Family-based whole-exome sequencing identifies novel loss-of-function mutations of *FBN1* for Marfan syndrome

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Background. Marfan syndrome (MFS) is an inherited connective tissue disorder affecting the ocular, skeletal and cardiovascular systems. Previous studies of MFS have demonstrated the association between genetic defects and clinical manifestations. Our purpose was to investigate the role of novel genetic variants in determining MFS clinical phenotypes. **Methods.** We sequenced the whole exome of 19 individuals derived from three Han Chinese families. The sequencing data were analyzed by a standard pipeline. Variants were further filtered against the public database and an in-house database. Then, we performed pedigree analysis under different inheritance patterns according to American College of Medical Genetics guidelines. Results were confirmed by Sanger sequencing. **Results.** Two novel loss-of-function indels (c.5027_5028insTGTCCTCC, p.D1677Vfs*8; c.5856delG, p.S1953Lfs*27) and one nonsense variant (c.8034C>A, p.Y2678*) of *FBN1* were identified in Family 1, Family 2 and Family 3, respectively. All affected members carried pathogenic mutations, whereas other unaffected family members or control individuals did not. These different kinds of LOF (loss of function) variants of *FBN1* were located in the cbEGF region and a conserved domain across species and were not reported previously. **Conclusions.** Our study extended and strengthened the vital role of *FBN1* LOF mutations in the pathogenesis of MFS with autosomal dominant inheritance pattern. We confirm that genetic testing by next-generation sequencing of blood DNA can be fundamental in helping clinicians conduct mutation-based pre- and postnatal screening, genetic diagnosis and clinical management for MFS.

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Abstract

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39

40 **Introduction**

41 Marfan syndrome (MFS) is an inherited connective tissue disorder with autosomal dominant
42 transmission. The clinical manifestations of MFS vary from individual to individual. More than
43 30 different signs and symptoms are variably associated with Marfan syndrome. The most
44 prominent of these affect the skeletal, cardiovascular, and ocular systems, but all fibrous
45 connective tissue throughout the body can be affected (Pyeritz & McKusick 1979). Clinically,
46 aortic dilatation and dissection are the most important and life-threatening manifestations of MFS
47 (Biggin et al. 2004). The estimated prevalence is 1 in 5,000 individuals, without gender
48 predilection (Sponseller et al. 1995; von Kodolitsch & Robinson 2007). An epidemiological study
49 in Taiwan revealed that the overall prevalence of MFS in Chinese population was 10.2 (95% CI,
50 9.8-10.7) per 100,000 individuals (Chiu et al. 2014).
51 Mutations in *FBN1* (OMIM #134797, encoding fibrillin-1) account for 70%-80% of MFS
52 (Stheneur et al. 2009). In addition to *FBN1*, there are other candidate genes functionally related to
53 MFS, such as *TGFBR1*, *TGFBR2*, *ACTA2, SMAD3, MYH11* and *MYLK*. Habashi *et al*. showed
54 that aortic aneurysm in a mouse model of MFS is associated with increased TGF-beta signaling
55 (Habashi et al. 2006) and noncanonical (Smad-independent) TGF-beta signaling may be a
56 prominent driver of aortic disease in Marfan syndrome mice (Holm et al. 2011).
57 Traditionally, the discovery of pathogenic genes for MFS has depended on locus mapping using a
58 candidate-gene strategy with family-based designs, while *FBN1* mutations have not been detected
59 in 10% of MFS patients from clinical diagnosis, implying that either atypical mutation types or
60 other genes may cause MFS-like disease (Li et al. 2017). Most cases inherit MFS from their parents

in an autosomal dominant fashion (Wieczorek et al. 1996). Marfan syndrome may also be caused by dominant negative-typemutations and haploinsufficiency (Hilhorst-Hofstee et al. 2011; Judge et al. 2004; Judge & Dietz 2005). Therefore, more pathogenic genes or atypical mutations in specific populations remain to be identified. Here, we performed a family-based study using whole-exome sequencing (WES) in 19 individuals, who were derived from three Han Chinese MFS families. We identified three novel LOF mutations in *FBN1* likely to cause MFS in these patients. Systematical evaluations and experimental replications were conducted to validate our findings.

Materials and Methods

Study Subjects

A total of 19 volunteers from three Han Chinese families were recruited from the First Affiliated Hospital of Nanjing Medical University between 2012 and 2016. The mean age of onset of cases was 24.6±6.8 years (Family 1: I-1 lost to follow-up). MFS was diagnosed through a medical record review, physical examination, and family history based on Ghent nosology: (i) Ectopia lentis; (ii) Systemic score ≥ 7; (iii) Aortic root Z-score ≥ 2, when there is history of Marfan syndrome in a primary relative (Loeys et al. 2010). The study was approved by the institutional ethical committee of Nanjing Medical University and complied with the principles of the declaration of Helsinki. Informed consent was obtained from all subjects.

Whole-exome sequencing

Genomic DNA was isolated from peripheral blood using the QIAamp™ DNA and Blood Mini kit (Qiagen™) according to the protocol. Total DNA concentration and quantity were assessed by measuring absorbance at 260 nm with NanoDrop 2000c Spectrophotometer (Thermo Scientific™).

WES library construction and sequencing were performed as below: 300ng genomic DNA was fragmented in a Covaris® M220 Focused-ultrasonicator™ to 100-500bp fragments followed by end repair, adding A-tailing, adaptor ligation, and 11 PCR cycles according to the manufacturer's protocols. After hybridization, exome enrichment was conducted with the Agilent XT SureSelect Human All Exon v5 Kit, which targets ~50 Mb of the human exonic regions. Five DNA libraries were multiplexed on every lane and 101 base paired-end sequencing was performed on Illumina HiSeq1500 (Illumina, Inc).

Quality control, mapping and variant calling

Raw sequencing reads were filtered to trim adapters and low quality reads using Trimmomatic-0.3.2 under PE module (ILLUMINACLIP: adapter. fa: 2:30:10; LEADING: 3; TRAILING: 3; SLIDINGWINDOW: 4:15; MINLEN: 20). All the qualified reads were processed with an in-house bioinformatics pipeline, which followed the best practice steps suggested by Genome Analysis Toolkit (GATK v3.5) (DePristo et al. 2011). Briefly, we first aligned the clean sequence reads to the human reference genome (UCSC Genome Browser hg19) using Burrows-Wheeler Aligner (BWA-MEM v0.7.12 with default parameters) (Li & Durbin 2010). PCR duplicates were removed by Picard v1.141. After initial quality control (QC), all eligible sequences were determined for regional realignment and base quality recalibration with GATK v3.5. Then variations including single nucleotide variants (SNVs), insertions and deletions were called using GATK v3.5 HaplotypeCaller module.

Gene Classification and Functional annotation

We used Annovar (2016Feb01) (Wang et al. 2010) for functional annotation with Online

Mendelian Inheritance in Man (OMIM), the Exome Aggregation Consortium (ExAC) Browser, MutationTaster2 and The Combined Annotation Dependent Depletion (CADD). Based on OMIM and MFS-related literature reported previously, genes were classified into three categories according to American College of Medical Genetics (ACMG) standards and guidelines (Richards et al. 2015): Category I: 8 MFS-causing genes reported directly; Category II: 125 MFS-related genes from GeneCards; Category III: Unknown genes not reported previously **(Supplementary Table 1)**.

Manual review and replication using Sanger sequencing

All remaining mutations were manually inspected using the Integrated Genome Viewer (IGV 2.3.80) (Thorvaldsdottir et al. 2013) before Sanger sequencing. PCR primers were designed for the target regions and were used to amplify these regions by PCR for Sanger sequencing. Primers are listed in the **Supplementary Table 2**. Mutations were validated according to the resulting data screened through Chromas 2.4.1 and Dnaman 6.0.

Results

One 18-year-old male (the proband, II-1, age of onset was 10) of Han Chinese ancestry from Family 1 was diagnosed with MFS. He presented with acute, anterior chest pain, on admission. His father (I-1) also had MFS **(Fig. 1A)**. Computed Tomography (CT) revealed that the proband had an aortic aneurysm with dissection (type III), ascending aortic root dilatation with the diameter of 4.9 cm. Family 2 is a large three-generation family with five family members affected (I-2, I-5, I-6, II-1 and III-1) **(Fig. 1B)**. The proband (II-1, age of onset was 30), a 31-year-old male, presented with elongated digits but no pectus excavatum. CT showed an aortic aneurysm with dissection

(type I). His mother (I-2), one aunt (I-5) and one uncle (I-6) all had MFS with aortic aneurysms. His mother underwent surgery for aortic dissection in 1993 and died in 2015. Family 3 spanned three generations with two family members affected (I-2 and II-2). The proband (II-2, age of onset was 27) was a 28-year-old male with a history of MFS. He had thoracic surgery for pectus excavatum at 2 years old. Although he had no clinical symptoms in the cardiovascular system, a CT scan showed ascending aortic dilatation, aortic regurgitation and mitral regurgitation. His mother (I-2) was also confirmed to have MFS **(Fig. 1C)**. Although case I-3 presented elongation of fingers and mild pectus excavatum, there were not sufficient clinical features to perform the diagnosis (systemic score = 4 and aortic root Z-score < 2) (**Table 1**).

Quality summaries from sequencing of the 19 samples is summarized in **Supplementary Table 3**. Each sample had an average of 69.46M raw reads, and over 99.60% of them were successfully mapped to the reference genome. The average of median insert size was 201 bp and percent of duplicate reads ranged from 1.44% to 8.11%. Totally 237,252 variants were kept for following evaluation. To identify qualified pathogenic mutations, stringent criteria according to ACMG guidelines were performed (**Supplementary Fig. 1**). First, we filtered these variants under the following criteria: (i) untranslated region, synonymous, intronic variants (except variants considered to be splicing variants and located at exon-intron junctions ranging from -5 to +5); (ii) variants with minor allele frequency (MAF) ≥ 1% based on 1000 Genomes (1KG) and ExAC databases; (iii) variants present in our in-house whole genome sequencing (WGS) database of 100 non-Marfan controls. Then, we classified these rare genetic variants into three categories: a. MFS-causing genes; b. MFS-related genes; c. Unknown genes. Then we assessed whether these variants were loss-of-function (nonsense, frameshift and essential splice-site variants). Three inheritance patterns were evaluated for the qualified mutations including autosomal dominant, autosomal

153 recessive and compound heterozygotes. Finally, we manually reviewed and selected variants in
154 Category I and II genes for validation. After replication by Sanger sequencing, three LOF
155 mutations in *FBN1* were identified in three families respectively. For Family 1, the insertion
156 (c.5027_5028insTGTCCTCC) was detected in both I-1 and II-1 individuals, which resulted in a
157 frameshift (p.D1677Vfs*8). For Family 2, a heterozygous deletion (c.5856delG) in exon 48
158 (NM_000138) was found in four patients (I-5, I-6, II-1 and III-1), also resulting in a frameshift
159 (p.S1953Lfs*27). I-2 and II-2 individuals from family 3 were heterozygous for the nonsense
160 variant (c.8034C>A), which gained an immediate stop codon (p.Y2678*). All mutations
161 (c.5027_5028insTGTCCTCC, c.5856delG and c.8034C>A) identified in three families were
162 predicted to be disease-causing using MutationTaster2 and CADD. In addition, all mutation sites
163 were located in a highly conserved amino acid region (Calcium-binding EGF domain) across
164 different species **(Supplementary Fig. 2)**. A summary of these mutations is presented in **Table 2**.
165 All healthy family members and 100 other non-MFS controls did not carry these *FBN1* variants.
166 It is worth noting that these mutations in *FBN1* have not been reported previously, but the variant
167 (c.5857dupT), near c.5856delG, was recorded by the Human Gene Mutation Database (HGMD).
168
169 **Discussion**
170 MFS is a systemic disorder of connective tissue with a high degree of clinical variability that
171 involves skeletal, ocular, and cardiovascular systems (Dietz 1993). In our study, massively parallel
172 sequencing was performed to identify genetic abnormalities in three MFS families, showing three
173 rare functional variations in *FBN1*.
174 Fibrillins are the major components of microfibrils in the extracellular matrix of elastic and non-
175 elastic tissues. Fibrillin-1 serves as a structural component of calcium-binding microfibrils and is

encoded by *FBN1* gene. *FBN1* is mapped to chromosome 15q21.1 and encodes a 2,871 amino acid protein. More than 1,800 different mutations have been identified in *FBN1*, most of which are associated with MFS, as seen in the UMD-*FBN1* mutations database (Collod-Beroud et al. 1997; Collod et al. 1996) using a generic software called Universal Mutation Database (UMD) (Collod-Beroud et al. 2003). Similar to this curated database, approximately 1,700 variants in *FBN1* are associated with MFS according to the professional version of The Human Gene Mutation Database (HGMD), and these accounts for more than 90% of MFS cases. In our study, all affected individuals also carried *FBN1* LOF mutations (c.5027_5028insTGTCCTCC, c.5856delG and c.8034C>A).

The mutated mRNAs in our study, although introducing premature termination codons (PTC), could in theory be stable. However, cellular recognition and degradation of mRNA that contains PTC via nonsense-mediated mRNA decay (NMD) is a process whereby potentially harmful effects of truncated proteins may be limited (Culbertson 1999; Frischmeyer & Dietz 1999). Accordingly to one study, in the majority of *FBN1* PTC (premature termination codon) MFS cases, synthesis of normal-sized fibrillin protein was ~50% of control levels, but much less matrix deposition occurred (Schrijver et al. 2002). They concluded that most PTC mutations have a major impact on the pathogenesis of type 1 fibrillinopathies but that it is not always through NMD. In our study, we found that two PTC mutations (p.S1953Lfs*27 and p.Y2678*) were quite near the mutations (p.Q1955X and p.I2681X) reported by Schrijver *et al* (Schrijver et al. 2002). In contrast, the relative amount of PTC-containing *FBN1* transcripts in blood was found to be significantly higher than in affected fibroblasts with experimental inhibition of nonsense-mediated decay, while in fibroblasts without NMD inhibition, no mutant alleles could be detected at all (Magyar et al. 2009), implying that tissue-specific degradation of transcripts also plays an important role in MFS

pathogenesis.

Along these lines, Faivre *et al.* found that patients with an *FBN1* PTC had a more severe skeletal and skin phenotype than did patients with an in-frame mutation. Mutations in exons 24-32 were also associated with a more severe and complete phenotype (Faivre et al. 2007). In our study, the LOF mutations were located in exon 41, 48 and 64, and patients did not have a complete MFS phenotype including ectopia lentis and skin striae. This expression of the MFS phenotype may also depend on the different ethnicity of our patient cohort from the above-cited studies.

In family 2, a suspected case of a 4-year-old boy (patient III-1) who had longer finger and anterior chest deformity (pectus excavatum), was slightly taller than his peers. It was difficult to make a clinical diagnosis owing to his age and uncertain status according to the clinical criteria, but our WES-based screening helped ease his diagnosis by excluding the *FBN1* mutation found in other affected members of his family. In family 3, we detected that clinically unaffected subject I-3 had a slight anterior chest deformity (pectus excavatum). Although she had this MFS-related symptom, she was clinically considered as a healthy individual, which was borne out by the result that she had no *FBN1* or other pathogenic mutations.

All family members above are followed up regularly to confirm their diagnoses. The identification of a causative gene variant by WES in those with an uncertain phenotype or complex subjects is of inestimable value for screening, clinical diagnosis and, ultimately, directing personalized patient care with development of specific small-molecule therapies.

Conclusions

In conclusion, our results may help further elucidate the genetic pathology of MFS, and these mutations could be included among probably pathogenic markers for pre- and postnatal screening

and genetic diagnosis for MFS.

Web Resources

Qiagen™, https://www.qiagen.com

Agilent™, https://www.agilent.com

Trimmomatic-0.3.2, http://www.usadellab.org/cms/index.php?page=trimmomatic

Genome Analysis Toolkit (GATK v3.5), https://software.broadinstitute.org/gatk

Burrows–Wheeler Aligner, BWA v0.7.12, http://bio-bwa.sourceforge.net

Picard v1.141, http://picard.sourceforge.net

Annovar (2016Feb01), http://annovar.openbioinformatics.org

Mendelian Inheritance in Man (OMIM), http://www.omim.org

Exome Aggregation Consortium (ExAC) Browser, http://exac.broadinstitute.org

MutationTaster2, http://www.mutationtaster.org/

The Combined Annotation Dependent Depletion (CADD), http://cadd.gs.washington.edu

GeneCards, https://www.genecards.org/Search/Keyword?queryString=marfan%20syndrom

Integrated Genome Viewer (IGV 2.3.80), http://software.broadinstitute.org/software/igv/

Human Gene Mutation Database (HGMD), http://www.hgmd.cf.ac.uk

Universal Mutation Database (UMD), http://www.umd

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Abbreviations:

Figure 1. Pedigree and mutations in *FBN1* for Marfan syndrome patients

1A: A *FBN1* insertion mutation (M1) was identified in two subjects with MFS (I-1 and II-1) from Family 1; **1B:** A *FBN1* deletion mutation (M2) was identified in four subjects with MFS (I-5, I-6, II-1 and III-1) from Family 2; **1C:** A *FBN1* nonsense mutation (M3) was identified in two subjects with MFS (I-2 and II-2) from Family 3. 3 individuals in the pedigrees were not sequenced including Family 2: I-2, I-7 and Family 3: I-4. W indicates wildtype allele. Circles represent female participants and squares male participants. Black symbols indicate patients with Marfan syndrome. A slash through the symbol indicates that the family member is deceased. Arrows indicate the proband.

Supplementary Figure 1. **Analytical pipeline for exome sequence filtration and prioritization**

Abbreviations: MAF, minor allele frequency; UTR, untranslated region.

Supplementary Figure 2 The locations of mutations in *FBN1* across different species

The positions of three LOF mutations in *FBN1* are shown. The affected amino acid residues are conserved across multiple species.

Table 1(on next page)

Clinical symptoms of all 19 members in three Marfan families

Facial features (3/5) = 1 (dolichocephaly, enophthalmos, downslanting palpebral fissures, malar hypoplasia, retrognathia) ND = not detected NA = not available *Suspected case

1 **Table 1. Clinical symptoms of all 19 members in three Marfan families**

Family ID	Member ID	Age of onset	Age	Wrist AND thumb sign	Pectus carinatum deformity(pectus excavatum or chest asymmetry)	Hindfoot deformity (plain pes planus)	Dural ectasia	Protrusio acetabuli	Pneumothorax	Reduced upper segment/lower segment ratio AND increased arm/height AND no severe scoliosis	Scoliosis or thoracolumbar kyphosis	Reduced elbow extension	Facial features	Skin striae (stretch marks)	Myopia > 3 diopters	Mitral valve prolapse	Systemic score	Aortic root Z-score	Case
Score				3	2(1)	2(1)	2	2	2	1	1	1	1	1	1	1			
F1	**I-1**	**25**	**46**	√	√	×	×	×	√	×	×	×	×	×	×	√	8	ND	1
F1	**I-2**		**43**	×	×	×	×	×	×	×	×	×	×	×	×	×	0	ND	0
F1	**II-1**	**10**	**21**	√	√	×	×	×	√	×	×	×	×	×	×	√	8	≥ 2	1
F2	**I-1**		**66**	×	×	×	×	×	×	×	×	×	×	×	×	×	0	ND	0
F2	**I-3**		**60**	×	×	×	×	×	×	×	×	×	×	×	×	×	0	ND	0
F2	**I-4**		**63**	×	×	×	×	×	×	×	×	×	×	×	×	×	0	ND	0
F2	**I-5**	**30**	**65**	×	×	√	×	×	×	√	×	√	√	×	×	√	6	ND	1
F2	**I-6**	**20**	**67**	×	×	√	×	×	×	√	×	√	√	×	×	√	6	ND	1

F2	**II-1**	**30**	**36**	×	×	√	×	×	×	√	×	√	√	×	×	√	6	≥ 2	1
F2	**II-2**		**30**	×	×	×	×	×	×	×	×	×	×	×	×	×	0	ND	0
F2	**II-3**		**50**	×	×	×	×	×	×	×	×	×	×	×	×	×	0	ND	0
F2	**III-1***	**2**	**5**	×	√	×	×	×	×	×	×	×	×	×	×	×	1	ND	0*
F3	**I-1**		**52**	×	×	×	×	×	×	×	×	×	×	×	×	×	0	ND	0
F3	**I-2**	**30**	**49**	×	√	√	×	×	×	×	×	×	×	×	×	√	5	ND	1
F3	**I-3**		**46**	√	√	×	×	×	×	×	×	×	×	×	×	×	4	<2	0
F3	**II-2**	**27**	**29**	×	√	√	×	×	×	×	×	×	×	×	×	√	5	≥ 2	1
F3	**II-3**		**28**	×	×	×	×	×	×	×	×	×	×	×	×	×	0	ND	0
F3	**II-4**		**22**	×	×	×	×	×	×	×	×	×	×	×	×	×	0	ND	0
F3	**III-1**		**6**	×	×	×	×	×	×	×	×	×	×	×	×	×	0	ND	0

2 Facial features (3/5) = 1 (dolichocephaly, enophthalmos, downslanting palpebral fissures, malar hypoplasia, retrognathia)

3 ND = not detected

4 NA = not available

5 *Suspected case

Table 2(on next page)

FBN1 variants identified for affected individuals in three Marfan families

1 **Table 2. *FBN1* variants identified for affected individuals in three Marfan families**

Family ID	F1	F2	F3
Chr.	chr15	chr15	chr15
Position	48,756,133	48,737,634	48,707,750
Ref allele	-	G	C
Alt allele	TGTCCTCC	-	A
Gene	*FBN1*	*FBN1*	*FBN1*
Mutation type	insertion	deletion	nonsense
Exon	41/66	48/66	64/66
Codon change	c.5027_5028insTGTCCTCC	c.5856delG	c.8034C>A
Amino acid change	p.D1677Vfs*8	p.S1953Lfs*27	p.Y2678*
Affected individuals	I-1/II-1	I-5/I-6/II-1/III-1	I-2/II-2
CADD Raw Score	9.18	7	16.63
PHRED scaled score†	35	33	56

2 †PHRED-like scaled C-scores = $-10 * \log_{10}(\frac{\text{rank}}{\text{total}})$, the recommended deleterious threshold was > 15 for scaled C-scores

3

Figure 1

Pedigree and mutations in *FBN1* for Marfan syndrome patients

1A: A *FBN1* insertion mutation (M1) was identified in two subjects with MFS (I-1 and II-1) from Family 1; **1B:** A *FBN1* deletion mutation (M2) was identified in four subjects with MFS (I-5, I-6, II-1 and III-1) from Family 2; **1C:** A *FBN1* nonsense mutation (M3) was identified in two subjects with MFS (I-2 and II-2) from Family 3. 3 individuals in the pedigrees were not sequenced including Family 2: I-2, I-7 and Family 3: I-4. W indicates wildtype allele. Circles represent female participants and squares male participants. Black symbols indicate patients with Marfan syndrome. A slash through the symbol indicates that the family member is deceased. Arrows indicate the proband.