Family-based Whole-exome Sequencing Identifies Novel Loss-of-function Mutations of *FBN1* for Marfan syndrome

Zhening Pu[1,2], Haoliang Sun[3], Junjie Du[3], Yue Cheng[1,2], Keshuai He[3], Buqing Ni[3], Weidong Gu[3], Juncheng Dai[1,2], Yongfeng Shao[3]

[1]Department of Epidemiology, School of Public Health, Nanjing Medical University, Nanjing 211166, China

[2]State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing 211166, China

[3]Department of Cardiovascular Surgery, the First Affiliated Hospital of Nanjing Medical University, Nanjing, China

Zhening Pu, Haoliang Sun and Junjie Du equally contributed to this work.

Corresponding author: Yongfeng Shao, yongfengshao30@hotmail.com; Juncheng Dai, Email: djc@njmu.edu.cn

Abstract

Background. Marfan syndrome (MFS) is an inherited connective tissue disorder ~~effecting~~ 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 ~~applied~~ sequenced the whole~~-~~ exome ~~sequencing for~~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 ~~conservative~~ a conserved domain across species and ~~was~~ 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 ~~G~~genetic testing by next-generation sequencing of blood DNA ~~could~~ can be fundamental in helping clinicians conduct mutation-based pre- and postnatal screening, genetic diagnosis and clinical management for MFS.

Keywords Marfan syndrome, Whole-exome sequencing, FBN1, Rare genetic variant

Introduction

Marfan syndrome (MFS) is an inherited connective tissue disorder with autosomal dominant transmission. The clinical manifestations of MFS vary from individuals to individuals. More than 30 different signs and symptoms are variably associated with Marfan syndrome. The most prominent of these affect the skeletal, cardiovascular, and ocular systems, but all fibrous connective tissue throughout the body can be affected (Pyeritz & McKusick 1979). Clinically, aortic dilatation and dissection are the most important and life-threatening manifestations of MFS (Biggin et al. 2004). The estimated prevalence is 1 in 5,000 individuals, without gender predilection (Sponseller et al. 1995; von Kodolitsch & Robinson 2007). An epidemiological study in Taiwan revealed that the overall prevalence of MFS in Chinese population was 10.2 (95% CI, 9.8-10.7) per 100,000 individuals, with peaks at the age of 15 to 19, 10 to 14, and 20 to 24 years. (Chiu et al. 2014).

Marfan syndrome is a genetic disorder. A mutation, or change, in the gene that controls how the body makes fibrillin causes Marfan syndrome (https://www.nhlbi.nih.gov/health/health-topics/topics/mar). Mutations in *FBN1* (OMIM #134797, encoding fibrillin-1) account for 70%-80% of MFS (Pinard et al. 2016; Stheneur et al. 2009). In addition to *FBN1*, there are other candidate genes functionally related to MFS, such as *TGFBR1*, *TGFBR2*, *ACTA2, SMAD3, MYH11* and *MYLK*. Habashi *et al*. showed that aortic aneurysm in a mouse model of MFS is associated with increased TGF-beta signaling (Habashi et al. 2006) and Holm *et al.* concluded that noncanonical (Smad-independent) TGF-beta signaling is may be a prominent driver of aortic disease in Marfan syndrome mice (Holm et al. 2011).

Traditionally, the discovery of pathogenic genes for MFS has depends depended on locus mapping using a candidate-gene strategy with family-based designs, and while the heritability of all known genes for MFS is about 90%*FBN1* mutations have not been detected in 10% of

MFS patients from clinical diagnosis, implying that either atypical mutation types or other genes may cause MFS-like disease (Li et al. 2017). However, inconclusive results exist in their association with MFS under complex pathogenesis. Most cases inherit MFS from their parents due to itsin an autosomal dominant nature fashion (Wieczorek et al. 1996). Approximately 15-30% of all cases are due to *de novo* genetic mutations (Cotran et al. 1999). Marfan syndrome is also reported to relate tomay also be caused by the dominant negative-type mutations and haploinsufficiency (Hilhorst-Hofstee et al. 2011; Judge et al. 2004; Judge & Dietz 2005). Therefore, more pathogenic genes or atypical mutations types in specific populations are warrantedremain 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* causing 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 voluntary family membersvolunteers from three Han Chinese families were recruited from the First Affiliated Hospital of Nanjing Medical University during between 2012 to and 2016. The mean age of onset of cases was 24.576 ± 6.84 years (Family 1: I-1 loss lost to follow-up). MFS was diagnosed by 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 forms werewas provided toobtained 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 ~~adopted~~ 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~~ly~~ 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 ~~P~~primers were designed for the target regions and were used to amplify these regions by PCR for Sanger sequencing. Primers ~~were~~ 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 ~~an~~ acute, anterior chest pain, ~~like tearing~~ 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 ~~the~~ 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 ~~received a~~ had thoracic surgery ~~on~~ for pectus excavatum ~~when he was~~at 2 years old. Although he had no clinical symptoms ~~of~~ 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)**. ~~In addition, albeit~~Although case I-3 presented elongation of fingers and mild pectus excavatum, ~~we had~~there were not sufficient clinical features ~~necessary~~ to perform the diagnosis (~~the~~ systemic score = 4 and aortic root Z-score < 2) (**Table 1**).

Quality ~~summary~~ summaries ~~of~~ from sequencing ~~for~~ of the 19 samples ~~was~~ is summarized in **Supplementary ~~–~~Table 3**. ~~Every~~ Each sample had an average of 69.46M raw reads, and ~~about above~~over 99.60% of them were successfully mapped to the reference genome. The average of median insert size was 201 bp and ~~duplicates~~ 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~~ly~~, we ~~removed~~ 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 recessive and compound heterozygotes ~~manners~~. Finally, we manually reviewed and selected variants in Category I and II genes for ~~technological~~ validation. After replication by Sanger sequencing, three LOF mutations in *FBN1* were ~~eventually~~ identified in three families respectively. For Family 1, the insertion (c.5027_5028insTGTCCTCC) was detected in both I-1 and II-1 individuals, which resulted in a frameshift (p.D1677Vfs*8). For Family 2, a heterozygous deletion (c.5856delG) in exon 48 (NM_000138) was found in four patients (I-5, I-6, II-1 and III-1), also resulting in a frameshift (p.S1953Lfs*27). I-2 and II-2 individuals from family 3 were heterozygous for the nonsense variant (c.8034C>A), which gained an immediate stop codon (p.Y2678*). All mutations (c.5027_5028insTGTCCTCC, c.5856delG and c.8034C>A) identified in three families were predicted to be disease-causing using MutationTaster2 and CADD. In addition, all mutation sites were located in a highly conserved amino acid~~s~~ region (Calcium-binding EGF domain) across different species **(Supplementary Fig. 2)**. A summary of these mutations ~~was~~ is presented in **Table 2**. All healthy family members and ~~other~~ 100 other non-MFS controls did not carry these *FBN1* variants. It is worth noting that these mutations in *FBN1* have not been reported previously, but the variant (c.5857dupT), near~~by~~ c.5856delG, was ~~collected~~ recorded by the Human Gene Mutation Database (HGMD).

Discussion

MFS is a systemic disorder of connective tissue with a high degree of clinical variability that involves skeletal, ocular, and cardiovascular systems (Dietz 1993). In our study, massively parallel sequencing was performed to identify genetic abnormalities in three MFS families~~. And we detected~~, showing three rare functional variations in *FBN1*. ~~There are no previous reports about all rare mutations.~~

Fibrillins are the major components of microfibrils in the extracellular matrix of elastic and non-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 ~~from~~ , as seen in the UMD-*FBN1* mutations database (Collod-Beroud et al. 1997; Collod-Beroud et al. 1998; Collod et al. 1996) using a generic software called Universal Mutation Database (UMD) (Collod-Beroud et al. 2003). Similar to ~~UMD~~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), ~~which~~ and these accounts for more than 90% of ~~related variants~~MFS cases. In our study, ~~a similar phenomenon was observed that~~ all affected individuals ~~could be attributed to~~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. ~~Also, the mutant proteins could be detected in blood and fibroblasts. Cellular~~ However, cellular recognition and degradation of mRNA that contains ~~premature termination codons~~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). ~~There are a lot of reports with regrading to pathogenic variants, which lead to haplo-insufficiency of FBN1. Patients' clinical phenotypes are also well illustrated.~~ Accordingly to ~~the~~ one study, ~~of *Schrijver I et al*,~~ in the majority of *FBN1* PTC (premature termination codon) ~~samples~~MFS cases, synthesis of normal-sized fibrillin protein was ~50% of control levels, but much less matrix deposition ~~was disproportionately decreased~~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 ~~the~~ two ~~similar~~ PTC mutations (p.S1953Lfs*27 and

p.Y2678*) were quite near the mutations (p.Q1955X and p.I2681X) reported by Schrijver ~~I~~ *et al* (~~Schrijver et al.~~ 2002). In contrast, ~~Istva´n *et al.* found that~~ the relative amount~~s~~ of PTC-containing *FBN1* transcripts in ~~fresh and PAXgene-stabilized~~ blood ~~samples were~~ was found to be significantly higher ~~(33.0+/-3.9% to 80.0+/-7.2%)~~ than in~~those detected in~~ affected fibroblasts with experimental inhibition of nonsense-mediated ~~mRNA~~ decay ~~(NMD) (11.0+/-2.1% to 25.0+/-1.8%)~~, ~~whereas~~ 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.

~~Further, we tend to focus on the genotype-phenotype correlation. Schrijver I *et al.* concluded that PTC mutations have a major impact on the pathogenesis of type 1 fibrillinopathies~~Along these lines, ~~and convey a distinct biochemical, clinical, and prognostic profile (Schrijver et al. 2002).~~ Faivre *et al.* found that patients with an *FBN1* ~~premature termination codon~~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 ~~such as~~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. ~~The difference between Chinese patients with *FBN1* PTC mutations and European-American patients could be the lower incidence of ectopia lentis or other eye-related diseases.~~

~~We have to note that III-1 in Family 2 and III-1 in Family 3 had no sufficient clinical features necessary to early diagnosis due to the age. Fortunately, our pedigree analysis showed an autosomal dominant pattern with high penetrance for *FBN1* gene.~~ In family 2, ~~one~~ a suspected case of a 4-year-old boy (patient III-1) ~~with~~ who had longer finger and anterior chest deformity (pectus excavatum), was slightly taller than ~~the~~ his peers ~~as a suspected case~~. It was difficult to

make a clinical diagnosis owing to ~~the~~ his age and uncertain ~~clinical~~ status ~~following~~ according to the clinical criteria, but our WES-based screening ~~may~~ helped ease ~~this process of~~ his ~~disease definition~~ diagnosis by excluding the *FBN1* mutation found in other affected members of his family~~for III-1~~. In family 3, we detected that clinically unaffected subject I-3 had a slight anterior chest deformity (pectus excavatum) ~~slightly~~. ~~In spite that~~Although she had this ~~Marfan Syndrome~~MFS-related symptom, ~~they were~~she was clinically considered as a healthy individual~~s~~, which ~~is consistent with the gene-based diagnosis~~was borne out by the result that she had no *FBN1* or other pathogenic mutations. ~~Similarly, III-1 would be healthy.~~

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

Conclusions

In conclusion, ~~these~~ our ~~interpreted~~ results ~~could~~ may help ~~us~~ further ~~understand~~ elucidate the genetic pathology of MFS, and these mutations could be ~~regarded~~ included among probably pathogenic ~~as genetic~~ markers for pre- and postnatal screening and genetic diagnosis for MFS.

Additional information and declarations

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

• Zhening Pu analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

• Haoliang Sun and Junjie Du analyzed the data, contributed reagents/materials/analysis tools.

• Yue Cheng, Keshuai He. Buqing Ni and Weidong Gu performed the experiments.

• Juncheng Dai and Yongfeng Shao initiated, conceived and supervised the study, reviewed drafts of the paper.

Data Availability

The following information was supplied regarding data availability:

The data is included in the manuscript (Table 1, 2; Fig. 1 and Supplementary data).

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, http://www.genecards.org

Integrated Genome Viewer (IGV 2.3.80), http://www.broadinstitute.org/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:** ~~*FBN1* the~~ 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.