INTRODUCTION

Dysferlinopathies are a group of autosomal recessive muscular disorders including limb-girdle muscular dystrophy type 2B (LGMD2B), Miyoshi myopathy (MM), and distal myopathy (Nguyen et al., 2007). LGMD2B is a progressive muscle weakness which affects predominantly pelvic and shoulder girdle muscles, usually starting in the second decade of life (Khadilkar, Faldu, Patil, & Singh, 2017).

Dysferlinopathy patients have myositis and are treated with immunosuppressants. Treatment of these disorders is limited to supportive measures, such as physical therapy.

METHODS: Eight unrelated Iranian families have been selected for this study. Sanger sequencing followed by haplotype analysis was performed to identify individual variations in DYSF sequence. Identified variants were analyzed, and their pathogenicity was interpreted according to the recommendations of the American College of Medical Genetics and Genomics.

RESULTS: We identified two new mutations in DYSF, the first one is a nonsense mutation c.2419C > T (p.Gln807*), which eliminates downstream part of the protein. Another novel mutation is c. (1,053 + 1_1,054-1)_(1,397 + 1_1,398-1)del, which causes deletion of the DNA segment from exon 12 to exon 15.

CONCLUSION: Two of the other six families are from the same ethnicity and share the same mutation and haplotype patterns, suggesting a founder mutation. Genetic analysis of dysferlinopathy can prevent a wrong diagnosis of myositis for these patients.

KEYWORDS
DYSF, founder effect, novel mutations, haplotype analysis, Iran

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### TABLE 1  clinical features of studied patients in the present study

| Family | Age at onset (yrs) | Loss of ambulation (yrs) | Calf hypertrophy | Ankle contractures | Abnormal pulmonary function | Cardiac involvement | Winging scapulae | Scoliosis | Lordosis | Serum CK (U/L) | Muscle biopsy | EMG with myopathic features |
|--------|-------------------|-------------------------|-----------------|-------------------|---------------------------|---------------------|-----------------|-----------|----------|----------------|--------------|----------------------------|
| 19,315 | 15                | Ambulant at age 20      | Yes             | No                 | No                        | No                  | No              | No        | No       | 11,726         | Dysferlinopathy         | Yes                         |
| 19,342 | 18                | Ambulant at age 21      | No              | No                 | No                        | No                  | Yes             | Yes       | No       | 12,000         | Dysferlinopathy         | Yes, BMD*                    |
| 17,132 | 19                | 29–28                   | No-NA           | No-NA              | No                        | Yes-No              | No-Yes          | Yes-Yes   | Yes-Yes  | 3,900          | Dysferlinopathy         | Yes                         |
| 16,935 | 13–19             | 29–30                   | No-No           | No-No              | No                        | No-No              | No-No           | No-No     | No-No    | 4,099–6506    | LGMD         | Yes-Yes                     |
| 17,298 | 35–23             | 43–25                   | Yes-Yes         | No-No              | No                        | No-No              | No-No           | No-No     | No-No    | 4,500          | Dysferlinopathy         | Yes                         |
| 16,938 | 20–17–18          | Ambulant at ages of 33 and 28 | No-No-No  | Yes-No-No | Yes-Yes-No | Yes-No-Yes | Yes-No-Yes | Yes-No-Yes | Yes-No-Yes | 3,000 | LGMD | LGMD |
| 17,124 | 22-NA             | Ambulant at ages of 33–33 | No-No            | No-No             | No-Yes                  | No-Yes            | No-Yes         | No-Yes     | No-Yes   | 4,586–3850     | Myositis     | Chronic myopathy in lower limbs- Miyoshi myopathy |
| 16,941 | 22–14             | 27–24                   | No-No           | Yes-Yes           | No-No                   | No-No             | No-No          | No-No     | No-No    | 3,000–2368     | Dysferlinopathy         | Yes                         |

CK, creatine phosphokinase.

*Becker Muscular Dystrophy.

### TABLE 2  previously reported mutations found in DYSF in the present study

| Family | Mutation at DNA level | Mutation at protein level | Intr/exon number | SIFT | Polyphen | CADD (Phred score) | Provean | UMD results (Pathogenicity) | Mutation taster | Mutant allele | Ethnicity of family |
|--------|-----------------------|--------------------------|------------------|------|----------|-------------------|---------|--------------------------|-----------------|---------------|-------------------|
| 19,315 | c.2706dupC            | p.Lys903Glnfs*4          | Exon 26          | —    | —        | 35                | —       | —                       | Disease causing | Homozygous    | Boyer-Ahmad       |
| 19,342 | c.2706dupC            | p.Lys903Glnfs*4          | Exon 26          | —    | —        | 35                | —       | —                       | Disease causing | Homozygous    | Boyer-Ahmad       |
| 17,132 | c.3112C > T           | p.Arg1038*               | Exon 29          | —    | —        | 39                | —       | —                       | Disease causing | Homozygous    | Sirjan            |
| 16,935 | c.3225delT            | p.Phe1075Leuf*45         | Exon 30          | —    | —        | 35                | —       | —                       | Disease causing | Homozygous    | Arak              |
| 16,938 | c.5804C > T           | p.Pro1935Leu             | Exon 52          | Damaging (0.001) | Probably damaging (0.997) | 34 | Deleterious (~9.13) | Presumably pathogenic (65)* | Disease causing | Homozygous    | Ardebil           |
| 17,124 | c.5633T > C           | p.Leu1878Pro             | Exon 50          | Damaging (0.000) | Probably damaging (0.993) | 32 | Deleterious (~6.34) | Presumably pathogenic (70) | Disease causing | Homozygous    | Tehran            |

*NM_003494.3.

*The closer the score is to 100, the more it will be pathogenic.
sequencing of all suspected genes for easier finding of the disease-causing mutations.

In this study, we aimed to detect mutations in eight Iranian families which presumably have LGMD2B and characterized them with autozygosity mapping and Sanger sequencing.

2 | MATERIAL AND METHODS

2.1 | Subjects

Twenty-five patients from eight unrelated Iranian families were referred to the Kawsar Human Genetics Research Center [KHGRC]. The affected male patients were ruled out for dystrophinopathy based on clinical, pathological, and molecular analysis of dystrophin gene. Clinical diagnosis of patients was made when they had weakness and atrophy of the pelvic and shoulder girdle muscles, and raised serum creatine phosphokinase concentration. Age at onset of dysferlinopathy is usually in the second decade of life. All examination and clinical procedure were made by neurologists.

The study protocol was approved by Ethics Committee of the Pasteur Institute of Iran (No: 91/0201/10425). Informed consent was received from all participants before sampling, and written informed consent was also obtained from the participants for the publication.

2.2 | Muscle biopsy

Fresh muscle biopsy samples were frozen instantly in isopentane precooled by liquid nitrogen. The samples were assayed by a standard panel of histochemical procedures (Dubowitz, 1973). Immunohistochemical studies were performed using monoclonal antibodies against dystrophin, dysferlin, and beta-spectrin. Beta-spectrin was applied as a positive control. All antibodies were purchased from Novocastra Laboratories (Newcastle, UK). Peroxidase method was used as a detection system.

2.3 | Molecular genetics studies

DNA was extracted by salting out procedure (Miller, Dykes, & Polesky, 1988). Autozygosity mapping was done by a multiplex set of four short tandem repeat (STR) markers flanking the \( DYSF \) (NM_003494.3). DNA sequencing, interpretation of data, and fragment analysis were performed as described previously (Mojbafan et al., 2016). Long-range polymerase chain reaction (PCR) was done using primers flanking the deleted region. Cycling conditions were as follows: 92°C for 2 min followed by 30 cycles of 92°C for 10 s, annealing temperature of 62°C for 15 s, and extension temperature of 68°C for 10 min. A final 7 min elongation step was performed at 68°C. The sequences of primers are available upon request.

2.4 | In silico analysis

Pathogenic effects of variants and mutations were evaluated using different softwares, such as SIFT (Kumar, Henikoff, & Ng, 2009), polyphen-2 (Polymorphism Phenotyping v2) (Adzhubei et al., 2010), PROVEAN (Protein Variation Effect Analyzer) (Choi & Chan, 2015), CADD (Combined Annotation Dependent Depletion) (Kircher et al., 2014), and mutation taster (Schwarz, Cooper, Schuelke, & Seelow, 2014).

3 | RESULTS

Clinical and histopathological characteristics of patients are listed in Table 1. The consanguinity rate of our studied families was 88%, and their haplotype analysis showed homozygosity in the \( DYSF \). Sanger sequencing of the gene in all eight families showed seven different mutations, two of which were not described previously (Tables 2 and 3). Pedigrees of all families are presented in Figure 1(a–g). Two non-consanguinous families (19,315 and 19,342) shared the same haplotypes (Figure 2) and the same frameshift mutation (c.2706dupC) which led to a premature termination of translation.

Three of the seven mutations were deletions/duplications causing the frameshift and two were nonsense mutations resulting in a premature stop codon. The other two mutations were missense ones (Tables 2 and 3).

One of the novel mutations detected in our study was a nonsense mutation (c.2419C > T, p.Gln807*) in exon 24, another new mutation was found in the family 16,941 with two affected members, which was a multi-exon deletion. Since one of the STR markers, located in intron 13, did not produce any fluorescent peak in the affected individuals (designated

| Family     | Mutation at DNA level | Mutation at protein level | Intron/exon number | CADD (Phred score) | Mutation taster | Mutant allele | ACMG interpretation | Ethnicity of family |
|------------|-----------------------|----------------------------|---------------------|---------------------|-----------------|---------------|---------------------|---------------------|
| 17,298     | c.2419C > T           | p.Gln807*                  | Exon 24             | 40                  | Disease causing | Homozygous    | Pathogenic          | Mashhad             |
| 16,941     | c.(1,053 + 1_1054−1)  | -                          | Deletion of exons 12−15 | -                   | -               | Homozygous    | Pathogenic          | Kermanshah          |
FIGURE 1  (a–g) Pedigrees of eight families with dysferlinopathy. Squares—males, circles—females, filled symbols affected individuals, open symbols—unaffected individuals. Double bars represent consanguineous unions. All family members tested in this project were abbreviated as Homo, Hete, and NL. Homo represents homozygote, Hete represents Heterozygote, and NL stands for Normal regarding to the mutation found in the family. Sequencing results are corresponding to the individuals in the pedigree who were shown by Homo, Hete, and NL.
as DNW, “Does Not Work”, in Figure 3) and showed a homozygous peak in the affected members’ parents, we suspected the presence of a possible deletion in this region (Figure 3). PCR performed in order to sequence the whole DYSF showed that all exons except 12–15 yield amplification products in the affected members. We repeated the PCR
of these exons with several primer pairs, various annealing temperatures, and cycle numbers, but it did not yield any amplification products suggesting a possible deletion of these exons. Further analysis of patient’s DNA had shown that the multiple primer sets flanking exons 12–15 failed to produce PCR product. Finally, a multiplex PCR with internal control, using exon 55, indicated that exons 12–15 have been deleted in the patients compared to a healthy control individual and patient’s parents (Figure 4).

Next, we used a long-range PCR to confirm this multi-exon deletion. We designed a primer set flanking exons 12 and 15 of DYSF for targets located in the middle of exons 11 and 16 which produced a predicted amplification product. The length of this region in a control individual was about 18.5 kb. We also used a primer pair near exon 13 as an internal control. The mutation in the affected individuals of family 16,941 caused a deletion of about 16.5 kb fragment including exons 12–15 and may additionally include some fragments of neighboring introns. As expected, exon 13 was deleted in the patient’s gene and therefore did not produce any bands; however, amplification of regions flanking exons 11 and 16 showed an about 2 kb PCR product which confirmed this deletion (Figure 5). When DNAs from parents heterozygous for this deletion were used as templates, amplification products with correct size were detected in both internal control and the deleted region. At the same time, in DNAs of control individuals without multi-exon deletion, we revealed only the internal band, while the region of 18.5 kb was too long to be amplified in control individual (Figure 5).

Immunohistochemical staining of muscle samples from a patient belonging to family 16,941 showed round and dispersed atrophic fibers with a wide variation in the fiber size when it was stained with hematoxylin and eosin (H & E) (Figure 6a). Endomysial connective tissue was significantly enlarged, which might be associated with slight adipose tissue replacement typical for dystrophic changes. Further immunohistochemical analyses were carried out using monoclonal antibodies against beta-spectrin as a positive control (Figure 6b), and dysferlin (Figure 6c). The loss of sarcolemmal labeling of all muscle fibers against dysferlin antibody confirmed our genetic results (Figure 6c). As it is mentioned in Table 1, muscle biopsy studies of the patient from family 17,298 showed the same features like family 16,941 which were compatible with dysferlinopathy.

4 | DISCUSSION

The prevalence of dysferlinopathy is not known but it has been reported in different countries and ethnicities (Gomez-Diaz et al., 2012; Magri et al., 2012; Moore et al., 2006; de Paula et al., 2002; Sveen, Schwartz, & Vissing, 2006; Tagawa et al., 2003; Walter et al., 2013). Autosomal recessive disorders like LGMDs are more prevalent in countries with high rate of consanguineous marriages including Iran, Turkey, and India (Dincer et al., 2000, 1997; Fatehi et al., 2015). Since DYSF is large, it is difficult to identify frequent mutations in this gene and different studies revealed that no hotspot is evident in this gene and all mutations are spread throughout the gene; but a
study in affected individuals of Spain could define a founder variant of Arg1905* (Vilchez et al., 2005), and another study showed that c.937+1G > A, c.1566C > G, c.2997G > T, and c.3373delG mutations account for half of all the mutations identified in Japanese LGMD2B patients (Hayashi et al., 2010). In Iran, it was just one genetic study on LGMD2B patients which was performed on nine families, and because of small sample size, no frequent mutation could be identified (Fatehi et al., 2015). Another investigation on muscle biopsy of 100 Iranian individuals who were suspected to have neuromuscular disorders revealed that only one patient in this study group suffered from dysferlinopathy (Dubowitz, 1973). In the present study, we have examined 25 LGMD2B patients from eight families, and detected novel mutations in two of eight. Previously reported mutations in DYSF were described in Italy (c.2706dupC) (Angelini, Grisold, & Nigro, 2011; Cacciottolo et al., 2011), France, and Japan (c.3112C > T, c.3225delT) (Krahn et al., 2009; Nguyen et al., 2005; Takahashi et al., 2013). Two other missense mutations (c.5804C > T and c.5633T > C) have been described in DYSF database (http://www.umd.be/DYSF/) as probable pathogenic mutations. These mutations have not been reported in Iranian population so far, and the results of in silico analyses shown in Table 2 revealed that they are deleterious.

One of the novel mutations described here was a nonsense mutation c.2419C > T (p.Gln807*) causing premature termination of translation producing a truncated protein that potentially can be removed from the cell by nonsense-mediated decay (NMD). According to the joint consensus recommendation of the American College of Medical Genetics and Genomics (ACMG) for the interpretation of DNA sequence variants [28],

FIGURE 3  STR markers surrounding DYSF in family 16,941. D2DYSFSU14.7 is a novel STR marker, where U means upstream. The numbers denote distance from the gene (e.g., 8.05 × 10^3 base pairs). Marker D2S1389 is located in intron 13 which does not produce any peak (DNW = Does Not Work). This marker reveals a homozygous peak in DNA from parents and in the heterozygote sibling

FIGURE 4  Evaluating exon deletion of exon 12 to exon 15 in DYSF on agarose gel by multiplex PCR. The deleted exons are shown in red in the schematic image. The PCR product size of each exon (s) is as follows: exon 12: 527 bp, exon 13:458 bp, exon 14–15: 641 bp, exon 15: 730 bp. The ladder used in this figure is a 100 bp ladder. P: Father, M: Mother, AC: Affected child, Co-: Negative control, PCR: Polymerase chain reaction

FIGURE 5  Evaluating deletion of exon 12 to exon 15 in DYSF on agarose gel by long-range PCR. Arrows show the location of primers: a primer pair (shown in orange) is designed for amplifying exon 13, while the other primer set is localized in a sequence surrounding exons 11 and 16 (shown in light blue). P: Father, M: Mother, AC: Affected child, Co-: Negative control, PCR: polymerase chain reaction
this novel variant demonstrates its pathogenicity because: 1. Null variants, such as nonsense mutations, are a strong evidence of pathogenicity (PVS1). 2. Absence of this variant in controls in different databases such as Exome Sequencing Project, 1,000 Genomes Project, or Exome Aggregation Consortium can be considered as a moderate proof for pathogenicity (PM2). 3. Haplotype analysis showed co-segregation of this variant with the disease in the family which provides an additional evidence for the pathogenicity of this variant (PP1). 4. Multiple lines of computational evidence support a deleterious effect on the gene product (PP3). 5. The phenotype of the patients in the family is compatible with LGMD2B (PP4).

The second novel mutation was c.(1,053 + 1_1054–1)_(1,397 + 1_1398–1)del, which causes deletion of DNA segment from exon 12 to exon 15. This deletion leads to a frameshift and truncation of dysferlin from 2,080 to 358 amino acids. This multi-exon deletion can potentially produce a truncated protein, which can be also discarded from the cell by NMD. According to the ACMG guideline, a single or multi-exon deletion may provide a strong evidence of pathogenicity (PVS1). Other evidence for pathogenicity includes PM2, PP1, and PP4 which have been described above. Functional analysis of the patients in both families, 17,298 and 16,941, by immunohistochemistry also showed lack of sarcolemmal labeling of all muscle fibers by dysferlin antibody confirming our genetic results.

Two families from Lurs of Boyer-Ahmad, an ethnicity in the south-west of Iran, had the same mutation (c.2706dupC) and the same haplotype (Figure 2). These data point to a possible founder effect for DYSF in this ethnicity. Larger sample size from patients of this ethnic group should be analyzed to confirm this hypothesis. Another evidence of founder mutation effect in LGMD2B patients (c.2779delG) was previously reported in Caucasus Jews isolate which was originated from an Iranian patient (Leshinsky-Silver et al., 2007).

In family 17,124, the initial diagnosis based on the immunohistochemistry data was myositis which can mislead the neurologist from the correct diagnosis and treatment. The genetic data could help the patient to avoid a wrong treatment by corticosteroids and immunosuppressive agents. Few researches have been done in Iranian population in the field of DYSF genetic analysis and diagnostics. This study contributes to our knowledge of the genetic abnormalities in the DYSF and improves our understanding of LGMD2B mechanism. Despite the fact that DYSF is a large gene lacking a hotspot, further studies in Iranian population may help to identify possible frequent mutations or founder effect in different ethnicities. We also could not find genotype-phenotype correlation in our studied patients; more sample size may help us to define the possible correlation in LGMD2B patients.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

Marzieh Mojbafan wrote the draft of manuscript and designed the primers and STR markers and did all PCRs. Tina Shirzadeh and Fatemeh Zafarghandi Motlagh carried out the analysis of some data. Andrei Surguchov edited the draft. Yalda Nilipour contributed in muscle biopsy study. Sirous Zeinali supervised the project, helping in the all process. All authors read and approved the paper.
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