Available online at www.sciencedirect.com
ScienceDirect
Taiwanese Journal of Obstetrics & Gynecology 50 (2011) 74–78
www.tjog-online.com

Case Report

Successful application of the strategy of blastocyst biopsy, vitrification, whole genome amplification, and thawed embryo transfer for preimplantation genetic diagnosis of neurofibromatosis type 1

Yi-Lin Chen [a,d,†], Chia-Cheng Hung [b,c,†], Shin-Yu Lin [a], Mei-Ya Fang [b], Yi-Yi Tsai [a], Li-Jung Chang [a], Chien-Nan Lee [a], Yi-Ning Su [a,b,c,*], Shee-Uan Chen [a,*], Yu-Shih Yang [a]

[a] *Department of Obstetrics and Gynecology, National Taiwan University Hospital, College of Medicine, Taipei, Taiwan*
[b] *Department of Medical Genetics, National Taiwan University Hospital, College of Medicine, Taipei, Taiwan*
[c] *Graduate Institute of Clinical Genomics, National Taiwan University Hospital, College of Medicine, Taipei, Taiwan*
[d] *Buddhist Tzu-Chi General Hospital, Taipei, Taiwan*

Accepted 11 November 2010

Abstract

Objective: Preimplantation genetic diagnosis (PGD) offers an alternative for women to carry an unaffected fetus risk of hereditary diseases. Trophectoderm biopsy may provide more cells for accurate diagnosis. However, the time allowed for transportation of the specimens to the laboratory and performance of molecular diagnosis is limited. We designed a PGD program of trophectoderm biopsy, vitrification of blastocysts, whole genome amplification (WGA), double confirmatory genotypings, and thawed embryo transfer.
Case Report: We conducted this strategy for a woman of familial neurofibromatosis type I (NF-1). She had a genotype of heterozygous c.6709C>T mutation of *NF1* gene. Trophectoderm biopsies were performed on 13 blastocysts. Then, individual blastocyst was vitrified. WGA was performed for the samples, followed by genotypings with both real-time polymerase chain reaction and sequencing. Eight embryos were diagnosed as unaffected, four were affected, and one was inconclusive because of allele drop-out. In the next cycle, two unaffected blastocysts were thawed and transferred, that resulted in a singleton pregnancy. The pregnancy was confirmed as unaffected by means of chorionic villi sampling.
Conclusion: We first demonstrate successful application of blastocyst biopsy, vitrification, WGA, and thawed embryo transfer for PGD of a monogenic disease. Vitrification of blastocysts after biopsy permits sufficient time for shipment of samples and operation of molecular diagnosis.

Keywords: Neurofibromatosis type 1; PGD; Vitrification; WGA

Introduction

Neurofibromatosis type 1 (NF-1), also known as Von Recklinghausen disease, is an autosomal dominant disorder with a birth incidence of 1 in 3,000–3,500 [1–3]. *NF1* gene is located on chromosome 17q11.2 and comprises 60 known exons. *NF1* gene encodes a protein, called neurofibromin. NF-1 is a neurocutaneous disorder with symptoms of multiple café-au-lait spots, cutaneous neurofibromas, optic gliomas, and iris nodules. The neurofibromas may transform into malignant tumors in 10–20%. More than 500 different *NF1* mutations have been found. In addition to conventional prenatal diagnosis, preimplantation genetic diagnosis (PGD) may provide an alternative to carry an unaffected fetus [1,3].

There are three potential sources of embryonic genetic material for PGD, including polar bodies from oocytes, blastomeres from cleavage-stage embryos, and trophectoderm

* Corresponding authors. Department of Obstetrics and Gynecology, National Taiwan University Hospital, 8 Chung-Shan South Road, Taipei, Taiwan.
E-mail addresses: ynsu@ntu.edu.tw (Y.-N. Su), sheeuan@ntu.edu.tw (S.-U. Chen).
† Yi-Lin Chen and Chia-Cheng Hung contribute equally to this study.

1028-4559/$ - see front matter
doi:10.1016/j.tjog.2011.01.040

cells from blastocysts. Cleavage-stage biopsy with aspiration of one blastomere is the most common approach for PGD [4]. The innate dilemma of polar body or blastomere biopsy is the limited genetic material available for analysis with risks of amplification failure and allele drop-out (ADO). Some misdiagnoses of PGD had been reported in the literature [4]. The biopsy of multiple trophoblast cells from blastocysts should theoretically lead to improving sensitivity and accuracy of PGD. Pregnancies after blastocyst biopsy for PGD have recently been reported and merit further investigation [5,6].

Polymorphic short tandem repeat (STR) markers close to mutated gene have been used for linkage analysis to provide an additional confirmation of genotyping and an evidence of ADO [7,8]. STR markers also help to detect contamination of exogenous DNA with different STR sizes. To detect mutated gene and STR markers, multiplex polymerase chain reaction (PCR) is commonly used for PGD [7,8]. It is critical to design compatible multiple primer sequences and conditions because several PCR reactions may interfere mutually in a tube. In addition, the procedures of multiplex PCR cannot be repeated when one of the PCR reactions failed. In contrast, the use of whole genome amplification (WGA) in PGD may provide sufficient DNA templates for independent PCR reactions and repeated confirmations [9]. The WGA for PGD of *NF1* gene and linked STR markers deserves further study.

Embryo biopsy and fresh embryo transfer are traditionally performed in the PGD cycle. However, before embryo transfer, the time allowed for transportation of the specimens to the reference laboratory and conduct of molecular diagnosis is limited, especially after blastocyst biopsy. Vitrification of blastocysts has been verified to achieve high-survival rate and pregnancy rate [10]. Accordingly, we establish the strategy of PGD for monogenic diseases using blastocyst biopsy, vitrification, WGA accompanied by double confirmatory genotypings, and thawed embryo transfer. Using this strategy, we achieve a successful unaffected pregnancy for a couple risk of carrying an NF-1 fetus.

Materials and methods

Patients

The couple, a 38-year-old male and a 32-year-old female, had a history of artificial abortion for an affected fetus of NF-1. The wife has NF-1 disease with a genotype of heterozygous c.6709C>T mutation of *NF1* gene. Her father and grandmother were also affected by this disease. They hoped to have an unaffected child.

PGD counseling

An experienced geneticist and a gynecologist provided comprehensive counseling to the couple. PGD is an alternative method for conventional prenatal diagnosis. The procedures of PGD, cryopreservation of the biopsied blastocysts, survival of thawed embryos, and pregnancy potential were explained. The odds of a misdiagnosis inherent to PGD and subsequent genotype confirmation of the pregnancy using chorionic villi sampling were discussed. This study was approved by the Ethic Committee of National Taiwan University Hospital. Informed consent was obtained from the couple.

Testing on single lymphocytes

To evaluate the reliability of the protocol of molecular diagnosis before the PGD cycle, the WGA, mutation analysis with real-time PCR, and STR detections were tested on single lymphocytes collected from the wife. Blank controls were also processed under the same conditions to check for the presence of contamination. The amplification efficiency, ADO rate, and contamination rate were measured.

WGA

The multiple displacement amplification (MDA) method using GenomiPhi V2 DNA amplification kit (GE Healthcare, Piscataway, NJ, USA) was performed for WGA, according to the manufacturer's instruction.

Informative linked STR marker

The STR marker, close to the *NF1* gene, was examined to avoid a possible misdiagnosis resulting from ADO. An informative intragenic STR marker of D17S1166 was used, which showed polymorphisms of 195, 218 bp for the husband and 193, 204 bp for the wife. The primers were 5′-CCC ATA CCT AGT TCT TAA AGT CTG T-3′ and 5′-TAA CAA TTG TGG AAC TGC AGC AAT TAT T-3′. One microliter of WGA product was examined for the selected STR marker. The procedures performed were based on the method described in Fiorentino et al.[11]

Real-time PCR with fluorescence resonance energy transfer hybridization

For mutation analysis, 1 μL of WGA product was used for real-time PCR using a LightCycler 480 instrument (Roche, Branchburg, NJ, USA). The primers, 5′-GCA GAT TTG CAT TCC AAT ATA-3′ (sense) and 5′-GCT TTA CAA CTT GAG AAC CAT-3′ (antisense), were used for the detection of c.6709C>T mutation. One probe was labeled with fluorescein at the 3′ end (5′-GAA TAC GGA TTA TCT GCT TTA TCT GCC C-3′) as the donor, and another probe (acceptor) was labeled with the LightCycler red fluorophore at the 5′ end (5′-TGA GAC ACT CAT TTG CTA ATA CAC C-3′). The procedures and conditions of real-time PCR and melting curve analysis had been described previously in details [12].

Sequencing analysis

PCR was performed in a total volume of 25 μL containing 1 μL of WGA products, 0.12 μM of each primer (5′-GCT ATT ACT GTA TGA TCA ATG-3′ and 5′-TAT GCT TTA CAA CTT GAG AAC-3′), 100 μM dNTPs, 0.5 units of AmpliTaq

Gold enzyme (PE Applied Biosystems, Foster City, CA, USA), 2 mM $MgCl_2$, and 2.5 μL of GeneAmp 10× buffer II (10 mM Tris-HCl, pH = 8.3, 50 mM KCl), as provided by the manufacturer. Amplification was performed in a multiblock system thermocycler (ThermoHybaid, Ashford, UK). PCR amplification was performed with an initial denaturation step at 95°C for 10 minutes, followed by 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 30 seconds, and then a final extension step at 72°C for 10 minutes. Sequencing reactions were separated on a PE Biosystems 373A/3100 sequencer (PE Applied Biosystems).

PGD using trophectoderm biopsy

Ovarian stimulation was performed with a long gonadotropin releasing hormone analog protocol as described previously [13]. Intracytoplasmic sperm injection was used to avoid contamination from sperm and cumulus cells. Trophectoderm biopsy was performed at the blastocyst stage, based on the method described by Kokkali et al [6]. A specimen containing approximately 5−10 trophoblast cells with clearly visible nuclei was removed from each blastocyst. The specimen was washed and transferred to a separate PCR tube. The last wash drop of each specimen served as a blank control.

Vitrification of blastocysts

After biopsy, individual blastocyst was treated with cryoprotectants and transferred onto the Cryotop (Kitazati Supply Co., Fujinomiya, Japan) [14]. It was vitrified and stored in liquid nitrogen separately.

Thawed embryo transfer

In the next cycle, using hormonal replacement treatment to prepare endometrium [15], two unaffected blastocysts were thawed and transferred. The pregnancy was followed by prenatal examinations.

Reanalysis of affected embryos

The affected embryos were thawed and reanalyzed to confirm the results of genetic analysis.

Results

Genetic analysis using single lymphocytes

Before performing the PGD cycle, 50 single lymphocytes of the wife were examined by MDA, genotyping using real-time PCR, and STR detection. The amplification efficiency of the *NF-1* gene for a single lymphocyte was 94% (47/50) with an ADO rate of 15% (7/47). There was no contamination in 50 reactions.

Trophectoderm biopsy and vitrification of blastocysts

In the PGD cycle, 26 oocytes were retrieved that 21 were mature and 16 were fertilized. Thirteen embryos developing to blastocysts were biopsied. Individual blastocyst was then vitrified in each Cryotop device.

Molecular diagnosis of the samples of trophoblast cells

The samples of trophoblast cells were processed by MDA, genotyping using both real-time PCR and sequencing, and STR detection. The results of real-time PCR (Fig. 1) revealed that eight embryos were unaffected, four were affected, and one was inconclusive diagnosis because of ADO. The data of sequencing (Fig. 2) were same that confirmed the results of real-time PCR. The amplification efficiency of the *NF-1* gene for the samples of trophoblast cells was 100% (13/13) with an ADO rate of 8% (1/13). There was no contamination in 13 reactions.

Transfer of thawed embryos and follow-up of pregnancy

In the next cycle, two unaffected blastocysts were thawed and all of them survived. Two blastocysts were transferred. The patient had a singleton pregnancy. The genotype of the fetus was confirmed as unaffected by NF-1 using chorionic villi sampling at 11 weeks' gestation. Cesarean section was performed at 26 weeks' gestation because of severe preeclampsia. A female baby was delivered, weighing 600 g with Apgar scores of 6−8.

Reanalysis of affected embryos

The four affected embryos were thawed and reanalyzed. The data were same as the results of trophectoderm biopsy.

Discussion

We demonstrate the successful application of blastocyst biopsy, vitrification, WGA with double confirmatory genotypings, and thawed embryo transfer for PGD of NF-1. Embryo biopsy and fresh embryo transfer are conventionally executed in the PGD cycle. However, before embryo transfer, the time allowed for shipment and genetic analysis of the specimens is restricted, particularly after blastocyst biopsy. Cryopreservation of biopsied blastocysts instead of fresh transfer permits sufficient time for accomplishment of molecular diagnosis with double confirmations. And then thawed embryo transfer is performed in the next cycle. In addition, cryopreservation of embryos may be beneficial for patients of high responder to circumvent risk of ovarian hyperstimulation syndrome and possible suboptimal endometrium [16,17].

Compared with cleavage-stage biopsy, trophectoderm biopsy providing more cells for genetic analysis may potentially reduce the risk of amplification failure and ADO [6]. In addition, trophectoderm biopsy would be relatively cost-effective and less labor-intensive, because only embryos

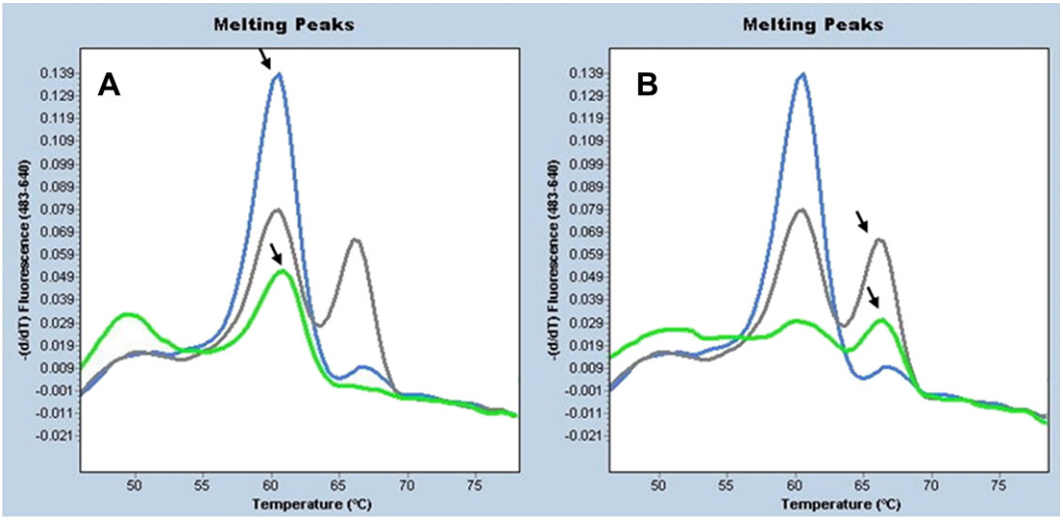

Fig. 1. Melting curve analysis of real-time polymerase chain reaction with fluorescence resonance energy transfer hybridization for detection of the c.6709C>T mutation of the *NF1* gene. (A) Blue curve: husband; gray curve: wife; green curve: embryos. The peak at the lower melting temperature (60.5°C) belonged to the wild-type allele, whereas the peak at the higher melting temperature (66°C) belonged to the mutated allele. The husband and an embryo with the wild-type genotype revealed one melting peak (arrow) at 60.5°C. (B) The wife and an embryo with the heterozygous c.6709C>T mutation showed one melting peak at 60.5°C and an additional melting peak (arrow) at 66°C.

competently developing to the blastocyst stage are biopsied. Kokkali et al [6] prospectively compared genotyping success and implantation rates in PGD cycles for β-thalassemia after biopsy at the blastocyst versus the cleavage stage. They found that the genotyping success rate was significantly higher in the blastocyst group (94%) than in the cleavage-stage group (75%). The implantation and pregnancy rates were not statistically different. With the higher genotyping success rates, trophectoderm biopsy may result in relatively more embryos available for transfer. However, the case number is still small in that series. In the present case of trophectoderm biopsy for NF-1, the amplification rate was 100% with an ADO rate of 8%. The value of blastocyst biopsy in reducing ADO and achieving accurate diagnosis needs further clarification.

The risk of ADO could lead to misdiagnosis in PGD. Polymorphic STR markers close to mutated gene have been used to provide an evidence of ADO [7,8]. MDA using Phi29

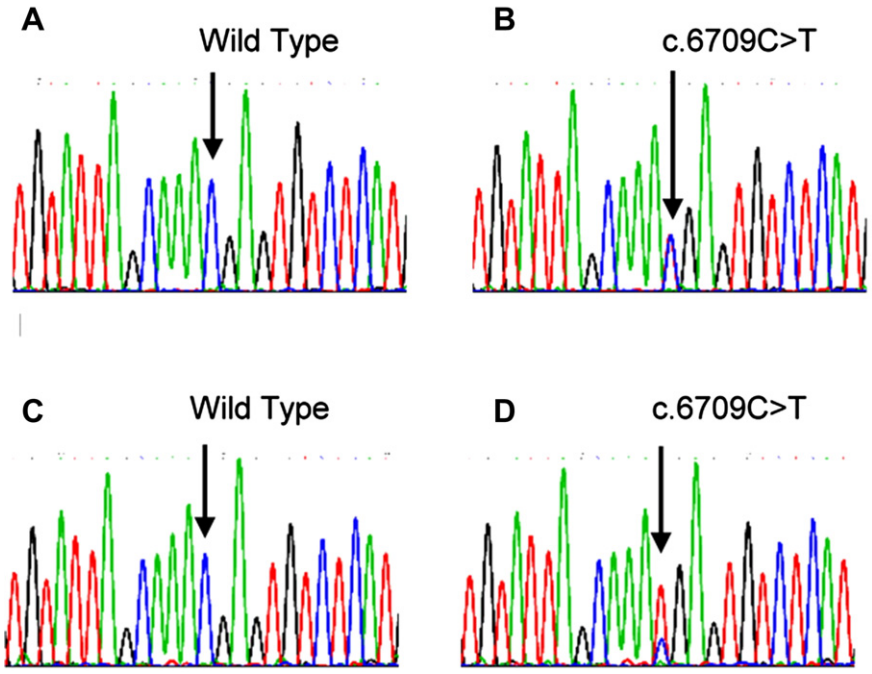

Fig. 2. The sequencing analysis for detection of the c.6709C>T mutation of the *NF1* gene. (A) The husband with the wild-type genotype revealed a single blue peak (arrow). (B) The wife with the heterozygous c.6709C>T mutation showed one blue peak and an additional red peak (arrow). (C) An embryo with the wild-type genotype revealed a single blue peak (arrow). (D) An embryo with the heterozygous c.6709C>T mutation showed one blue peak and an additional red peak (arrow).

DNA polymerase and random hexamer primers is recently developed for WGA of scanty DNA material with unbiased amplification [18]. In this study, we used MDA for WGA of the sample of approximately 5−10 trophoblast cells. After WGA, the *NF1* gene and the selected STR marker can be examined independently. We applied two genotyping methods for double confirmations of diagnosis and obtained consistent results. In addition, WGA allows reassuring examinations for an ambiguous result. A few clinical applications of MDA for PGD have been reported, such as for Marfan syndrome, Duchenne muscular dystrophy, cystic fibrosis, β-thalassemia, and Hungtington chorea [19]. Here we present the clinical application of MDA for PGD of NF-1.

We first validate the successful application of blastocyst biopsy, vitrification, WGA accompanied by double confirmatory genotypings and STR detection, and thawed embryo transfer for PGD of NF-1. Cryopreservation of blastocysts after biopsy permits more sufficient time for shipment of the samples and performance of molecular diagnosis. Trophectoderm biopsy provides more cells for analysis that may reduce amplification failure and ADO. After WGA, the quality and quantity of DNA may facilitate multiple independent examinations of gene mutations and polymorphic STR markers as well as repeating confirmations for an uncertain result. Further studies may merit to clarify an overall advantage of this strategy regarding efficiency, diagnostic accuracy, and pregnancy potential.

Acknowledgment

We thank the National Science Council of Taiwan (Grants NSC 95-2314-B-002-280-MY3 and NSC 96-2628-B-002-063-MY3) for financial support. The authors would like to thank Ms Yi-Lin Yao and Ms Chia-Jen Shieh for their technical assistance and Ms Ling-Li Liu for her nursing assistance.

References

[1] Friedman JM. Epidemiology of neurofibromatosis type 1. Am J Med Genet 1999;89:1−6.
[2] Spits C, De Rycke M, Van Ranst N, Joris H, Verpoest W, Lissens W, et al. Preimplantation genetic diagnosis for neurofibromatosis type 1. Mol Hum Reprod 2005;11:381−7.
[3] Vanneste E, Melotte C, Debrock S, D'Hooghe T, Brems H, Fryns JP, et al. Preimplantation genetic diagnosis using fluorescent in situ hybridization for cancer predisposition syndromes caused by microdeletions. Hum Reprod 2009;24:1522−8.
[4] Dreesen J, Drüsedau M, Smeets H, de Die-Smulders C, Coonen E, Dumoulin J, et al. Validation of preimplantation genetic diagnosis by PCR analysis: genotype comparison of the blastomere and corresponding embryo, implications for clinical practice. Mol Hum Reprod 2008;14:573−9.
[5] de Boer KA, Catt JW, Jansen RPS, Leigh D, McArthur S. Moving to blastocyst biopsy for PGD and single embryo transfer at Sydney IVF. Fertil Steril 2004;82:295−8.
[6] Kokkali G, Traeger-Synodinos J, Vrettou C, Stavrou D, Jones GM, Cram DS, et al. Blastocyst biopsy versus cleavage stage biopsy and blastocyst transfer for preimplantation genetic diagnosis of beta-thalassaemia: a pilot study. Hum Reprod 2007;22:1443−9.
[7] Gutiérrez-Mateo C, Sánchez-García JF, Fischer J, Tormasi S, Cohen J, Munné S, et al. Preimplantation genetic diagnosis of single-gene disorders: experience with more than 200 cycles conducted by a reference laboratory in the United States. Fertil Steril 2009;92:1544−56.
[8] Fiorentino F, Kokkali G, Biricik A, Stavrou D, Ismailoglu B, De Palma R, et al. Polymerase chain reaction-based detection of chromosomal imbalances on embryos: the evolution of preimplantation genetic diagnosis for chromosomal translocations. Fertil Steril 2010;94:2001−11.
[9] Chen SU, Su YN, Fang MY, Chang LJ, Tsai YY, Lin LT, et al. PGD of β-thalassaemia and HLA haplotypes using OmniPlex whole genome amplification. Reprod Biomed Online 2008;17:699−705.
[10] Liebermann J. Vitrification of human blastocysts: an update. Reprod Biomed Online 2009;19(Suppl. 4):4328.
[11] Fiorentino F, Biricik A, Karadayi H, Berkil H, Karlikaya G, Sertyel S, et al. Development and clinical application of a strategy for preimplantation genetic diagnosis of single gene disorders combined with HLA matching. Mol Hum Reprod 2004;10:445−60.
[12] Hung CC, Chen SU, Lin SY, Fang MY, Chang LJ, Tsai YY, et al. Preimplantation genetic diagnosis of β-thalassaemia using real-time PCR with FRET hybridization probes. Anal Biochem 2010;400:69−77.
[13] Ho CH, Chen SU, Peng FS, Chang CY, Lien YL, Yang YS. Prospective comparison of the short and long GnRH agonist protocols using recombinant gonadotropins for IVF/ICSI treatments. Reprod Biomed Online 2008;16:632−9.
[14] Chen SU, Yang YS. Slow freezing or vitrification of oocytes: their effects on survival and meiotic spindles and the time schedule for clinical practice. Taiwanese J Obstet Gynecol 2009;48:15−22.
[15] Chen SU, Lien YL, Tsai YY, Chang LJ, Ho HN, Yang YS. Cryopreserved sibling oocytes and intracytoplasmic sperm injection rescue unexpectedly poor fertilization in conventional in vitro fertilization. J Assist Reprod Genet 2004;21:367−9.
[16] Chen SU, Chen CD, Yang YS. Ovarian hyperstimulation syndrome (OHSS): new strategies of prevention and treatment. J Formos Med Assoc 2008;107:509−12.
[17] Liu Y, Lee KF, Ng EH, Yeung WS, Ho PC. Gene expression profiling of human peri-implantation endometria between natural and stimulated cycles. Fertil Steril 2008;90:2152−64.
[18] Glentis S, SenGupta S, Thornhill A, Wang R, Craft I, Harper JC. Molecular comparison of single cell MDA products derived from different cell types. Reprod Biomed Online 2009;19:89−98.
[19] Chow JF, Yeung WS, Lau EY, Lam ST, Tong T, Ng EH, et al. Singleton birth after preimplantation genetic diagnosis for Huntington disease using whole genome amplification. Fertil Steril 2009;92:828.e7−828.e10.