The clinical application of preimplantation genetic diagnosis for the patient affected by congenital contractural arachnodactyly and spinal and bulbar muscular atrophy

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Abstract

Purpose To investigate the usefulness of preimplantation genetic diagnosis (PGD) for the patient affected by congenital contractural arachnodactyly (CCA) and spinal and bulbar muscular atrophy (SBMA).

Methods Multiple displacement amplification (MDA) was performed for whole genome amplification (WGA) of biopsied trophoctoderm (TE) cells. Direct mutation detection by sequencing and next-generation sequencing (NGS)-based single nucleotide polymorphism (SNP) haplotyping were used for CCA diagnosis. Direct sequencing of the PCR products and sex determination by amplification of sex-determining region Y (SRY) gene were used for SBMA diagnosis. After PGD, the unaffected blastocyst (B4) was transferred in the following frozen embryo transfer (FET).

Results In this PGD cycle, sixteen MII oocytes were inseminated by ICSI with testicular spermatozoa. Four blastocysts

(B4, B5, B10, B13) were utilized for TE cell biopsy on day 5 after ICSI. After PGD, B4 was unaffected by CCA and SBMA. B5 was affected by CCA and carried SBMA. B10 was unaffected by CCA and carried SBMA. B13 was affected by CCA and unaffected by SBMA. B4 was the only unaffected blastocyst and transferred into the uterus for the subsequent FET cycle. The accuracy of PGD was confirmed by amniocentesis at 21 weeks of gestation. A healthy boy weighing 2850 g was born by cesarean section at the 38th week of gestation.

Conclusions PGD is a valid screening tool for patients affected of CCA and SBMA to prevent transmission of these genetic diseases from parents to children.

Keywords Congenital contractural arachnodactyly · Spinal and bulbar muscular atrophy · PGD · Frozen embryo transfer

Capsule A description of a PGD cycle for the diagnosis of CCA and SBMA, resulting in the birth of a healthy boy.

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Introduction

Preimplantation genetic diagnosis (PGD) has been widely applied to the diagnosis of monogenic disorders, X-linked disorders, and structural and numerical chromosomal abnormalities since 1990 [1], and to date, more than 200 monogenic disorders and X-linked disorders have been diagnosed by PGD [2]. Additionally, it has been reported that the neonatal outcomes of PGD are comparable to those of ICSI treatment [3, 4]. PGD has been used to screen unaffected embryos for couples with a known genetic disease to avoid the emotional and financial burden associated with an affected child, pregnancy termination of the affected fetuses by prenatal diagnosis or recurrent miscarriages. A couple, the male affected by congenital contractural arachnodactyly (CCA) and spinal and bulbar muscular atrophy (SBMA) with a 10-year-old son affected by CCA, was enrolled in this study and wanted to prevent the transmission of the genetic diseases to their offspring by PGD.

Congenital contractural arachnodactyly (CCA; MIM 121050), also known as Beals syndrome, is an autosomal dominant heritable connective tissue disorder characterized by crumpled ears, arachnodactyly or camptodactyly, joint contractures (especially elbow and knee joints), narrow body habitus, and scoliosis [5, 6]. However, the incidence and prevalence of CCA is difficult to estimate due to the overlap in clinical features with Marfan syndrome (MFS; MIM 154700). Although there are overlapping clinical features between CCA and MFS, they have different molecular mechanisms and result from two homologous gene mutations: CCA is caused by a mutation in the fibrillin 2 gene (FBN2) on chromosome 5q23-31, whereas MFS is caused by a mutation in the fibrillin 1 gene (FBN1) on chromosome 15q15-21.3. Forty-eight mutations (including nonsense and missense mutations, insertion/duplication, and splice-site mutations) have been reported in the middle region of the FBN2 gene, specifically from exons 22 to 36 [7, 8]. Most mutations in FBN2 are missense mutations that disrupt one of the calcium-binding EGF (cbEGF) domains, which are thought to be essential for the conformation and stability of fibrillin-2 [9, 10]. The FBN2 gene is thought to be the only gene associated with CCA. Thus far, only prenatal diagnoses by ultrasonography have been used to detect CCA for families at risk for this disease [11, 12].

Spinal and bulbar muscular atrophy (SBMA; MIM 313200), also known as Kennedy's disease, is an X-linked recessive and adult-onset motor neuron disease characterized by slowly progressive weakness and atrophy in the limb and bulbar muscles [13, 14]. SBMA is caused by an expansion of trinucleotide cytosine adenine guanine (CAG) repeats in exon 1 of the androgen receptor (AR) gene on chromosome Xq11-12 [15]. The mutant AR protein is prone to aggregation and forms intracellular inclusions in SBMA, resulting in toxicity to neurons [16]. It has been reported that the prevalence of this disorder is

approximately 1 in 40,000 in many different ethnic groups. To date, there is no available treatment or cure for SBMA.

However, PGD for CCA associated with SBMA has not yet been reported. In this study, we applied two genotyping methods for each monogenic disorder and prenatal diagnosis for PGD of CCA and SBMA to ensure the accuracy of PGD. This is the first report of using PGD to screen the unaffected blastocysts of a patient affected by CCA and SBMA, resulting in the birth of a healthy boy.

Materials and methods

Patient

A couple, of which the male was affected by CCA and SBMA and who had a 10-year-old son affected by CCA (Fig. 1), attended our reproductive medical center for PGD. The couple signed informed consent forms for ICSI treatment, PGD, and follow-up. Luteal phase downregulation, controlled ovarian stimulation, oocyte retrieval, and ICSI procedures were performed according to the routine protocols of our reproductive medical center. This project was approved by the Ethics Committee of the Drum Tower Hospital, which is affiliated to Nanjing University Medical College.

Embryo culture, TE biopsy, and vitrification

All embryos were cultured in sequential media (G1 and G2, Vitrolife) at 37 °C and high humidity under 6 % CO₂, 5 % O₂, and 89 % N₂ in a Labo C-Top mini-incubator. For the trophoblast (TE) biopsy, a 12-μm hole was made in the zona pellucida of the embryos using a laser on the morning of day 3 after ICSI. The TE biopsy was performed when the TE cells had herniated out of the zona pellucida. In our diagnosis, four blastocysts (B4, B5, B10, B13) were utilized for TE biopsy on day 5 after ICSI. Approximately five to ten TE cells

Fig. 1 Pedigree of the family with CCA and SBMA. Squares and circles indicate males and females, respectively. Arrow indicates the probands, the solid symbols represent those affected by CCA, black symbols represent those affected by SBMA (X-linked), and circles with the blank circle represent those females carried SBMA

away from the inner cell mass were aspirated into a biopsy pipette (internal diameter, 20 μm) and dissected using a laser. Biopsied TE cells were washed three times in PBS (SAGE) and transferred to a 0.2-ml PCR tube for whole genome amplification (WGA). The blastocysts were vitrified after the TE biopsy using a Kitazato vitrification kit (Kitazato Biopharma). Each blastocyst was loaded onto an individual cryotop and stored in liquid nitrogen. The vitrification procedure was performed according to the manufacturer's protocols.

WGA and PCR

Multiple displacement amplification (MDA) was performed using a REPLI-g Single Cell Kit (Qiagen, Germany) for WGA. PBS was added to the samples and blanks to give a volume of 4 μl , which was mixed with 3 μl of buffer D2 and then incubated at 65 °C for 10 min followed by the addition of 3 μl of stop buffer. The obtained solution (10 μl) was mixed with 40 μl of master mixture for WGA, incubated at 30 °C for 8 h, and heat inactivated at 65 °C for 3 min. The MDA products were used for PCR identification immediately or stored at -20 °C. PCR was performed in a 50- μl reaction system containing 5 μl of 1/100 diluted MDA products or 100 ng genomic DNA (gDNA), 5 μl of $\times 10$ PCR buffer, 1.5 μl of 50 mM MgCl_2 , 1 μl of 10 mM dNTP mix each, 1 μl of 10 μM primer each, and 1.5 units of Taq DNA polymerase (Invitrogen). The DNA was denatured at 96 °C for 3 min followed by 40 cycles of denaturation at 96 °C for 30 s, annealing with primers at 55 °C for 30 s and elongation at 72 °C for 1 min, and a final elongation at 72 °C for 5 min. The PCR products were then analyzed by agarose gel electrophoresis and DNA sequencing.

CCA diagnosis procedure

Direct mutation detection by sequencing of PCR-amplified exon 33 of the FBN2 gene

For sequencing analysis, a pair of primers was designed (5'-caccctcagtagaagtcagaatg-3', 5'-tacatagtcacctgcaagtcaag-3') for amplification of exon 33 of the FBN2 gene. PCR products were gel separated and cut out individually for purification and then sequenced by Shanghai Sangon.

PGH analysis by NGS-based SNP haplotyping

A panel of 58 high-frequency single nucleotide polymorphism (SNP) (Minor Allele Frequency, MAF > 0.2) markers located 5 Mb upstream and downstream of the FBN2 gene in the genomes of CHB (Han Chinese in Beijing) and CHS (Southern Han Chinese) from the 1000 Genomes Project and a pathogenic mutation site in exon 33 of the FBN2 gene were chosen for next-generation sequencing (NGS)-based

SNP haplotyping. These 59 sites were submitted to a primer design website. The MDA products and designed primers were used for library preparation by an Ion Torrent AmpliSeq panel (Life Technologies). Library enrichment was then performed and followed by chip loading for Personal Genome Machine (PGM) (Life Technologies) sequencing. The detailed procedure was performed according to the manufacturer's protocol (Life Technologies). The data from the PGM sequencing was analyzed by Peking Jabreho Med Tech., Ltd.

SBMA diagnosis procedure

Direct sequencing of the PCR-amplified CAG repeat region

For the sequencing analysis, a pair of primers from exon 1 of the androgen receptor (AR) gene was synthesized (5'-gggaagtaggtggaagattcagcca-3', 5'-tggcctcgtcaggatgtcttaag-3') for the amplification of the CAG repeat region. Both the upper and lower bands were gel-separated and cut out individually for purification and then sequenced by Shanghai Sangon.

Sex determination by amplification of the SRY gene

For amplification of the sex-determining region Y (SRY) gene, a pair of primers from the SRY gene coding sequence was synthesized (5'-agcgatgattacagtcagctgtgc-3', 5'-caattctcggcagcatcttcgcct-3'). PCR products were then analyzed by agarose gel electrophoresis and sequencing.

FET and follow-up

Five months after oocyte retrieval, the patient was treated with oral Progynova to prepare the endometrium for frozen embryo transfer (FET). When the endometrium reached adequate thickness (≥ 8 mm), this phase was complemented by administration of intramuscular progesterone. The vitrified-warmed blastocyst (B4) was transferred on day 6 of progesterone administration. Serum β -hCG levels were measured at 14 days after FET. Clinical pregnancy was defined as the presence of a gestational sac and fetal heartbeat by sonography at 6 weeks after FET. Amniocentesis was performed at 21 weeks of gestation.

Results

IVF and TE biopsy

Seventeen cumulus-oocyte complexes were collected, and sixteen MII oocytes were inseminated by ICSI with testicular spermatozoa. The injected oocytes were checked for

fertilization at 16–18 h post-ICSI, and nine had fertilized normally with the presence of two pronuclei. Eight embryos developed to 6–10 cells on day 3 after ICSI. Four blastocysts (B4, B5, B10, B13) were utilized for TE biopsy on day 5 after ICSI. The biopsied TE cells were used for WGA.

CCA diagnosis results

Direct sequencing analysis was successful for 4/4 (100 %) of the biopsied TE cells from four blastocysts (B4, B5, B10, B13) (Fig. 2a). The results of the direct sequencing indicate that B4 and B10 have the wild homozygote c.4274G/G (unaffected blastocysts) in exon 33 of the FBN2 gene, whereas B5 and B13 have heterozygous c.4274 G > A (p.Cys1425Try) mutations (affected blastocysts) in exon 33 of the FBN2 gene. For the preimplantation genetic haplotyping (PGH) analysis, we applied NGS-based SNP haplotyping. Based on the SNPs in the affected child (III-1), we selected heterozygote SNPs in the affected father (II-2) and homozygote SNPs in the healthy mother (II-1) at the same loci to construct the blastocyst haplotypes. The haplotypes were constructed from 12 informative SNPs from 58 selected high-frequency SNP markers and a pathogenic site (Fig. 2b). B4 and B10 demonstrated an

unaffected haplotype with the normal paternally inherited allele present, whereas B5 and B13 demonstrated an affected haplotype pattern similar to the affected child. There was consistency between the direct sequencing and the PGH analysis results from the four blastocysts. The above results showed that B4 and B10 were unaffected by CCA, whereas B5 and B13 were affected by CCA.

SBMA diagnosis results

The lengths of the normal and expanded parental alleles in exon 1 of the AR gene were determined by direct sequencing of the PCR-amplified CAG repeat region (Fig. 3a). The father (II-2) had an expanded allele of 53 repeats, whereas the mother (II-1) had two normal alleles of 25 repeats. B4 and B13 had a normal allele of 25 repeats, whereas B5 and B10 had a normal allele of 25 repeats and an expanded allele of 53 repeats. SRY amplification in the MDA products of the biopsied TE cells from the four blastocysts (B4, B5, B10, B13) showed that B4 and B13 were male blastocysts, whereas B5 and B10 were female blastocysts (Fig. 3b). There was no discordance between the number of CAG repeats by direct sequencing and the SRY amplification results from the four blastocysts. B4

Fig. 2 Direct mutation detection by sequencing and NGS-based SNP haplotyping for CCA diagnosis. **a** Direct sequencing showed heterozygous c.4274 G > A (p.Cys1425Try) mutations in exon 33 of the FBN2 gene from II-2, III-1, B5, and B13. However, II-1, B4, and B10 had the wild homozygote c.4274G/G. **b** Diagram showing the haplotype analysis of mother (II-1), father (II-2), III-1, and four preimplantation blastocysts (B4, B5, B10, B13). III-1, B5, and B13 demonstrated an affected haplotype (red-line allele) from the father, whereas B4 and B10 demonstrated a normal haplotype (blue-line allele). The pink circle marks recombination on the SNP marker between the mother and the affected blastocyst (B13)

Fig. 3 Direct sequencing of the PCR products and sex determination by amplification of the SRY gene for SBMA diagnosis. **a** Analysis of the PCR products by amplification of the CAG repeat region in exon 1 of the AR gene. *N* and — indicate a normal man and blank control, respectively. B4 and B13 had a normal allele of 25 CAG repeats, whereas B5 and B10 had a normal allele of 25 CAG repeats and an expanded allele of 53 CAG repeats. **b** Sex determination by amplification of the SRY gene. B4 and B13 were the male blastocysts, whereas B5 and B10 were female blastocysts. XY, XX, and — represent positive, negative, and blank controls, respectively

and B13 were male blastocysts unaffected by SBMA, whereas B5 and B10 were female blastocysts that carried SBMA.

Follow-up results

The level of serum β -hCG was 319 IU/l at 14 days after FET. A successful pregnancy was achieved, and a positive fetal heartbeat was measured at 6 weeks by sonography after FET. There were no mutations observed in exon 33 of the FBN2 gene from the cultured amnion cells gDNA (Fig. 4a). A normal male karyotype with 46, XY was shown by a cytogenetic analysis of cultured amnion cells (Fig. 4b). At 38 weeks, the patient delivered by cesarean section a healthy boy with normal fingers (Fig. 4c) weighing 2850 g and there were no mutations observed in exon 33 of the FBN2 gene from peripheral blood gDNA (Fig. 4a).

Discussion

This study described the successful clinical utility of trophoctoderm biopsy and blastocyst vitrification, whole genome amplification followed by two genotyping methods for each monogenic disorder, FET, and prenatal diagnosis for PGD of CCA and SBMA. To our knowledge, this is the first report of a healthy birth following the use of PGD to select an unaffected blastocyst for FET from a patient affected by CCA and SBMA.

Although PGD can be used for almost all genetic disorders with a recognized mutation, allele dropout (ADO), where one of the two alleles is preferentially amplified and the other allele is amplified under a detectable level, is the primary inherent pitfall of single-cell PCR and can lead to misdiagnoses [17, 18]. For autosomal dominant disorders, ADO of the affected allele would cause a serious misdiagnosis for the transfer of an affected embryo. In recent years, trophoctoderm

cell biopsies from the blastocyst, which provide more cells than blastomere biopsies from cleavage-stage embryos (5–10 cells vs. 1–2 cells), have been used for genetic analyses to reduce the incidence of ADO and amplification failure and to increase the accuracy of genetic diagnosis [19, 20]. The genotyping success rate was higher in the trophoctoderm cell biopsy group (94 %) than in the blastomere biopsy group (75 %), and the rates of amplification failure and ADO were 6 and 0 % in the former group, whereas they were 10 and 15 % in the latter group, respectively [19]. In our study, there was no ADO happened. Moreover, polymorphic markers with short tandem repeats (STR) or SNPs close to the mutated gene for PGH have been used for linkage analyses to find ADO and avoid misdiagnosis [20–22]. Cryopreservation of the biopsied blastocysts without fresh transfer can provide sufficient time for genetic analyses [23]. Furthermore, WGA can provide sufficient DNA templates for genetic analyses of gene mutations, PGH analyses by polymorphic markers. Therefore, trophoctoderm biopsy, the cryopreservation of biopsied blastocysts, and WGA followed by genotyping comprise a viable strategy for PGD [23, 24].

CCA is an autosomal dominant connective tissue disorder, although the PGD of CCA has not been reported. In the present family, this disease was caused by a heterozygous point mutation (c.4274 G > A) in exon 33 of the FBN2 gene of the father. Now, several linked polymorphic markers (STRs or SNPs) in conjunction with pathogenic mutations have been used in preimplantation genetic haplotyping (PGH) analyses for PGD [20, 25]. Therefore, we performed direct mutation detection by sequencing and a PGH analysis for PGD of CCA (Fig. 2) to prevent misdiagnosis by the single genotyping method. Our method for this detection is similar to Qubbaj's report [21]. In their study, they applied direct mutation detection by sequencing combined with PGH for PGD of congenital hyperinsulinism caused by a homozygous point mutation. First, they analyzed the MDA products by sequencing the blastomeres of biopsied embryos. Then, they applied seven informative linked STR markers for the PGH analysis to validate the results of the sequencing, and they found that one out of four embryos was normal according to the sequencing. However, the embryo showed only the normally derived paternal allele from the PGH analysis, which suggested a haploid or monosomy pattern for that chromosome. PGH can distinguish the chromosome of the pathogenic gene from the normal chromosome, haploid, and diploid embryos. All these can not be obtained from direct mutation detection by sequencing. Therefore, PGH analysis combined with a mutation analysis can successfully identify misdiagnoses and prevent an affected embryo transfer. However, meiotic recombination could impair accurate diagnoses [26]. Because the linked polymorphic marker is not the pathogenic mutation gene, a misdiagnosis is possible if recombination occurs and separates the pathogenic allele and the linked marker [27]. Although

Fig. 4 Genetic analysis of the cultured amnion cells and the shapes of fingers of members of the family. **a** DNA sequencing analysis. The DNA sequencing results showed the wild homozygote c.4274G/G in exon 33 of the FBN2 gene from the gDNA of cultured amnion cells and peripheral blood of male baby. **b** Karyotype analysis of the cultured amnion cells. G-

band analysis at a 400–500 band level showing a normal male karyotype with 46, XY. **c** The shapes of fingers of members of the family. The mother (II-1) and the male baby exhibited normal fingers, whereas the father (II-2) and the baby's affected brother (III-1) exhibited camptodactyly fingers (the *arrows* in the diagram)

Fig. 4 continued.

loci 1 Mb apart demonstrate approximately 1 % recombination, recombination between the pathogenic allele and the linked marker can never be excluded, even for very tightly linked markers [28]. Therefore, it is necessary to select more polymorphic markers away from the pathogenic mutation site for haplotyping. Considering the more large number and wide distribution of SNP in human genome, we chose NGS-based SNP haplotyping, which was first used to provide more informative genetic markers per embryo in high throughput and to overcome the problem of recombination to ensure the accuracy of the PGD. Because there are genetic variations among human populations, an SNP allele that is common in one geographical or ethnic group may be much rarer in another. Therefore, we selected a panel of 58 high-frequency SNP markers located 5 Mb upstream and downstream of the FBN2 gene in the genome of the Han Chinese population for NGS-based SNP haplotyping. Twelve informative SNP markers and the pathogenic mutation site were finally used for haplotype construction. Recombination only occurred in one (B13) out of four blastocysts and derived from maternal chromosome. This recombination did not impair accurate diagnosis (Fig. 2b), which was consistent in the sequencing results. According to our findings, the PGH analysis combined with the mutation analysis successfully prevented misdiagnosis and discerned an unaffected embryo.

SBMA is an X-linked recessive disease. Based on the genetic analysis, the male blastocysts were unaffected by SBMA, whereas the female blastocysts were carriers of SBMA. Therefore, sex detection of the blastocysts was sufficient for the identification of SBMA. However, it has been reported that the detection of sexing for X-linked disease by PCR could result in a misdiagnosis [17]. To prevent misdiagnoses, we simultaneously applied two genotyping methods: a direct analysis for the number of CAG repeats in exon 1 of the AR gene and sex determination by amplification of the SRY gene to diagnose SBMA. SBMA is caused by an expansion of the CAG repeat in exon 1 of the AR gene, and individuals with more than 40 CAG repeats should be diagnosed with SBMA; the average CAG repeat length from patients with SBMA is 47 CAGs [29]. Patients with SBMA have two important clinical features, one is slowly progressive weakness and atrophy in the limb and bulbar muscles and the other one is androgen insensitivity. Androgen insensitivity mainly shows gynecomastia, hypogonadism, and reduced fertility (azoospermia, oligospermia) [30, 31]. Patients with SBMA usually present muscle weakness in their mid-forties [32]. However, the age of disease onset correlates inversely with the CAG repeat length, i.e., greater CAG repeat lengths correspond to earlier disease onset [29]. Christopher Grunseich et al. reported a 29-year-old patient with SBMA who had 68 CAG repeats in the AR

gene and who had presented weakness since age 18 [14]. In our study, the father (II-2), 37 years old, had 53 CAG repeats (Fig. 3a) in the AR gene, which exceeded the average CAG repeat (47 CAG repeats). Although the father did not present muscle weakness, he presented another clinical feature of SBMA, which was azoospermia. There was no sperm in the semen when the couple came to our reproductive center for PGD treatment, and only a small amount of sperm in his testis by testicular biopsy, so testicular spermatozoa were used for ICSI. After diagnosis, the mother (II-1) and the affected child (III-1) had the normal 25 CAG repeats in the AR gene. The male blastocysts (B4, B13) were unaffected by SBMA, exhibiting a normal allele of 25 repeats, and the female blastocysts (B5, B10) were carriers of SBMA with a normal allele of 25 repeats and an expanded allele of 53 repeats (Fig. 3a). As a result, we obtained consistent results for both of the genotyping methods (Fig. 3).

Although PGD has been applied successfully in the clinic, misdiagnoses discovered by prenatal diagnosis have been reported [2, 18]. The European Society of Human Reproduction and Embryology (ESHRE) PGD consortium reported that the misdiagnosis rates of the PCR-based and FISH-based PGD cycles were 0.14 % (13/9317) and 0.06 % (21/34,855), respectively [2]. Prenatal diagnosis should be recommended for pregnancy with PGD to prevent the birth of an affected fetus due to misdiagnosis. In our study, prenatal diagnosis was performed at the 21st week of gestation. The results of amniocentesis showed that the fetus was not affected by CCA and had a normal male karyotype with 46, XY (Fig. 4). The results of the prenatal diagnosis confirmed the accuracy of the PGD performed in this study.

In conclusion, two different genotyping methods for each monogenic disorder and prenatal diagnosis comprise a viable strategy to ensure the accuracy of PGD. PGD can successfully prevent the transmission of CCA and SBMA from the father to his child. Although it has not been reported that PGD induces any adverse effect on the newborn, a long-term and multiple-center follow-up of the children born after PGD is necessary.

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Compliance with ethical standards The couple signed informed consent forms for ICSI treatment, PGD, and follow-up.

This project was approved by the Ethics Committee of the Drum Tower Hospital, which is affiliated to Nanjing University Medical College.

Conflict of interest The authors declare that they have no conflict of interest.

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OMIM, <http://www.ncbi.nlm.nih.gov/omim/>
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