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De Novo Paternal *FBN1* Mutation Detected in Embryos Before Implantation

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Background: Marfan syndrome (MFS) is an autosomal dominant disease caused by mutations in the *Fibrillin* (*FBN*)*1* gene and characterized by disorders in the cardiovascular, skeletal, and visual systems. The diversity of mutations and phenotypic heterogeneity of MFS make prenatal molecular diagnoses difficult. In this study, we used preimplantation genetic diagnosis (PGD) to identify the pathogenic mutation in a male patient with MFS and to determine whether his offspring would be free of the disease.

Material/Methods: The history and pedigree of the proband were analyzed. Mutation analysis was performed on the couple and immediate family members. The couple chose IVF treatment and 4 blastocysts were biopsied. PGD was carried out by targeted high-throughput sequencing of the *FBN1* gene in the embryos, along with single-nucleotide polymorphism haplotyping. Sanger sequencing was used to confirm the causative mutation.

Results: c.2647T>C (p.Trp883Arg) was identified as the *de novo* likely pathogenic mutation in the proband. Whole-genome amplification and sequencing of the 3 embryos revealed that they did not carry the mutation, and 1 blastocyst was transferred back to the uterus. The amniocentesis test result analyzed by Sanger sequencing confirmed the PGD. A premature but healthy infant free of heart malformations was born.

Conclusions: The *de novo* mutation c.2647T>C (p.Trp883Arg) in *FBN1* was identified in a Chinese patient with MFS. Embryos without the mutation were identified by PGD and resulted in a successful pregnancy.

MeSH Keywords: Haplotypes • Marfan Syndrome • Preimplantation Diagnosis

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Background

Marfan syndrome (MFS) is a connective tissue disorder inherited in autosomal dominant fashion that mainly involves the cardiovascular, skeletal, and visual systems, but sometimes affects the lungs, skin, and dura [1]. The leading cause of death is cardiovascular symptoms, including dilatation of the aortic root, aortic dissection, mitral valve prolapse, and mitral and aortic regurgitation [2–4]. Skeletal manifestations include disproportionately tall stature, chest and spinal deformity, arachnodactyly, and joint laxity. The major abnormalities in the visual system are lens dislocation, myopia, cataracts, and retinal detachment [5]. The neonatal birth rate of MFS is 2–3/10 000 [6]; approximately 75% of cases are inherited and 25% arise from *de novo* mutations, with a spontaneous mutation rate of approximately 1/20 000 [7]. MFS is diagnosed in accordance with Ghent nosology criteria [8].

Fibrillin (*FBN*)*1* (NM_000138), the main causative gene of MFS, is located on the long arm of chromosome 15 (15q21) and consists of 65 exons distributed along 200 kb. *FBN1* encodes the extracellular matrix protein profibrillin-1, the main structural component of elastin-associated microfibers [9]. As of August 28, 2014, 1847 different mutations had been identified in *FBN1*, including missense (66%) and nonsense (10%) mutations; small insertions, deletions, or duplications (13%); and splicing errors (13%) [10].

The primary structure of *FBN1* gene is 3 cysteine-rich repeats [11]. There are 3 main domains in *FBN1* including 147 epidermal growth factor (cbEGF)-like domains, 27 latent transformation growth factor-binding protein(LTBP) domains and a unique microfibrillar protein structure. The hybrid motif has characteristics of classic cbEGF and LTBP domains; mutations in either one are common and frequently result in a single amino acid substitution that can influence calcium binding and protein stability [12].

Molecular diagnosis of MFS is made difficult by the diversity of mutations in the *FBN1* gene [11]. The offspring of an individual with MFS has a 50% risk of inheriting the disorder. Previously, prenatal diagnoses for MFS families were made by invasive procedures – i.e., chorionic villus sampling and amniocentesis performed at 12 and 16 weeks of gestation, respectively. Although the disease shows complete penetrance, there is still considerable clinical variability between and within families [13,14]. *FBN1* gene mutations are inherited to the offspring by autosomal dominantly, and in theory there is a 50% chance to transmit to the offspring. But during the embryo development and morphogenesis after fertilization, there will be some factors that affect the phenotype outside the gene, such as genomic imprinting which is the allele-specific expression of several genes [15,16] and epigenetic modification, the most known of which is DNA methylation. Even if the chromosomes carrying the mutation are inherited to the offspring, genomic imprinting may also occur, and the allele of the maternal may modify the dominant degree of the mutated gene, which may be the cause of heterogeneity of clinical manifestations. This is similar to Markandona's research on MLH3, a MutL homolog protein in mammals playing a role in DNA mismatch repair, whose wild genotype is GG, the heterozygotic genotype is GA, the homozygotic genotype is AA. When genotype was AA, the function of the repairing mechanism didn't completely eliminate because of the compensatory molecular repairing mechanisms of the oocyte [17,18]. In addition, epigenetics during the embryo development before and after implantation and some possible self- modification of embryos will lead to some diverse gene expression pattern even apoptosis or chromosomal abnormalities [19].

Many couples find it difficult to terminate a pregnancy when the fetus has been identified as carrying the mutated gene. To this end, pre-implantation genetic diagnosis (PGD) provides a means of determining the heritability of MFS before embryo implantation. Meanwhile the effect of abnormal sperm on embryonic development, IVF clinical outcome and the health of the offspring should be studied. Genomic imprinting and epigenetic modification is needed to take into account when making a clinical decision in PGD which is a means of blocking gene mutation inheritance. To do this, blastocyst biopsy is better than the blastomere biopsy in the process of PGD, because blastocyst development further with less embryonic self-corrective repair and chimerism, PGD test results will be more credible.

Study review of MFS PGD

A total of 12 studies have reported PGD of MFS since an initial report using reverse transcription (RT-)PCR [20]. Direct PCR analysis of mutations [21–26] has also been used along with linkage analysis [27–30]. A recent study described a successful PGD for an MFS couple based on karyomapping and direct mutation detection combined with linked short tandem repeat (STR) marker analysis that resulted in the birth of healthy twins [31]. However, linkage analysis requires customization with respect to the particular polymorphism in the family before PGD, whereas PCR has a high misdiagnosis rate due to technical limitations. In response to a questionnaire given to 857 patients with MFS, about half indicated that they would be willing to undergo PGD [24]. Examples of cases where PGD has been used in MFS families are listed in Table 1.

Material and Methods

Patient history

The present study describes the case of a couple, in which the male harbored an *FBN1* mutation, who requested genetic counseling and PGD at the reproductive center of the Chinese PLA

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General Hospital. The proband was 34-year-old male who developed tall stature, chest and spinal deformity, arachnodactyly, and typical cardiovascular and visual symptoms at an early age. He was diagnosed with MFS and underwent BentaU (prosthetic replacement of ascending aorta and aortic valve) surgery at An Zhen Hospital of Capital Medical University in Beijing in 2003. The proband's wife did not show symptoms of MFS, but had a history of chronic hypertension and diabetes. The proband was found to have 5 mutations in the coding region of the *FBN1* by targeted next-generation sequencing (NGS) at the Beijing Genomics Institute (BGI) (Table 2 and Figure 1). One of these was a heterozygous c.2647T>C (p.Trp883Arg) missense mutation. An analysis using SIFT software indicated the mutation was likely pathogenic, with a low frequency in the general population. Additionally, the proband had 2 missense mutations (p.Pro1148Ala and p.Cys472Tyr), a synonymous mutation (p.Asn625Asn), and a splice variant (c.3464-5G>A) at polymorphic sites in the coding region of *FBN1*.

The pedigree of this family was mapped based on family history information. We determined that c.2647T>C (p.Trp883Arg) in the proband was likely a *de novo* mutation according to the new American College of Medical Genetics and Genomics (ACMG) guidelines. We carried out a PGD by targeted NGS based on gene capture technology and linkage analysis of single-nucleotide polymorphism (SNP) haplotypes for the family, which had no reference for haplotyping. A premature but healthy girl without MFS was born to the couple. This study was approved by the Ethics Committee of Chinese PLA General Hospital. Written, informed consent was obtained from the couple and other participants.

Mutation analysis of the MFS family

The peripheral blood of patients and their immediate family members was collected for mutation analysis; peripheral blood of a normal people was used as control. Genomic DNA as extracted from blood samples using a kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions, and 3 µl were used as a template for PCR amplification with a kit (Takara Bio, Dalian, China). The total reaction volume was 50 µl and contained 0.5 µl each primer (20 pM; forward, 5'GATCTAAAAACATGTGAAGACATTG3' and reverse, 5'TGCACTCCTCGTCCTCGTAC3'); 4 µl dNTP (2.5 mM each); 10 µl of 10× buffer; and 0.25 µl Taq (5 U/µl) in and 31.75 µl sterilized distilled water. The reaction conditions were 95°C for 5 min; 31 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 30 s; and 72°C for 7 min. PCR products were sequenced by Life Technologies Invitrogen Trading (Shanghai, China).

In vitro fertilization (IVF) and embryo biopsy

The oral contraceptive (OC) Diane-35 and the long protocol were used for ovarian stimulation. After OC pretreatment for

Table 1. Review of MFS studies.

No.	Date	Author	Proband	Reproductive history	Mutation	Familial *de novo*	PGD method	No. of biopsied blastomeres	No. of biopsied embryos	No. of normal embryos	No. of implanted embryos	Pregnancy outcome
1	1995	Eldadah	Father	G3P0A3	c.4987T>C (p.Cys1163Arg)	CVS samples	RT-PCR mutation analysis	1–2 on day 3	No embryo transfer			
2	1996	Harton & Kilpatrick	Father	G1P0A1	NR	Familial	Linkage analysis: CA, mts-4	1–2 on day 3	12	6	5	Singleton pregnancy confirmed by CVS
3	1998	Grifo	NR	G2P0A2	NR	CVS samples	Fluorescence PCR mutation analysis	1–2 on day 3	5	1	1	No pregnancy
4	1998	Sermon	Father	G0P0A0	NR	Familial	Linkage analysis: mts-1, fluorescence PCR	1–2 on day 3	19 (3 cycles)	9	NR	No pregnancy
5	1998	Blaszczyk	Father	NR	Exon 12 (p.Cys1585Thr)	Familial	Nested PCR mutation analysis	1 on day 3	9	4	4	Normal ongoing pregnancy
6	2001	Toudjarska	Father	NR	c.1735G>A (p.Cys534Tyr)	NR	Nested PCR mutation analysis	1–2 on day 3	10	NR	2	Singleton pregnancy confirmed by umbilical cord blood test
7	2002	Loeys	Father	NR	Duplication of 15 nucleotides in exon 44	Familial	Nested PCR, CSGE, SSC	1–2 on day 3	1 cycle	NR	NR	Ongoing pregnancy
8	2006	Lledo	Father	NR	c.2446T>C (p.Cys816Arg)	Familial	MDA, linkage analysis: mts-2, -4	1 on day 3	7	4	2	Singleton pregnancy; healthy baby with no confirmed mutation

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Table 1 continued. Review of MFS studies.

No.	Date	Author	Proband	Reproductive history	Mutation	Familial *de novo*	PGD method	No. of biopsied blastomeres	No. of biopsied embryos	No. of normal embryos	No. of implanted embryos	Pregnancy outcome
9	2006	Spits	Father	G1P1A0	c.1868G>C (exon 15)	*De novo*	Linkage analysis: D15S1028, D15S992, D15S576		Spontaneous pregnancy			
			Mother	G0P0A0	c.4786C>T (exon 36)	Familial	Linkage analysis: D15S992, D15S196, D15S143		19 (3 cycles)	10	5	Healthy boy
			Father	G0P0A0	c.5208T>A (exon 39)	Familial	Linkage analysis: D15S1028, D15S992, D15S576		Delayed implantation			
			Father	G0P0A0	c.8213C>T (exon 62)	*De novo*	c.8213C>T D15S196		2	1	1	Ongoing pregnancy
			Mother	G0P0A0	c.1571_ 1572insA (exon 12)	Familial	Linkage analysis: D15S992, D15S196, D15S123	1–2 on day 3 or the first polar body	18 (4 cycles)	7	3	No pregnancy
			Father	G1P0A1	c.266G>T (exon 3)	*De novo*	Linkage analysis: D15S1028, D15S992, D15S576		6	6	1	No pregnancy
			Mother	G0P0A0	c.1122-1G>C (exon 8)	Familial	Linkage analysis: D15S992, D15S196, D15S143		5	4	1	Ongoing pregnancy
			Father	G1P0A1	c.5063C>T (exon37)	*De novo*	Linkage analysis: D15S992, D15S196, D15S143		23 (3 cycles)	13	2	Ongoing pregnancy
			Father	G0P0A0	c.2645C>T (exon 20)	*De novo*	c.2645C>T D15S992		14 (2 cycles)	6	3	No pregnancy
			Mother	G2P0A2	c.4786C>T (exon 36)	Familial	Linkage analysis: D15S1028, D15S992, D15S196, D15S143		Delayed implantation			
10	2010	Smith	Father	NR	c.6388G>A (p.Glu2130Lys)	*De novo*	PCR mutation analysis	1 on day 3	11	4	2	Twin pregnancy
11	2013	Vlahos	Mother	NR	c.2049C>A (p.Cys683X)	Familial	PCR mutation analysis	1–2 on day 3	9	1	1	Healthy boy
12	2015	Thornhill	Father	G2P1A1	c.235C>T (p.Gln79X); c.3089A>G (p.Asn1030Ser)	Familial	Karyomapping, linkage, direct mutation analysis	1–2 on day 3	8	4	2+1 (implanted twice)	Twin birth (one twin boy died), no pregnancy

NR – not reported.

one menstrual cycle, the 31-year-old female was given daily intramuscular injections (0.1 mg) of the gonadotropin-releasing hormone analog triptorelin acetate (Diphereline; Ipsen Pharma Biotech, Signes, France) from the mid-luteal phase of the cycle preceding the treatment. Recombinant follicle-stimulating hormone (FSH; Serono, Geneva, Switzerland) was then administered. Ovarian response was monitored based on transvaginal scanning and FSH, luteinizing hormone, and estrogen levels. When the leading follicle was 18 mm in diameter and there were at least 3 follicles with diameters of 15 mm, human

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Table 2. Five mutations in the coding region of *FBN1* (NM_000138) detected in the proband by targeted NGS.

Variant name	RS-ID	dbSNP frequency[a]	HapMap frequency[b]	1000 Genomes Project frequency[c]	Local frequency[d]
c.3464-5G>A	rs11853943	0.043	0.109	0.054	0.1795
p.Pro1148Ala	rs140598	0.143	0.429	0.1108	0.4154
p.Trp883Arg	novel	0	0	0	0
p.Asn625Asn	rs25458	0.085	0	0.1117	0.1744
p.Cys472Tyr	rs4775765	1	1	1.0	1.0

[a] Frequency information about this SNP was obtained from the dbSNP database; [b] Frequency information about this SNP among Asians was obtained from the HapMap database; [c] Frequency information about this SNP in all sequenced samples was obtained from the 1000 Genomes Project; [d] Frequency information about this SNP was obtained from 625 locally collected normal human samples. The table shows that the frequency information of the 5 mutations in the coding region of the proband's *FBN1*detected by NGS. The allele frequencies of the heterozygous c.2647T>C (p.Trp883Arg) missense mutation were 0 in the dbSNP, HapMap, 1000 Genomes Project, and 625 locally collected normal human samples, and the site was not found in the Human Gene Mutation. The other 2 missense mutations (p.Pro1148Ala and p.Cys472Tyr), a synonymous mutation (p.Asn625Asn), and a splice variant (c.3464-5G>A) were polymorphic change obtained by some normal people.

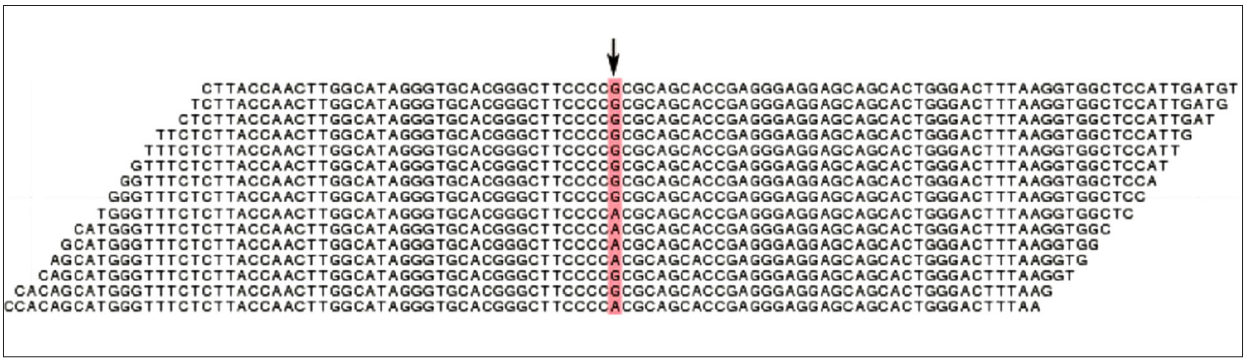

Figure 1. c.2647T>C mutation of the proband detected by targeted NGS by BGI. The figure shows the result of the antisense chain reads.

chorionic gonadotropin (HCG; Livzon Pharmaceutical, Shanghai, China) (10,000 U by intramuscular injection) was administered to trigger ovulation. Transvaginal ultrasound-guided oocyte retrieval was performed 36–38 h after HCG injection. Ten oocytes were harvested in G-GAMETE medium (Vitrolife AB, Goteborg, Sweden). After removal of granulosa cells, 9 mature oocytes were subjected to intracytoplasmic sperm injection and then incubated in G1 medium (Vitrolife AB). Fertilization was confirmed after 16–18 h by the presence of 2 pronuclei. Embryos were cultured for 48 h to the cleavage stage, transferred to G2 medium, and incubated for 48–72 h until they reached the blastocyst stage. Five to 8 trophectoderm cells herniating from the hole in the zona pellucida created by a noncontact laser (Saturn Research Instruments, Falmouth, UK) were biopsied from expanding blastocysts using the rubbing dissection method; the cells were washed in phosphate-buffered saline (PBS) and transferred to a PCR tube containing 2–4 µl sterile PBS. Each biopsied embryo was cryopreserved by vitrification. Solutions (Kitazato Medical Supply, Tokyo, Japan) were prepared according to the manufacturer's protocol. Four blastocysts were ultimately biopsied and trophectoderm cells were sent to the Clinical Laboratory of BGI-Shenzhen for gene sequencing on November 30, 2013.

Multiple displacement amplification (MDA)

The MDA reaction was performed using the REPLI-g Single Cell kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Briefly, 4 µl of biopsied trophectoderm cells (if less than 4 µl, the volume was made up to 4 µl with PBS) and 3 µl of denaturation buffer were mixed and incubated at 65°C for 10 min. A 3-µl volume of stop solution was added, and the 10-µl reaction mixture was combined with 40 µl of reaction master mix followed by incubation at 30°C for 8 h. The enzyme was inactivated at 65°C for 3 min and then maintained at 4°C.

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Targeted gene capture and NGS

MDA products of embryonic cells and genomic DNA extracted from the peripheral blood of the parents using the QIAamp DNA Blood Midi kit (Qiagen) were quantified, and 1 μg of each was fragmented into pieces ranging from 200–300 bp using a Covaris S2 ultrasonoscope (Woburn, MA, USA). DNA fragments were purified using Ampure Beads. T4 DNA polymerase, T4 phosphonucleotide kinase, and the Klenow fragment were used to repair the cohesive ends and terminal A bases were added using dATP and Klenow 3'-5' exonuclease (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. T4 DNA Ligase was used to connect the index linker to each library. The linker template was purified using magnetic beads. Purified DNA fragments were amplified and purified by ligation-mediated PCR, and the target sequence was captured using a custom human microarray chip; the 10-well marked DNA library was hybridized for 22–24 h at 47°C. Captured DNA fragments were enriched using streptavidin magnetic beads, and free fragments were eluted. Fragment sizes and concentrations of the sequencing DNA library were determined with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and ABI StepOne (Applied Biosystems, Foster City, CA, USA), and eligible DNA libraries were hybridized to the complementary linker on the Illumina sequencing flow cell. DNA libraries were amplified using high-fidelity DNA polymerase on the Illumina HiSeq 2500 platform and subjected to high-throughput sequencing. The average sequencing depth of each sample was greater than 100× to meet requirements for information analysis. Sequencing data were analyzed according to the manufacturer's protocol (Illumina). Raw image data were processed using Illumina Basecalling v.1.7 software. The sequencing procedure yielded bi-directional sequencing read lengths of 90 bp [32].

Sequence analysis

Low quality data and connector-contaminated reads were removed. Sequences were analyzed according to HG19 with the Burrows Wheeler Aligner [33]. At the same time, single-nucleotide variant and insertion/deletion queries were performed with SOAP SNP [34] and Samtools [35] software to identify polymorphisms in the target region. Results were compared to existing databases (National Center for Biotechnology Information SNP database (dbSNP), HapMap, 1000 Genomes Project, and 625 locally collected normal human samples), and suspected mutations were annotated and screened.

Sanger sequencing

To determine whether the embryo carried the father's pathogenic mutation, primers were designed up- and downstream of the c.2647T>C site. Puncture specimens were obtained from the amniotic fluid. PCR products were sequenced, and results were compared with the standard sequence of the *FBN1* gene (NM_000138) to verify results obtained by gene chip capture and high-throughput sequencing.

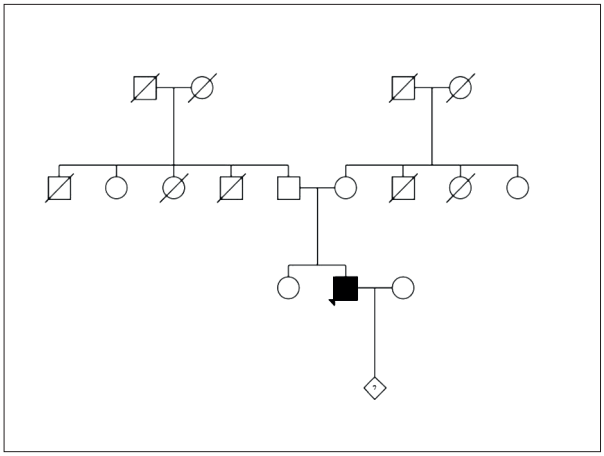

Figure 2. Pedigree of the MFS family reported in this study. Only the proband had the MFS.

Results

Pedigree analysis

The pedigree analysis of this family revealed that the proband's father (60 years, 174 cm), mother (61 years, 160 cm), sister (28 years, 170 cm), and wife (31 years, 165 cm) were in good physical condition with no obvious manifestations of MFS (Figure 2). The proband's father had family history of diabetes and myopia and his mother had no family history of disease. There was no MFS-related history among other family members of the proband and his wife.

Mutation analysis of patients and their families

Sanger sequencing of the *FBN1* gene in the patients and their family members revealed that only the proband had a bimodal (C/T) mutation in the gene of interest, which is in accordance with the targeted NGS results obtained at the BGI. The other family members showed a single peak (T) (Figure 3, where A–F are the sequencing results of *FBN-1* exon 21 of the proband, his mother, his father, a normal control, the proband's wife, and his sister, respectively).

Identification of the *de novo* mutation

The causative mutation c.2647T> C (p.Trp883Arg) in the proband was located in the hybrid motifs. It was predicted that the mutation would cause a change in protein conformation as a result of substitution of tryptophan by arginine. The allele

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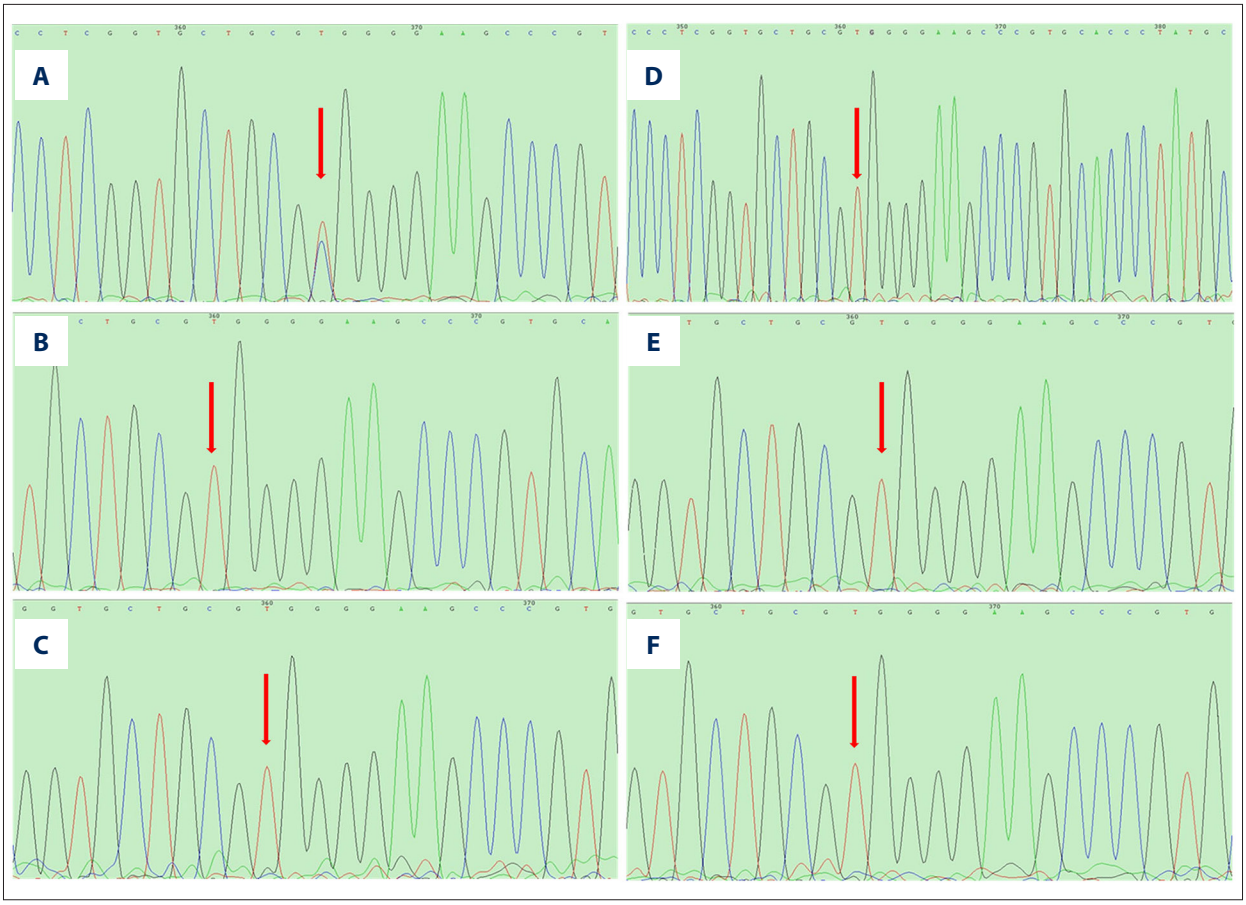

Figure 3. Mutation analysis of the MFS family by Sanger sequencing, where **A–F** are the sequencing results of *FBN-1* exon 21 of the proband, his mother, his father, a normal control, the proband's wife, and his sister, respectively. (**A**) Only the proband had a bimodal (C/T) mutation in the *FBN1*. (**B–F**) The other family members showed a single peak (T).

frequencies of this mutant site were 0 in the dbSNP, HapMap, 1000 Genomes Project, and 625 locally collected normal human samples, and the site was not found in the Human Gene Mutation Database (Table 2). None of the proband's immediate family members were carriers of this mutation, which showed evidence of PS2 + PM2 + PP1 + PP3 + PP4 according to the most recent version of the ACMG guidelines [36]; it was therefore classified as a *de novo* pathogenic mutation that had a 50% probability of being transmitted to the proband's offspring and the pre-implantation diagnosis of the c.2647T>C (p.Trp883Arg) site of *FBN1* for the couple is consistent with the ACMG guidelines.

Result of PGD

For the IVF treatment, 10 oocytes were harvested and fertilized, yielding 4 blastocysts that were biopsied. Whole-genome amplification (WGA) of one embryo failed. Mutation analysis performed by targeted high-throughput sequencing of the *FBN1* gene revealed that none of the 3 remaining embryos carried the paternal mutation (Table 3). Six samples were analyzed, yielding 171.24M of raw data of which 93.11% were of high quality (>Q20 and N ≤5) with an average utilization rate of 61.22%. The average coverage and depth of the targeted region were 100% and 97.49-fold, respectively. Of the 505 detected SNPs, we selected 225 informative *loci* linked to the *FBN1* gene for haplotype analysis.

Due of the absence of the reference proband, analysis of the family was not the same as conventional linkage analysis, which cannot distinguish between the parents of the haplotype. In the present case, we obtained the results according to the following information. 1) We knew the father was also the proband, and by sequencing we identified the mutation site in the father and the genotype. 2) By sequencing, we found that 3 of the 4 biopsied embryos were homozygous at this site; since the mother was homozygous while the father was heterozygous, the 3 embryos did not inherit the father's mutation. 3) In order to eliminate the possibility of allele dropout (ADO), we also analyzed SNPs within and up- and downstream of the *FBN1* gene, focusing on loci at which the mother as homozygous and the father heterozygous. The reason

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Table 3. Result of targeted NGS*.

Samples	Test result (c.2647 site)	Depth
Father	Mutation (T/C)	24: 23
Mather	Normal (T/T)	47: 0
Embryo 1	Normal (T/T)	75: 0
Embryo 2	Normal (T/T)	31: 0
Embryo 7	Test failed	test failed
Embryo 8	Normal (T/T)	33: 0

* None of the 3 embryos carried the paternal mutation(T/C), all of them were homozygous (T/T), as determined by targeted high-throughput sequencing.

Table 4. ADO in the PGD*.

Embryo	No. of candidate SNP *loci*	No. of ADO SNPs	Rate of ADO
E1	111	1	0.90%
E2		0	0.00%
E8		0	0.00%
Overall rate of ADO		1	0.30%

* ADO in the PGD was low and the results were credible.

for this criterion was that if there was ADO (for example, if the father was T/C and the mother was T/T, when the embryo was found to be T/T, it was likely to reflect ADO since the C may have been lost, or else it may be a true T/T; however, if the embryo was T/C or C/C, then it would have inherited the father's haplotype). In this manner, we found that the 3 embryos had the same paternal SNPs. The overall ADO rate of the 3 embryos was low (Table 4). Since the embryos had the same normal paternal haplotype (Figure 4), we deduced that they were normal.

Clinical outcome

The results of the PGD and other analyses were explained for the couple, and they chose to transfer an embryo. The remaining blastocysts are cryopreserved for future use. Due to their physical conditions, transplantation was delayed until October 8, 2015. A frozen embryo diagnosed as normal was thawed and cultured for 2 h before transfer into the woman's uterus for implantation, which resulted in pregnancy; HCG level 14 days after transplantation was 769.9 mIU/L. An amniocentesis test result performed by Sanger sequencing later confirmed the embryo as normal (Figure 5). Despite maternal gestational diabetes and pregnancy-induced hypertension, the woman gave birth to a healthy girl weighing 1445 g at 31 + 3 weeks of gestation. Although premature, the infant did not have cardiac malformations caused by the c.2647T>A mutation. We confirmed that the embryo haplotype inherited from the father was not linked to this mutation.

Discussion

In this study, the causative mutation c.2647T>C (p.Trp883Arg) of the proband was identified as a potential *de novo* pathogenic mutation. We carried out several analyses to determine whether this mutation was the cause of MFS in the proband. Firstly, this mutation has not been reported in the literature and the mutation frequency in various databases was 0, indicating that it should not result in a polymorphic change. Secondly, the mutated amino acid was located in the second hybrid motif of the protein and was predicted to be deleterious. On the other hand, c.2645C>T (p.Ala882Val) and c.2648G>A (p.Trp883X) were reported to be located near c.2647T>C (p.Trp883Arg) in the Universal Mutation Database. The former was located in the second hybrid motif and affected protein structure, and was classified as an MFS pathogenic mutation, whereas c.2648G>A – which encodes the same amino acid as the 2647 site – was also identified as a pathogenic mutation. The fact that only the proband had MFS and that none of his immediate family members were carriers of the mutation along with the evidence of PS2 + PM2 + PP1 + PP3 + PP4, c.2647T>C (p.Trp883Arg) is a novel mutation that can be used to diagnose MFS.

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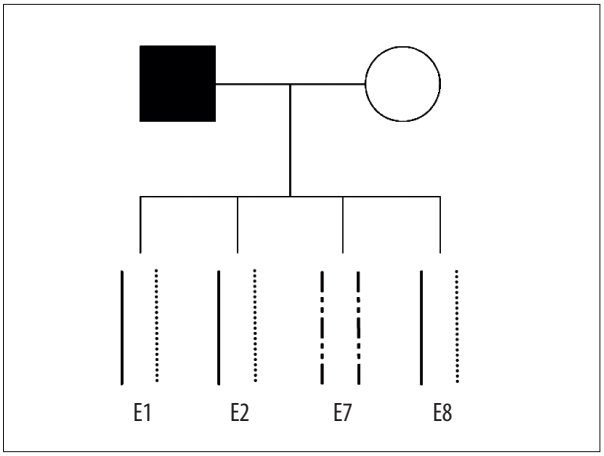

Figure 4. SNP haplotyping of embryos*. * The discontinuous lines indicate that the WGA of embryo 7 failed. All the other 3 embryos had the same paternal haplotype showed by the solid lines, while the dotted lines represent the maternal haplotype.

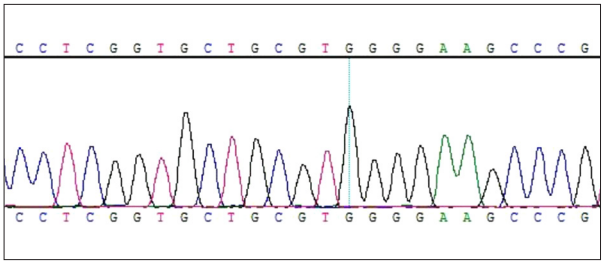

Figure 5. Sanger sequencing of the fetal DNA obtained by amniocentesis. The result showed a single peak in the antisense chain without the heterozygousc.2647 mutation.

The inheritance of monogenic diseases can be prevented by PGD. The recent development of single-cell WGA methods such as PCR, MDA, and multiple annealing and looping-based amplification has enhanced the application of PGD [37], which can be divided into 2 categories [38]: the first is linkage and comparative analyses including fluorescence *in situ* hybridization(FISH), array comparative genomic hybridization(aCGH), SNP arrays, karyomapping; and the second is direct mutation analysis and sequencing by PCR (RT-PCR, nested PCR, fluorescence PCR, and NGS). However, PGD has certain disadvantages in terms of the detection of aneuploidy and chromosomal mosaicism, contamination, ADO, amplification failure, and preferential amplification (PA), among others. Families with a history of monogenic disease can be analyzed for specific polymorphisms, although these must be identified before linkage analysis, which is labor-intensive. Direct sequencing can be used for *de novo* mutations, but this approach can lead to misdiagnosis. However, more reliable results can be obtained if it is combined with haplotyping of discarded embryos or single sperm [39].

In this study, PGD of the pathogenic mutations likely causing MFS in the proband was carried out by NGS based on gene capture technology and linkage analysis of SNP haplotypes. Gene capture technology employs a specific DNA or RNA probe corresponding to the region of interest to capture the target gene via base complementarily, thereby avoiding the detection of redundant non-target regions. The gene capture chip contained a large number of SNP *loci* covering the *FBN1* gene, eliminating the need for a priori customization of polymorphic markers for the family. Captured DNA was subjected to high-throughput NGS, which has the advantages of high accuracy, throughput, and sensitivity [40,41]. We performed a linkage analysis based on the sequenced SNPs, which are more widely distributed and are present at a higher density in the genome than STRs. SNP haplotyping can identify ADO, PA, and contamination to enhance the accuracy of PGD. Only STR or SNP haplotype analysis for this *de novo FBN1* mutation is impossible because of the absence of the reference in the family. So, the mutation must be analysis directly by the sequencing, and SNP locus for the haplotype are also determined by the NGS at the same time. The method used in this study was proved to be efficient and sensitive single gene disease diagnosis method through analysis of 6 genetic diseases [32].

Although we used PGD to select embryos that did not carry the parental mutant gene, in view of the presence of genomic imprinting, epigenetic and embryo self-correction in embryo development, the whole-genome sequencing of the blastocyst and genome-wide testing in prenatal diagnosis after pregnancy may be more helpful in obtaining better clinical outcomes. The study group account for this situation to patients and their families, the patient refused to do the whole-genome detection during pregnancy, only the detection of pathogenic genes.

After implantation of the embryo diagnosed as normal by PGD, the woman became pregnant and fetal DNA obtained by amniocentesis was confirmed by conventional Sanger sequencing as normal. The birth of a normal infant without the cardiac abnormalities of her MFS father also confirmed that the PGD results were correct.

In summary, the PGD method used in this study has the following advantages. 1) NGS with SNP haplotyping reduced ADO, PA, and other limitations of PGD and consequently, the rate of misdiagnosis. 2) It can be used to identify *de novo* mutations in families in which there is no reference. 3) It is suitable for all types of family-specific linkage analyses and does not require the labor-intensive identification of polymorphic markers. 4) Gene target capture eliminated the risk of analyzing redundant genes. However, a disadvantage of PGD is that it is only suitable for monogenic diseases, and cannot be used to detect other chromosomal abnormalities. This shortcoming can be mitigated by concomitant whole-genome exon

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sequencing. Meanwhile, given the technical limitations associated with single-sperm sequencing and the accuracy of sequencing methods in general, we did not perform pre-PGD single-sperm haplotyping, nor did we analyze the haplotype of the discarded embryos, although this may have helped to confirm the causative haplotype in the absence of a reference in an MFS family. The proband gene sequencing results showed the C/T heterozygous mutations, which may have been caused by gonadal mosaicism in the proband. The sperm haplotype analysis can be used to analyze whether the proband has gonadal mosaicism. And if the proband is gonadal mosaicism, the offspring would not inherit the paternal mutation when the normal *FBN1* genotype sperm is used for fertilization. But the detection of sperm will hurt the sperm. The existing detection technique cannot identify the sperm haplotype without damaging the spermatozoa, so only through the embryo PGD method to get healthy offspring.

Conclusions

We described here the identification of a novel likely pathogenic mutation of MFS and the use of PGD to screen embryos for this mutation so that an MFS couple could avoid giving birth to a child with the disease. The PGD method can also be applied to other autosomal dominant monogenic diseases and could be especially effective in combination with third-generation sequencing technology in the future.

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