (p.Thr189Ile) and c.1319delT (p.Leu440Argfs*19) mutations in *TECPR2* were reported.[20] Despite having similar clinical features of previously reported individuals, all the patients showed extra clinical manifestations of autonomic neuropathy. The authors, as a conclusion, believed that this disorder should be classified as a form of HSAN not HSP, owing to the fact that none of complicated forms of HSP shows autonomic neuropathy. Interestingly, two affected siblings in current study represented with identical clinical manifestations to those who were previously reported in the 2012 study, showing no sign of autonomic neuropathy except having grescilli appearance due to c.1135_1136del genetic alteration in *MLPH* . Moreover, detailed clinical and paraclinical examinations revealed additional symptoms including elevated levels of platelets and SGOT in blood and triangular face. All in all, because of diverse multisystem signs and symptoms of this disorder, affecting autonomic neurons in limited individuals, we believe that it should still be classified as complicated SGP49 with or without sensory autonomic neuropathy. Another interesting point is that co-incidence of two monogenic disorders in an individual can sometimes misguide clinicians in diagnosis, as in our study, we initially misdiagnosed the disorder as PKU with combination of immunodeficiency.*TECPR2* plays a critical role in autophagy by associating with distinct cellular components such as *COPII* , *SEC24D* ,*HOPS* and *BLOC-1* .[19] The most possible role of *TECPR2* is that it probably acts as an anchor point for multiple cellular components. In one example, it stabilizes*SEC24D* to ensure the efficient function of endoplasmic reticulum in exporting several secretory elements. In general, more functional studies are required to throw light on the exact mechanism of*TECPR2* in neuronal cell development and maintenance.

Turning to *FA2H* , the detected variant was predicted as likely pathogenic, due to the protein changing effect of the deletion of conservative amino acid Isoleucine (p.I229del). *FA2H* contains seven exons and encodes an integral membrane enzyme of smooth endoplasmic reticulum (ER), which catalyzes galactosylceramide and sulfatide hydroxylation in the myelin sheath. The enzyme contains two conserved domains. First, is the N- terminus cytochrome b5-like heme-binding domain (residues 15–85), responsible for the redox activity of the enzyme. Second, is the sphingolipid fatty acid hydroxylase domain, spanning residues 124-366 in the C-terminal. The identified deletion is located in the latter domain. The domain is known as sterol desaturase domain, composed of a catalytic di-iron cluster and four transmembrane domains, mediating

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anchoring the enzyme to the ER membrane.[1, 4, 25]With this regard, the variant may interfere with the catalytic activity of the protein. In previous reports, several pathogenic alterations, which are scattered along the sterol desaturase domain, including p.V149L, p.L130F, p.R235C and p.H260Q, have been examined. The result indicated their pathogenic effects on the hydroxylase activity of the FA2H. The enzyme activity of p.V149L and p.R235C variants each separately was 60%-80%, p.L130F approximately 52% and p.H260Q nearly 0%.[5, 26, 27]However, additional consequences of *FA2H* variants in post transcriptional level, such as p. R154C variant, which, reduces the mRNA or protein stability should not be excluded. In such a case, western blot for measuring protein abundance and enzyme-linked immunosorbent assay (ELISA) or gas chromatography–mass spectrometry for quantifying the enzyme activity are applied.[10]Since we were unable to determine the enzyme activity and protein abundance in our patient, we predicted the variant's pathogenicity and its effect on protein structure in silico. Our findings showed on the surface that the mutation is pathogenic and leads to change of the helix structure of the enzyme to a moderate extent, proposing a possible role of the conserved amino acid Isoleucine in the catalytic activity. Further functional studies in terms of patient's cell culture or animal models are necessary to confirm our findings.

The SPG35 affected patients show a complicated genotype-phenotype correlation. Similar to the majority of cases with early onset spastic paraplegia (mean age of onset 5.76 years ±3.20; 36 patients out of 38[28]), our proband revealed early onset spastic paraplegia at age 2. Dystonia, with a high prevalence among the SPG35 patients (89.7%; 26 patients out of 29), was also observed in the proband. SPG35 is considered as a subtype of neurodegeneration with brain iron accumulation (NBIA).[10, 29]According to some research, iron accumulation is not always present in all of the patients, whilst the affected individuals share the same mutation.[10, 11]In our proband's MRI, no sign of brain iron deposition was found. This is suggestive of the phenotype being associated with iron accumulation as a consequence of FA2H variation seeming variable in the patients, or T2 MRI not being a fully conclusive technique for detection of iron deposition. However, progressing cognitive decline and epileptic seizures as typical symptoms of SPG35 which are seen in almost 90% and 30% of patients, respectively.[1, 30]

In summary, WES analysis allowed us to identify two homozygous deletions, p.I229del and c.1568delC in *F2AH* and *TECPR2*respectively, in two patients with symptoms of complicated spastic paraplegia. Nonetheless, functional studies in terms of enzyme testing, for assessing the enzyme activity in patients and also animal models are required to validate such a deduction. Further investigations are needed to precisely describe the range of phenotypes in the SPG35 and SPG49 cases in Iran.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

M. Edizadeh performed WES data analysis, variant interpretation, bio-informatics measurements and wrote the manuscript. N. Chegeninezhad performed WES, Sanger sequencing and co-segregation analysis. S. Akbari made clinical diagnosis. R. Pakmanesh designed primers and extracted DNA from peripheral blood samples. M. Salehirad performed PCR and Sanger sequencing. S. Ahmadipour made patient work-up and management. K. Hayatigolkhatmi wrote and revised the manuscript. H. Khodadadi supervised the study, revised and finalized the manuscript, supported the study financially.

ETHICAL APPROVAL

This study was approved by the ethics committee of Lorestan University of Medical Sciences (IR.LUMS.REC.1399.328) and adhered to tenets of the Declaration of Helsinki. Prior to study, written informed consent form was obtained from the participants' parents for publication of this study.

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Figure 1. Pedigree and molecular findings of family A. A: There are two affected siblings (IV-2 and IV-3) with clinical features of complicated hereditary spastic paraplegia, indicated with blue color. Proband's father, aunt and uncles (III-1,2,3) represented with Griscelli appearance, green color. C: The nucleotide and amino acid sequences in the mutation site (c.1568delC, p.S523Ffs*12) are shown. In normal protein, TCT codon codes serin residue at 523th amino acid, while c.1568delC changes this amino acid to phenylalanine and creates premature stop codon exactly 11 amino acids after mutated residue, causing truncated protein. B and D illustrate nucleotide sequences in *TECPR2* and *MLPH* respectively. Mother is carrier for both mutations, while the affected sibling is homozygote for identified alterations. In addition, the mutation in TECPR2 gene was not found in the healthy elder sibling, however, the mutation in MLPH gene was identified in heterozygote manner.

Figure 2. Pedigree and molecular findings of family B. A: MRI imaging at age 5 which shows very mild abnormal deep white matter periventricular (yellow arrows). B: Pedigree of the family. The proband has been indicated by the arrow. C: The mutation segregation by Sanger sequencing has been done for the proband's relatives. As can be seen in sequence chromatograms, his parents (III-3 and III-4) are heterozygotes for c. del685-687 and the proband (IV-1, colored in red) is homozygous. D: The deletion of three nucleotides leads to non-frameshift deletion of highly conserved amino acid Isoleucine at 229th residue as shown by the orthologous sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi). E: Schematic representation of 7 exons of the FA2H and its two highly conserved domains; cytochrome b5-like heme-binding domain (residues 15–85) and sterol desaturase domain (residues 124-366). The identified variant in this study was shown.

Table 1. Primers

Gene	Forward Primer	Reverse Primer

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FA2H	GCTATGCACCGGTCCTCA	GCTATGCACCGGTCCTCA
TECPR2	TTAGCTGCCCTCAAACCCAT	ACATCTTCCCTCGCTCCATT
MLPH	GGTCTTAGATAGCCAGGGGT	TTGGTGTGCTCCTGTGGG