INTRODUCTION

Marfan syndrome (MFS; OMIM: 154700) and Marfan-associated disorders such as Loeys–Dietz syndrome (LDS; OMIM: 609192) are autosomal dominant connective tissue diseases (Cao et al., 2018). MFS with an incidence rate of at least one per 5,000 individuals is a pleiotropic genetic disorder with systemic involvement (Coucke, Van Acker, & De Paepe, 2006; Summers et al., 2012). The clinical diagnosis is primarily based on well-defined clinical
manifestations often involving cardiovascular, ocular, and also skeletal systems with broad clinical variability (Coucke et al., 2006).

Over 1,800 mutations in the fibrillin-1 (FBN1) gene (NM_000138.4) associated with MFS have been described. Various studies reported that around 91% of MFS cases were associated with FBN1 mutations (MFS-1; OMIM: 134797) (B. Loeys et al., 2004), while a further small percentage of the patients had a mutation in the transforming growth factor β receptor 1 or 2 (TGFBR1 or 2) (MFS-2; OMIM 190182) (Waldmüller et al., 2007). MFS-2 is also known as LDS type II (Cao et al., 2018). The estimated incidence of LDS is not clear but it seems to be less frequent than MFS-1.

FBN1 encodes a large cysteine-rich glycoprotein known as fibrillin-1; this protein is a principal component of microfibril macromolecules of the extracellular matrix (ECM), which can be associated with elastin fibers (Frédéric et al., 2009). Fibrillins play essential roles as calcium-binding microfibrillar structural molecules and also function as a regulator of TGF-β signaling (Cao et al., 2018). Loss-of-function mutations, especially those that affect cysteine residues, cause disulfide-bond disruption and subsequently misalignment of microfibrils which lead to the aberrant TGF-β signaling (Shi et al., 2011). Disregulation of the TGF-β signaling pathway is also involved in MFS-2 (Cao et al., 2018; Dong et al., 2012; Kirby, Dietz, & Sponseller, 2018).

TGF-β receptor 2, encoded by TGFBR2 (NM_003242.6), is a transmembrane serine-threonine kinase. To function appropriately, TGFBR2 forms a homodimer. Mutations affecting the intracellular protein kinase domains can disturb TGF-β signaling and subsequently lead to similar clinical manifestations to MFS-1. In actual fact, TGF-β signaling regulates many key cellular processes such as proliferation, cell differentiation, apoptosis, cell cycle arrest, ECM formation, and cell remodeling (Cao et al., 2018). Bone abnormalities in MFS and MFS-related disorders can cogently be associated with the deficits of the TGF-β signaling pathway which is crucial for chondro- and osteogenesis (Kirby et al., 2018; Razmara et al., 2019).

Although clinical evaluations have remained a cornerstone in the diagnostic decision-making process (Baetens et al., 2011), molecular genetic testing is becoming increasingly important. In other words, the clinical diagnosis of MFS and Marfan-related diseases can be challenging due to the age-related entity of some of the clinical manifestations, the variable phenotypic expression of the diseases, and their phenotypic similarities. Besides, it has been reported that the identified mutations scattered over the entire FBN1 gene, are nearly always specific to each family and there are not located in a particular hot spot region (Coucke et al., 2006). Studies have revealed that around 25% of the patients show de novo mutations, often with more severe phenotypes in comparison with familial cases (Cao et al., 2018). Thus, molecular analysis can be helpful to make an accurate robust diagnosis (Coucke et al., 2006; Seo et al., 2018).

In this study, by using targeted next-generation sequencing, seven likely causative variants in seven unrelated Iranian families were identified in FBN1 and TGFBR2, of which three variants were novel yet have not been reported. Further clinical assessments confirmed MFS in the patients.

2 | MATERIALS AND METHODS

2.1 | Patients and ethics statement

In this study, we enrolled seven unrelated Iranian families suspected with MFS who were referred to the Department of Medical Genetics, DeNA Laboratory, Tehran, Iran. The study protocol was approved by the local medical ethics committee of Tarbiat Modares University, Tehran, Iran. Written informed consent obtained either from all subjects or from their legal representatives. Furthermore, informed consent was obtained from the parents when patients were under 18.

Some cases were sporadic in which the familial history and also other family members’ genotypes were negative for MFS and the variants, while other families had multiple affected members with a positive MFS history. The diagnosis of the disease was made based on the revised Ghent nosology for MFS (Cao et al., 2018; Chandra et al., 2015; B. L. Loeys et al., 2010). All of the clinical information and the medical histories were collected at the Department of Medical Genetics, DeNA Laboratory, Tehran, Iran.

2.2 | DNA extraction

Genomic DNAs were extracted from the peripheral blood of the patients and also all available family members by the High Pure PCR template preparation kit (Roche: Product No. 11814770001). Genetic screening using a panel to test 14 genes including ACTA2, CBS, FBN1, FBN2, MYH11, COL3A1, SMAD3, TGFBR1, TGFBR2, MYLK, MSTN, COL5A2, TGFBR2, and SLC2A10 was performed on the probands from seven unrelated Iranian families in BGI clinical laboratories.

2.3 | Targeted next-generation sequencing

The sequencing was performed according to a custom-designed NimbleGen chip capturing the aforementioned genes. In general, the test platform examined >95% of the target gene with sensitivity >99%. Point variants, micro-insertions, deletions, and duplications (<20 bp) could be simultaneously detected. For analysis of the sequencing results, the
international publicly available mutation and polymorphism databases such as 1000 genome project, ExAC (Exome Aggregation Consortium), ESP (Exon Sequencing Projects), Iranome (http://www.irano me.ir/), and also BGI self-developed local database were utilized. Only variants with minor allele frequency below 1 percent (MAF <1%) were selected. Previously reported mutations that have been described in HGMD (Human Gene Mutation Database) and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar) as pathogenic or likely pathogenic were given the highest priority.

2.4 | Co-segregation analysis

Samples from all available family members were subjected to Sanger sequencing to show whether the potential variants in the causative genes co-segregate with the MFS phenotype. Primers surrounding the region of the identified variant were designed by using Primer3.0 (version 0.4.0; http://bioinfo.ut.ee/prime r3-0.4.0) (Table S1). PCR reaction was included 10–40 ng of genomic DNA with Taq DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany). PCR was performed in a standard condition (Esmaeilzadeh-Gharehdaghi, Razmara, Bitaraf, Mahmoudi, & Garshasbi, 2019), then PCR products were sequenced by ABI 3130XL, using the conventional capillary system; sequence traces were analyzed using the Sequencher 4.7 program (Gene Codes Corporation).

2.5 | In silico prediction

Prediction for the consequence of the variants was obtained from at least three online databases including SIFT, PolyPhen, and Provean. In terms of nonsense variants, we used ENTRPRISE-X (http://cssb2.biology.gatech.edu/entprise-x/) to predict the pathogenicity of the variants. Additionally, to better show the potential pathological identity of the variants, the ConSurf (http://www.consurf.tau.ac.il) was applied to check the evolutionary conservation profile for FBNI and TGFBR2 proteins. Additionally, UCSC databases (https://genome.ucsc.edu) were used to check the genomic conservation.

To predict the possible effects of the missense variants on protein conformation, the protein families and domains were analyzed using ScanProsite (Gattiker, Gasteiger, & Bairoch, 2002). Sequence alignments of the human TGFBR2 and FBNI were recruited by using ClustalW (http://www.ebi.ac.uk/clustalw). Also, a BLAST sequence search against the protein data bank (PDB) was performed to select a template structure with the closest sequence similarity to the domains of TGFBR2 and FBNI. The protein structures were depicted by PyMOL (DeLano, 2002) after the structures were

| TABLE 1 | Identified variants in seven unrelated families. We summarized some of the key clinical findings |

| FID | Age | Sex | Gene | Exon | Nucleotide | Protein | Domain | F.H | N/R | Ref. | C | S | O | SK | O.M | Cys | Diagnosis |
|-----|-----|-----|------|------|------------|---------|--------|-----|-----|------|----|----|---|----|-----|-----|----------|
| 1   | 4   | F   | TGFBR2 | 4   | c.1085A>G | p.(His362Arg) | PKD VIA | N | — | — | R | Zarate, Sellars, Lepard, Tang, and Collins II (2016) | + | + | + | − | − | − | MFS-2 |
| 2   | 5   | F   | TGFBR2 | 5   | c.1258G>A | p.(Gly420Arg) | PKD DVIII | R | Y | Yang, Gan, Jiang, Gong, and Qiu (2019) | + | + | + | − | − | − | MFS-1 |
| 3   | 48  | M   | FBN1 | 15  | c.1879C>T | p.(Arg627Cys) | cb EGF-like | 06 | R | | Yang, Guo, Jiang, Gong, and Qu (2019) | + | + | + | − | − | − | MFS-1 |
| 4   | 43  | M   | FBN1 | 6   | c.643C>T | p.(Arg215*) | Hyd module 01 | NA | R | | Jin et al. (2007) | + | − | + | − | − | − | MFS-1 |
| 5   | 8   | F   | FBNI | 30  | c.385C>G | p.(Arg128 Tyr) | cb EGF-like | 16 | R | | | + | + | + | − | − | − | |
| 6   | 33  | M   | FBNI | 50  | c.6288C>A | p.(Gly2096*) | TGFBP | 06 | R | | Frank et al. (2016) | + | + | + | − | − | − | |
| 7   | 24  | F   | FBNI | 55  | c.6791G>T | p.(Gly2264 Glu) | cb EGF-like | 35 | R | | | + | + | + | − | − | − | |

Abbreviations: C, cardiovascular; Cys, affected Cysteine; F, Family ID; F.H, Family history; Hyd, hybrid module; H, Hidroxyproline; ID, F.H. Family history; PKD, Protein kinase domain; Ref., Reference; S, Skeletal; SK, Skin.
recruited from SWISS-PROT (Boutet, Lieberherr, Tognolli, Schneider, & Bairoch, 2007) in a distinct PDB file.

2.6 | Prediction of single-point variation on protein stability

The I-Mutant2.0, which computes the ΔΔG values of protein variant, was utilized to predict and annotate the effect of the single nonsynonymous variants on the stability of the proteins from their sequences. I-Mutant2.0 (https://www.folding.biofold.org/i-mutant/i-mutant2.0.html) is based on the Support Vector Machine and ProTherm database (Bava, Gromiha, Uedaira, Kitajima, & Sarai, 2004) and is trained to predict the thermodynamic free energy change upon single-point variations in protein sequences.

2.7 | Prediction of effects of the variants on disulfide bonding state

Some of the identified variants influence Cysteine amino acids; to predict the possible effects of such substitutions on disulfide bonding state, the DISULFIND server (Ceroni, Passerini, Vullo, & Frasconi, 2006) was utilized. Just in case, DiANNA 1.1 webserver (Ferrè & Clote, 2006) was applied as well.

3 | RESULTS

3.1 | Clinical outcomes

We evaluated seven unrelated Iranian families suspected with MFS according to the revised Ghent criteria. Clinical manifestations are summarized in Table 1. All patients showed typical clinical features of MFS. Additionally, they underwent mutational screening using a panel involving 14 genes. The list of the identified variants in all patients is shown in Table 2. Blood samples from at least one other affected family member(s) available in seven families were investigated to find the segregated variants (Figures 1 and 2).

3.2 | In silico prediction

Various in silico predicting tools such as SIFT, Polyphen, Mutation taster, Provean, and ENTPRISE-X (Zhou, Gao, & Skolnick, 2018) were used to evaluate the possible pathogenicity of all variants. All detailed results are described in Table 2.

All variants were located in the extremely conserved protein regions, while p.(Arg627Cys) was an exception.

### Table 2

| Gene          | NM            | Nucleotide change | AA change | Variant Type | dbSNP ID | SIFT | Polyphen | Mutation Assessor | Provean | I-Mutant2.0 (DDG) | PhastCons | ENTPRISE-X | Iranome 1K Genome | EXAC |
|---------------|---------------|-------------------|-----------|--------------|----------|------|----------|-------------------|---------|-------------------|-----------|-------------|---------------------|------|
| TGFBR2        | NM_003242.6   | c.1085A>G         | p.(His362Arg) | Nonsynonymous | —        | D    | P.D      | D                 | D       | −0.59              | 1         | N.R         | N.R                 | N.R  |
| TGFBR2        | NM_003242.6   | c.1258G>A         | p.(Gly420Arg) | Nonsynonymous | —        | D    | P.D      | N                 | D       | −0.83              | 1         | N.R         | N.R                 | N.R  |
| FBN1          | NM_000138.4   | c.1879C>T         | p.(Arg627Cys) | Nonsynonymous | —        | D    | P.D      | D                 | D       | −0.52              | 1         | N.R         | N.R                 | N.R  |
| FBN1          | NM_000138.4   | c.643C>T          | p.(Arg215*)  | Stop Gain    | —        | D    | —        | —                 | —       | —                 | —         | N.R         | N.R                 | N.R  |
| FBN1          | NM_000138.4   | c.3833G>A         | p.(Cys1278Tyr) | Nonsynonymous | —        | D    | P.D      | D                 | D       | −0.15              | 1         | N.R         | N.R                 | N.R  |
| FBN1          | NM_000138.4   | c.6288C>A         | p.(Cys2096*) | Stop Gain    | —        | D    | —        | —                 | —       | −0.15              | 1         | N.R         | N.R                 | N.R  |
| FBN1          | NM_000138.4   | c.6793T>G         | p.(Cys2265Gly) | Nonsynonymous | rs105752222 | D    | P.D      | D                 | D       | −0.32              | 1         | N.R         | N.R                 | N.R  |

### Abbreviations
- D, Damaging
- P.D, probable Damaging
- M, moderate
- N, Neutral
- N.R, Not Reported
p.(Arg627Cys) is a highly conserved amino acid in primates, not invertebrates. All identified variants change the high conserved nucleotides, confirmed by the UCSC database, although in amino acid levels there were some fluctuations (Figure 3).

Data of I-Mutant2.0 demonstrated all missense identified variants cause to reduce protein flexibility and stability (DDG < 0; Table 2).

DISULFIND and DiANNA 1.1 web servers were used to predict the possible effects of the variants on the disulfide bonding state. Data showed that p.(Cys1278Tyr), p.(-Cys2096*), and p.(Cys2265Gly) variants may cause to disrupt the conformation of fibrillin-1. Thus, these cysteines are essential in forming disulfide bonds.

4 | DISCUSSION

According to the UMD-FBN1 mutation database (Collod-Béroud et al., 2003), which is the currently the most extensive FBN1 mutation database, in total 1,847 different mutations in FBN1 have been reported in which missense, small deletions, nonsense, and splice sites mutations with 1,015, 268, 210, and 200 reports, respectively, are the frequent alternation types (www.umd.be/FBN1). Studies have revealed that mutations are scattered throughout the entire length of the FBN1 gene (Coucke et al., 2006). Some mutations in fibrillin lead to the disruption of microfibril formation and fibrillin protein abnormalities, so causing the connective tissue disorders (Child, Aragon-Martin, & Sage, 2016; Ferrè & Clote, 2006). Moreover, to date, 111 different mutations including 93 missense mutations have been reported in the UMD-TGFBR2 mutations database (www.umd.be/TGFBR2). Spontaneous cases of MFS justifies around 25% of the total MFS cases; it has been proven that these spontaneous cases show severe clinically important phenotypes compared to the familial ones (reviewed in [Child et al., 2016]).

The fibrillin-1 is a multi-domain protein composed of 47 epidermal growth factor (EGF)-like domains, 43 calcium-binding (cb-EGF), seven TGF (transforming growth factor)
factor)-β-binding (TB) domains, two hybrid domains, an N-terminal, and a C-terminal domain (Figure 2). Each cb-EGF and TGF-beta binding domain contains six and eight highly conserved cysteine residues, respectively. Disulfide bonds between these cysteines play a key role in forming a proper three-dimensional structure of the protein. It has been shown that mutations affecting these cysteines and consequently the disulfide bonds most likely affect the function of the protein and make it more vulnerable to proteolysis (Liu et al., 2015; Muiño-Mosquera et al., 2018). Thus, substitutions in which cysteines would be removed or added have detrimental functional consequences. The loss or addition of cysteine residues will result in misfolded protein which may be degraded by intracellular proteases or retained within the cell (reviewed in [Child et al., 2016]).

All the missense variants found in the FBN1, namely p.(Arg627Cys), p.(Cys1278Tyr), and p.(Cys2265Gly), affect the cysteine amino acids which are important to the three-dimensional structure of fibrillin proteins. It has been reported that p.(Cys2265Gly) is associated with MFS (Franken et al., 2016) and is categorized as likely pathogenic variant. To support this notion, we provided further clinical evidence and data derived from in silico prediction. According to the in silico predictions, both p.(Cys1278Tyr) and p.(Cys2265Gly) are potentially disrupting disulfide bonds with high predicted confidence. This leads to changing the conformation of TGFBR2 which in turn can affect the proper functions of this protein (Dietz, Saraiva, Pyeritz, Cutting, & Francomano, 1992). Although according to the DiANNA and DISULFIND databases p.(Arg627Cys) could not affect any structural cysteines, it seems that this variant is located adjacent to Ca\(^{2+}\) binding EGF-like domain which in turn may affect the protein’s affinity to the Ca\(^{2+}\) (Collod-Béroud et al., 2003); however, further investigations are necessary to validate the kinds of conclusions that can be drawn from this study.

The cb-EGF domains contain specific calcium-binding sites playing crucial roles such as maintaining the structural stability of the protein, protecting against degradation, and regulating interaction with other components of the extracellular matrix (Muiño-Mosquera et al., 2018). Thus, they can lead to the formation of an unstable protein with a tendency to undergo degradation. Accordingly, the majority of the missense mutations reported in previous studies affect highly conserved cysteines in the cb-EGF modules (Dong et al., 2012; Seo et al., 2018).

Consistent with previous studies, variants identified in this study were spreading throughout all exons of FBN1, not in a hotspot region (Coucke et al., 2006). The two nonsense variants, p.(Arg215*) and p.(Cys2096*), affect the Hybrid module and TGFβP domains of FBN1, respectively. We propose two mechanisms by which these variants can impose their effects: haploinsufficiency and impaired downstream functional protein. The variants may lead to the production of a truncated protein with loss of downstream functional domain
in the cell; as a result, cells should forestall this process by Nonsense-Mediated Decay response (NMD response) which is increasingly appreciated as one of the central mechanisms of RNA surveillance, with a great role in the physiological control of gene expression. Secondly, the main cause of MFS is thought to be haploinsufficiency of fibrillin proteins; these proteins provide stretch and elasticity to the connective tissues and are also involved in TGF-β1 signaling. Decreased fibrillin in the ECM leads to weakening the tissues and causes MFS phenotypes (Cao et al., 2018).

TGFBR2 encodes a receptor that plays a substantial role in TGF-β signaling. The TGFBR2 protein composed of several subdomains including the N-terminal domain or a signal peptide from codon 1 to 22, a cysteine-rich extracellular domain from codon 51 to 142, a transmembrane serine/threonine-protein kinase catalytic domain from codons 246–544, and the C-terminal. The kinase catalytic domain is encoded by exons 4–7. Seven kinase subdomains, I to VII, are coded by exon 4, two kinase subdomains, VIII and IX are coded by exon 5, and subdomains X and XI are coded by exon 6 and...
7, respectively (Takenshita et al., 1996). Loss-of-function mutations in the TGFBR2 cause disturbing TGF-β signaling. Mutations, affecting the intracellular kinase domain of this protein, lead to similar clinical features to those of MFS-1 (Cao et al., 2018).

The two identified variants in the TGFBR2 gene, p.(His362Arg) and p.(Gly420Arg), affect the evolutionarily conserved amino acids of TGFBR2 and can influence protein stability or affect the protein functions (Pannu et al., 2005). We conjectured that these variants may affect the serine/threonine-protein kinase catalytic domains (codons 246–544). The p.(His362Arg) is located inside the protein kinase domain VI, while the p.(Gly420Arg) is placed in protein kinase domain VIII. They may disrupt the serine/threonine kinase domain of the protein which in turn leads to diminishing TGF-β signaling (Takenshita et al., 1996) and causes MFS-2. On the other hand, these variants can also change the protein’s affinity to bind each other and form a homodimer that is required for the proper function. However, further investigations (functional analysis) are necessary to find the underlying mechanisms.

We believe that the molecular characterization of patients with MFS would give novel insights into the complex role of fibrillins and TGF-β signaling in other human disorders. It can also help to improve the development of drugs by better understanding the pathophysiology of MFS (van der Feltz, ).

5 | CONCLUSION

In this study, we investigated seven families suspicious of MFS-1, even though molecular studies indicated that two of them affected by MFS-2. This study identified two novel variants in FBN1 and also a novel variant in TGFBR2. We believe that these variants can broaden the mutation spectrum of Iranian patients. We also are optimistic that early precise molecular diagnosis can be helpful to reduce morbidity and mortality from life-threatening manifestations such as cardiovascular complications.

ACKNOWLEDGEMENT

We thank the families for their contribution to this study. We are especially grateful to the staff of the DeNA laboratory for helping us in this research. This research received no specific grant from any funding agency, commercial or not for profit sectors.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHORS’ CONTRIBUTIONS

MG and FB conceived and designed the experiments. MG and MK contributed to data collection. FB, ER, MKH, AK, and EJ wrote the paper. MG supervised the work. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

ORCID

Fatemeh Bitarafan https://orcid.org/0000-0002-4489-2923
Ehsan Razmara https://orcid.org/0000-0001-9926-3975
Mohammad Keramatipour http://orcid.org/0000-0002-5803-4209
Masoud Garshasbi https://orcid.org/0000-0002-5508-7903

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Bitarafan F, Razmara E, Khodaeian M, et al. Three Novel Variants identified in FBN1 and TGFBR2 in seven Iranian families with suspected Marfan syndrome. Mol Genet Genomic Med. 2020;8:e1274. https://doi.org/10.1002/mgg3.1274