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RESEARCH ARTICLE

Application of single-sperm sequencing in a male with Marfan syndrome: a case report and a literature review

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Abstract. Marfan syndrome (MFS) is caused by a *FBN1* mutation. Many organ systems are affected in patients with MFS, including the skeletal, ocular, cardiovascular and pulmonary systems. Cardiovascular manifestations are the main cause of mortality in patients with MFS. The mode of inheritance of MFS is autosomal dominant inheritance and the offspring are at great risk for the disease. Thus, the genetic testing for monogenic disease during preimplantation (PGT-M) is routinely advised for patients with MFS. PGT-M is a clinical genetic method to obtain normal embryos which are not affected by the monogenetic disorder. However, allele drop out (ADO) typically results in misdiagnosis during the PGT-M in the autosomal dominant disorder. Thus, a linkage analysis of polymorphic sites is used to identify ADO and improve the accuracy of PGT-M. However, when there are no family members affected, or the patients carry a *de novo* mutation, a linkage analysis cannot be performed to position the abnormal chromatid. Here, we performed single-sperm sequencing of preimplantation genetic testing in a male patient with MFS with a *de novo* mutation in *FBN1*. We constructed the chromosomal haplotype of the male patient by analysing information at the mutation site and at polymorphic sites. Next, the normal embryos were selected based on the results of high-throughput sequencing and haplotyping, and the one frozen embryo was transferred to the uterus. Finally, the preimplantation genetic testing results were confirmed by the prenatal genetic diagnosis during pregnancy, which showed that the foetus did not carry the pathogenic mutation. In conclusion, our research showed that single-sperm sequencing and haplotype analysis can be used in male patients with monogenetic disorders caused by *de novo* mutations to improve the accuracy of the preimplantation genetic diagnosis.

Keywords. single-sperm sequencing; haplotyping; preimplantation genetic diagnosis; Marfan syndrome; single-nucleotide polymorphism.

CL and FZ performed the experiments. JT, YM and LK analyzed the data. YZ, YZ, BL, JL and CL contributed reagents/materials/analysis tools. CL and YL wrote the manuscript.

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Introduction

Marfan syndrome (MFS) is one of the most common connective tissue disorders inherited in an autosomal-dominant manner. Patients with MFS have multisystem complications typically involving the ocular, cardiovascular and skeletal systems (Bitterman and Sponseller 2017). The leading cause of death in patients with MFS is rupture of an aortic aneurysm (Faivre *et al.* 2007). Additionally, the clinical features of MFS are age-dependent, and a partial manifestation has been observed in the general population (Judge and Dietz 2005). Moreover, many clinical manifestations in patients with MFS overlap with other connective tissue disorders, including Loeys–Dietz syndrome, Ehlers–Danlos syndrome, Beals syndrome, and MASS syndrome (Child *et al.* 2016). Therefore, diagnosing patients with MFS depends on the newly revised Ghent criteria, which require a clinical multisystem assessment. Approximately 99% of MFS cases are caused by mutations in *FBN1*, which is on chromosome 15 (15q15-q21.1). In total, 75% of patients with MFS inherit the mutation, whereas 25% develop a *de novo* mutation (Cañadas *et al.* 2010). To date, over 1000 FBN1 mutations have been identified, which are distributed throughout the whole gene sequence. The pathogenetic mutations between exons 24 and 32 tend to create a severe phenotype that results in death due to cardiopulmonary failure within the first year of life (Child *et al.* 2016). *FBN1* encodes fibrillin-1, which interacts with other extracellular matrix proteins to construct the microfibril; thus, reduced or abnormal fibrillin-1 expression due to a *FBN1* mutation results in tissue weakness (Castellano *et al.* 2014). Further, abnormal fibrillin-1 expression results in abnormal activation of transforming growth factor-β signalling, which regulates cell activities including cell apoptosis, proliferation, and differentiation and is critical for cellular matrix homeostasis (El-Hamamsy and Yacoub 2009). Abnormalities in cellular matrix homeostasis results in vascular remodelling, which is characterized by exaggerated elastolysis due to overexpression of matrix metalloproteinases (MMP-2 and MMP-9) and an increased hyaluronan content (Nataatmadja *et al.* 2006). As the hereditary mode of MFS is autosomal dominant, with complete penetrance, the prevalence in offspring with MFS is approximately 50% (Robinson *et al.* 2002). Thus, it is necessary to perform prenatal diagnostic or preimplantation genetic testing to avoid abnormal pregnancy. PGT for monogenic disease (PGT-M) is a clinical technology used to prevent pregnancy in women affected by monogenetic disorders (Sullivan-Pyke and Dokras 2018). A cell biopsy at the cleavage or blastocyst stage is crucial during PGT-M. Whole-genomic amplification is routinely performed because of the small amount of DNA in a single cell. Polymerase chain reaction (PCR) is the primary method used to amplify the mutation sites. Amplification efficiency, contamination, and allele drop out (ADO), which is characterized by an unamplified allele, affect the accuracy of PGT-M. The prevalence of ADO is 10–20% (Lee *et al.* 2017). Thus, polymorphic markers, including short tandem repeats and single-nucleotide polymorphisms (SNPs), which are unique sequences located on either side of an allele, are used to avoid ADO and combine the mutation sites to construct a haplotype for linkage analysis (Dolan *et al.* 2017). However, linkage analysis is not feasible in patients with *de novo* mutation or without affected relatives. In this study, we report a male patient with MFS who carried a *de novo* mutation site, and there was no affected relatives for molecular studies. To establish linkage between *FBN1* mutation site and SNPs, we performed single-sperm sequencing to identify the genotype of selected SNPs, establish the haplotype of FBN1 mutation site, and identify the chromatid carrying pathogenic mutation.

Materials and methods

The MFS pedigree involved four people from two generations. The proband (figure 1, IIa) and his mother (figure 1, IIb) were diagnosed with MFS. The clinical manifestations of his mother were abnormal, she was tall and had large hands and feet. The results of an electrocardiogram revealed abnormal cardiac structure. We diagnosed the mother of the proband with MFS based on a multiple system manifestation. The height of the proband was 190 cm, which was higher than the average height for that age group and the results of an electrocardiogram revealed aortectasia. The proband was also diagnosed with MFS according to the clinical manifestations and the positive family history. We carried out genetic testing on the family members to identify the carrying condition and origin of the mutation sites. First, we performed whole-exome sequencing in proband and his family to screen the suspicious mutations, and carried out Sanger sequencing to validate the suspicious mutations. The mutation site of the proband, as identified by the genetic diagnosis, was c.2370T> G (NM_000138.4, p.Cys790Trp) in *FBN1* (figure 2a) (Traven *et al.* 2017). Further, we referred to the ACMG Standards and Guidelines to determine whether the proband likely carried a pathogenic mutation (PS2 + PM1 + PM2 + PM5 + PP4) (Richards

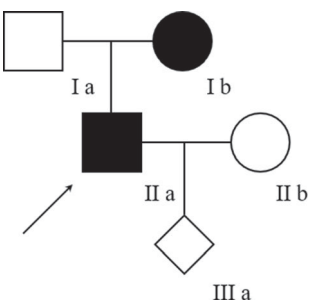

Figure 1. Marfan syndrome pedigree. (Ia) father of the proband; (Ib) mother of the proband; (IIa) proband with Marfan syndrome; (IIb) wife of the proband; IIIa, foetus during pregnancy.

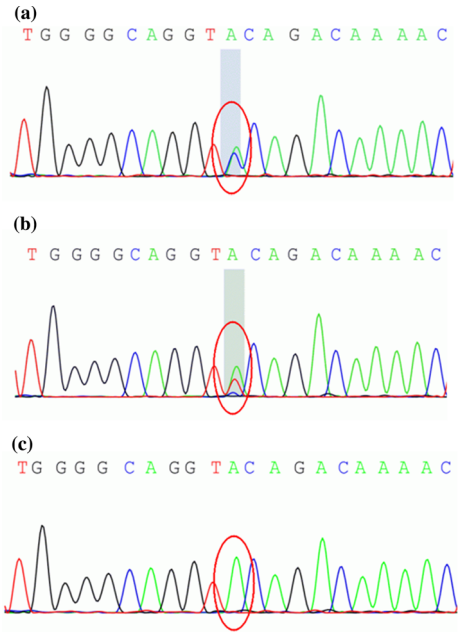

Figure 2. Results of pedigree validation indicated the genotype of *FBN1* gene site by Sanger sequencing. (a) Results of Sanger sequencing of the proband revealed that he carried the c.2370T>G(p.C790W) in *FBN1*. (b) Results of Sanger sequencing of the proband's mother revealed that she carried the c.2370T> A(p.C790R) at *FBN1*. (c) Results of Sanger sequencing of the proband's father revealed that he carried the c.2370T=T at *FBN1*.

et al. 2015). The genetic testing revealed that the mother carried the c.2370T> A (NM_000138.4, p.Cys790Ter) *FBN1* mutation (figure 2b). The father of the proband was confirmed to carry c.2370T= T (figure 2c). We could not perform a linkage analysis to identify the abnormal chromatid because of the different mutation sites carried by the proband and his mother. After genetic counselling, the couple decided to undergo single-sperm sequencing in PGT-M to avoid an abnormal pregnancy. The couple submitted their informed consent to PGT-M. The research was approved by the Ethics Committee of the Shengjing Hospital, China Medical University.

Single sperm separation and pathogenic site testing

We obtained the spermatozoa by the density gradient centrifugation. The spermatozoa was washed using the Sperm-Rinse and swim-up in G-IVF with 10% human serum albumin. The spermatozoa were transferred into the phosphate buffer saline (PBS) with 10% serum substitute supplement under mineral oil. Then we selected the single sperm with a micromanipulator pipette. PVP was used to immobilize the selected sperm and transfer them to another fresh PBS. Whole genome amplification (WGA) was performed for the five sperm cells by single cellular amplification technology (PicoPLEX WGA, Rubicon Genomic, USA). We performed PCR using the specified primers to amplify the pathogenic site region (5′-GAGGTCCCT-GAATCATGAAGCT-3′, 5′-GGTTTGTAGA-TAAATCCCTTGG-3′). Sanger sequencing was further carried out to screen the sperm cells with the wild-type and mutant pathogenic sites.

Haplotype construction

SNPs flanking 1 Mb of the pathogenic sites were selected for linkage analysis. We performed multiplex PCR technology for the genomic DNA of the proband, his wife and the single cellular amplification product of two single sperm cells. We constructed DNA library using the amplified products, and further complete DNA sequencing reaction. We analysed the genotype sites to screen the informative SNPs loci where the proband was heterozygous and his wife was homozygous.

Embryo culture and biopsy, WGA

A sperm with normal form was injected into oocytes by intracytoplasmic sperm injection (ICSI) to produce zygotes, which were cultured in sequential culture media for 5–6 days to reach the blastocyst stage. Nine surviving embryos reached the blastocyst stage. Next, 3–10 trophoblast ectoderm (TE) cells were collected from the embryo during the blastocyst stage as biopsy samples. The embryos were frozen immediately after biopsy. Elective transplantation of normal embryos was validated by PGT-M and PGT-A after thawing. The TE biopsies were subjected to cell lysis followed by whole genome amplification. The WGA products were quantified using the Qubit dsDNA HS Assay.

PGT-M and PGT-A

The WGA products were subjected to SNP site testing. We further analysed the sequence data to identify the SNP site genotype. Combining the haplotype results, we determined whether there were pathogenic mutant sites at the chromosome inherited from the proband. We further performed PGT-A of the embryos. A 300 ng portion of whole-nomic DNA was subjected to fragmentation using NEBNext dsDNA Fragmentase. We completed the DNA library construction and sequencing reaction utilizing the fragmentation products. And the sequencing data were aligned to the human genome database for the embryo aneuploid analysis.

Prenatal diagnosis

A total of 10 mL amniotic samples was collected from the wife of the proband at 19 weeks of pregnancy. Fetal

genomic DNA was separated from the amniotic sample and Sanger sequencing was performed to confirm whether the foetus carried the pathogenic mutation sites.

Results

Sperm separation and Sanger sequencing results

Five sperm samples were selected to perform the following analysis. The genomic DNA amplified products of the five sperm samples were obtained by WGA. We performed the Sanger sequencing to detect the genotype of pathogenic sites of amplified products. The Sanger sequencing results indicated that one wild-type sperm and one mutant-type sperm were successfully detected among the five sperms (figure 3, a & b).

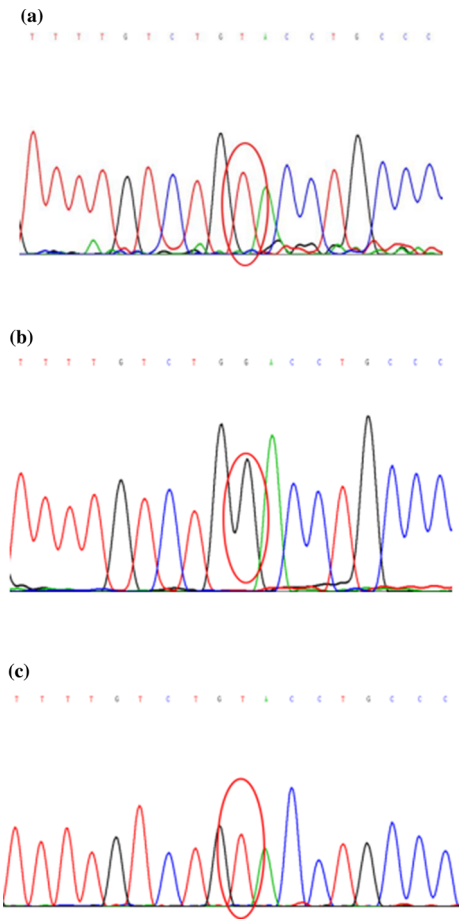

Figure 3. Results of Sanger sequencing of sperms and cultured amnion cells revealed the genotype of *FBN1* gene site. (a) Results of Sanger sequencing revealed that one sperm carried the wild-type allele (c.2370T=T) in *FBN1*. (b) Results of Sanger sequencing revealed that one sperm carried the mutant-type allele (c.2370T> G; p.C790W) at *FBN1*. (c) Results of amniocentesis at 19 weeks of pregnancy. Results of Sanger sequencing revealed that the genotype at the pathogenic site of the foetus was c.2370T=T.

Haplotype analysis results

We identified the genotype of the 79 SNP sites using NGS technology, whose site information and designed primer sequences are listed in table 1 in electronic supplementary material at http://www.ias.ac.in/jgent/. Of these SNP sites, 40 were located upstream of the pathogenic site and 39 were located downstream of the pathogenic site. We simultaneously tested 33 SNP sites in wild-type and mutant-type sperm cells to ensure testing accuracy. The haplotypes of the two sperm samples with wild-type and mutant site were consistent with the SNPs in the same region of the proband genomic DNA. Finally, 12 informative SNP markers were selected to construct the haplotype of the paternal disease-causing allele. We can identify abnormal embryos carrying the chromatid with FBN1 mutation site from the proband according to the haplotyping information. A total of three embryos (EmB326, EmB322 and EmB321) carried the mutated allele, which was consistent with the direct sequencing results of the mutation site (table 1).

PGT-M and PGT-A results

The PGT-A results indicated that four embryos (EmB325, EmB324, EmB320 and EmB319) were euploid (table 2). By combining the information of the chromosome haplotype with the PGT-M and PGT-A results, we detected four embryos that were completely normal (EmB325, EmB324, EmB320 and EmB319) (table 2).

Prenatal diagnosis results

We selected the high-level, euploid embryo (EmB324) without the pathogenic mutation to transfer into the uterus. An amniocentesis was performed at 19 weeks of pregnancy. The results of the amniocentesis revealed that the foetus did not carry the pathogenic mutation c.2370T> G in FBN1 (figure 3c).

Discussion

In the present study, we successfully applied a single sperm-based SNPs haplotyping for the PGT of the MFS family to help provide a healthy infant birth without the pathogenic mutation. PGT-M is the most common method to detect MFS and avoid abnormal pregnancy. However, MFS is an autosomal dominant disorder, and the ADO can result in a misdiagnosis during PGT-M (Piyamongkol *et al.* 2003). Linkage analysis during PGT-M was used to construct the haplotype to guarantee the accuracy of PGT-M. Hao *et al.* (2018) performed PGT for a couple carrying the nonsyndromic sensorineural hearing loss gene mutation. They established the haplotype based on NGS information from

Table 1. The result of linkage analysis to confirm the carrying status of paternal mutation.

CHROM	Position In CHROM	Wife	Husband	Sperm (mutation)	Sperm(wild-type)	EmB327		EmB326		EmB325		EmB324		EmB323		EmB322		EmB321		EmB320		EmB319	
						W	H	W	H	W	H	W	H	W	H	W	H	W	H	W	H	W	H
Chr15	48592060	A	A	A	G	A	G	A	A	A	G	A	G	A	G	A	A	A	A	A	G	A	G
Chr15	48592083	A	A	A	G	A	G	A	A	A	G	A	G	A	G	A	A	A	A	A	G	A	G
Chr15	48713018	G	G	G	A	G	A	G	G	G	A	/	/	G	A	G	G	G	G	G	A	G	A
Chr15	48788346	T	T	G	T	T	T	T	G	T	T	T	T	T	T	T	G	T	G	T	T	T	T
Chr15	49262185	G	G	G	A	G	A	G	G	G	A	G	A	G	A	G	G	G	G	G	A	G	A
Chr15	49276416	G	G	G	T	G	T	G	G	G	T	G	T	G	T	G	G	G	G	G	T	G	T
Chr15	49279406	A	A	A	G	A	G	A	A	A	G	A	/	A	G	A	A	A	A	A	G	A	G
Chr15	49291527	T	T	T	TA	T	TA	T	T	T	TA	T	TA	T	TA	T	T	T	T	T	TA	T	TA
Chr15	49389347	A	A	A	G	A	G	/	/	A	G	A	G	A	G	A	A	A	A	A	G	A	G
Chr15	49450944	T	T	T	C	T	C	/	/	/	/	T	C	T	C	/	/	/	/	/	C	T	C
Chr15	49455608	G	G	G	A	/	/	/	/	/	/	G	A	G	A	G	G	G	G	G	A	/	/
Chr15	49456526	A	A	A	G	/	/	A	A	A	G	A	G	A	G	A	A	A	A	A	G	A	G

CHROM, chromosome. The red Pos indicates the mutation from the proband C.2370T>G; / indicates the MISS; the allele carrying the mutation from the proband is indicated in blue; 'W' indicates the chromatid from the wife of the proband; 'H' indicates the chromatid from the proband.

Table 2. PGT-A and PGT-M results of embryos.

Embryo	Embryo level	Results of PGT-M	Results of PGT-A
EmB327	4AA	Wild type	dup(mosaic)(8)(q22.1-q24.3)
EmB326	4BC	Heterozygous mutation	dup(6)(q13-q24.2) del(mosaic)(7)(q11.22-q36.3)
EmB325	4BC	Wild type	Euploid
EmB324	4BB	Wild type	Euploid
EmB323	4BB	Wild type	-(mosaic)(4), -(mosaic)(9)
EmB322	4BB	Heterozygous mutation	− 16
EmB321	4BB	Heterozygous mutation	+ 13
EmB320	4BB	Wild type	Euploid
EmB319	4BB	Wild type	Euploid

PGT-A, preimplantation genetic testing of aneuploidy; PGT-M, preimplantation genetic testing of monogenic disorder.

the couple and their affected daughter. The authors surveyed the biopsy results of normal embryos and performed a haplotyping analysis; the results showed one normal embryo and five embryos with ADO that were not at pathogenic sites. They also found one embryo carrying a monosomic chromosome and one embryo showing simultaneous ADO and SNP site recombination. These results further revealed the advantages of haplotyping for PGT.

In the current study, the proband with MSF carried a different mutation from his mother. The two mutation sites were located in exon 20, which encodes the calcium-binding epidermal growth factor-like domain. The changes in the amino acid sequence caused by the mutations resulted in failed calcium binding. As a result, the protein could breakdown in this domain to cause abnormalities in fibrillin-1 function. An abnormal protein could result in abnormal biological processing and further lead to a multiple system manifestation.

Due to the different mutation in the proband and his mother, we could not perform a linkage analysis to identify the chromatid carrying the pathogenic mutation. We conducted single-sperm haplotyping based on NGS for the proband to identify the chromosomal haplotype. We selected informative SNP sites as markers to construct the haplotype in the current study. Compared to short tandem repeat sites, SNP sites have advantages including large number, wide distribution, stable inheritance, and uniform coverage. Chen *et al.* (2019) used a similar method for a male patient with osteogenesis imperfecta. They selected mutation sites and sufficient SNP sites flanking the gene region containing the pathogenetic sites to establish the haplotype. We successfully constructed the haplotype by combining the 11 SNP sites and the pathogenic sites. We also successfully selected the normal embryos to transplant to the uterus according to the haplotype information of the embryos. Finally, the wife of the proband had a successful pregnancy. The result of the amniocentesis at 19 weeks of pregnancy revealed that the foetus did not carry the same mutation at the pathogenic site.

In summary, single-sperm-based haplotyping by NGS can be used to determine patient's haplotype when this information cannot be accurately established by linkage analysis due to *de novo* mutations. This method decreases the rate of misdiagnosis due to ADO during PGT-M while increasing the accuracy of PGT-M.

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