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Running Title: PGD for Huntington's disease

Singleton birth after preimplantation genetic diagnosis for Huntington's disease using whole genome amplification

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Capsule: A healthy girl was born after preimplantation genetic diagnosis for Huntington's disease using multiple displacement amplification and polymerase chain reaction.

Abstract

Objective: To report a successful case of preimplantation genetic diagnosis (PGD) for Huntington's disease using whole genome amplification

Design: Case report

Setting: University assisted reproduction unit

Patient(s): A couple with family history of Huntington's disease; the husband was carrying the expanded allele of the *IT15* gene, while the wife had the normal allele.

Intervention(s): PGD with whole genome amplification for identification of genetically normal embryos

Main Outcome Measure(s): Live birth

Result(s): In an IVF cycle, 15 oocytes were retrieved, of which 13 were mature and 11 were fertilized. On Day 3, embryo biopsy and PGD were performed on 10 good quality embryos. Multiple displacement amplification was conducted followed by polymerase chain reaction (PCR) with fluorescence primers. Three pairs of primers were used for the amplification of the *IT15* gene at the 1) trinucleotide expansion site; 2) trinucleotide expansion site plus the polymorphic site situated on its 3'-end; and 3) polymorphic marker located downstream of the trinucleotide repeats. Two normal blastocysts were replaced on day 5 and another two good quality blastocysts were cryopreserved. The woman gave birth to a normal baby girl whose normal genetic status was confirmed by prenatal diagnosis. **Conclusion(s):** Whole genome amplification by multiple displacement amplification can be used for PGD of Huntington's disease. (207 words)

Key Words: Huntington's disease, live birth, whole genome amplification, PGD

Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder affecting 1 in 10,000 individuals. It is associated with the expansion of the CAG repeats in exon 1 of the *IT15* gene (*Huntingtin* gene) on the short arm of chromosome 4 (4p16.3). The penetrance of the disease depends on the number of CAG repeats: <27, normal; 27-35, intermediate; 36-39, reduced penetrance; >39 full penetrance (1). Individuals with 27-35 repeats will not develop HD but their children are at risk of having the disease due to potential increase in the number of repeats in successive generations. People with 36-39 repeats may or may not develop the disease. Those with >39 repeats will develop the disorder with a variable age of onset usually around the late forties and early fifties. The symptoms of the disease usually develop progressively with the physical signs appearing first to be followed by cognitive and psychiatric signs. Pathogenesis of the disease is thought to be associated with intranuclear inclusion and cytoplasmic aggregation of huntingtin protein in the brain (2).

Because HD is inherited in an autosomal dominant fashion, there is a 50% chance of transmitting the disease to the next generation. Preimplantation genetic diagnosis (PGD) can avoid couples from having a fetus with a known genetic disease, and thus prevents the couples from the psychological trauma associated with carrying an affected child, termination of pregnancy or recurrent miscarriage. PGD has been applied clinically on HD by direct determination of the CAG repeats and/or by indirect linkage analysis of microsatellite markers (3-5). All these reports have used multiplex PCR for diagnosis, which necessitates a careful choice of primers and tedious optimization process for the multiplex PCR.

Whole genome amplification using phi 29 DNA polymerase (also known as multiple displacement amplification, MDA) is a recently developed method for producing DNA of high molecular weight (>10kb) with unbiased amplification, good reproducibility (6) and low error rate (7) from a small quantity of starting DNA material such as from single cells. A few

clinical applications of MDA resulting in pregnancies in PGD cycles have been reported, such as X-linked retinoschisis (8), Marfan syndrome (9), Duchenne muscular dystrophy (10), fragile X syndrome (11), cystic fibrosis and β -thalassemia (12). This is the first report on PGD for HD using whole genome amplification.

MATERIALS AND METHODS

Patients

A couple requested PGD because the husband had a family history of HD. The husband, aged 33, was known to be carrying the expanded allele containing 38 CAG repeats, while the wife, aged 32, was confirmed to have the normal alleles. They had had a previous therapeutic termination of pregnancy for an affected baby. The genetic status of the couple was confirmed by the Clinical Genetic Service in the Department of Health, Hong Kong Special Administrative Region, Hong Kong. The couple was extensively counseled about potential risks of in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI) and PGD. Consent was obtained from them; they were also advised to have a confirmatory prenatal diagnosis for the ensuing pregnancy. Ovarian stimulation was performed using the long protocol of pituitary downregulation as described previously (13).

Pre-PGD preparative work

Before treatment, the genetic status of couple was re-confirmed in our laboratory using their genomic DNA extracted from 8 ml of sodium citrate-treated peripheral blood by QIAamp DNA Blood Maxi Kit (Qiagen, Upsala, Sweden) according to manufacturer's protocol. Single cell assay was optimized on the couples' lymphocytes, which were isolated from whole blood by Ficoll-Paque Plus separation according to manufacturer's instructions (GE Healthcare, Germany). Ficoll-processed lymphocytes were resuspended in RPMI 1640

medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma, St Louise, MO). The PCR condition was optimized using single isolated lymphocytes. Individual lymphocyte in 0.5 µl of PBS/1.5% polyvinylpyrrolidone (PVP) was transferred into a 0.5 ml thin-walled PCR tube using finely-drawn Pasteur pipette under dissection microscope (14). PCR was performed on at least 20 individual lymphocytes (10 from the wife and 10 from the husband).

Blastomere Biopsy

Blastomere biopsy was performed using standard procedure (15). A hole was made on the zona pellucida of Day 3 cleavage-stage embryos with an infrared laser. One nucleated blastomere was biopsied from each embryo. The biopsied blastomeres were washed 3 times in PBS/1.5% PVP and transferred into a PCR tube before whole genome amplification. The solution for final wash was also collected as negative control. PCR was performed with couple's DNA as positive control and water blank as negative control. Duplicated PCR were performed for each reaction.

Whole Genome Amplification and Single-cell PCR

Whole genome amplification was performed on single lymphocyte or blastomere according to the manufacturer's instruction (REPLI-g Ultrafast Kit, Qiagen, Uppsala, Sweden). Briefly, cells were lysed in 1.5 µl of denaturation buffer. Denaturation was stopped by the addition of the neutralization buffer. A master mix containing reaction buffer and REPLI-g UltraFast DNA Polymerase was then added. The reaction proceeded for 1.5 hours at 30°C and was stopped by incubation at 65°C for 3 minutes.

Three pairs of primers were used for amplification of the *IT15* gene. A fully informative marker flanking the site of CAG repeats and a downstream CCG polymorphic

site was chosen (Hu4: 5'-FAM- ATG GCG ACC CTG GAA AAG CTG ATG A-3' and Hu3: 5'- GGC GGC TGA GGA AGC TGA GGA- 3') (3). PCR was performed in a 25 µl reaction mixture containing 1X Expand Long Template Buffer 2, 0.2 µM of each primers, 5% of DMSO (Sigma), 0.5 mM dNTPs (Roche), 1.4 U of Expand Long Template DNA polymerase (Roche) and 1 µl of whole genome amplified DNA. The second pair of primers (A1: 5'- FAM-CCT TCG AGT CCC TCA AGT CCT TC -3' and A2: 5'- GGC TGA GGA AGC TGA GGA G-3') amplified the CAG repeats site in a 13 µl reaction mixture containing 1X GC-RICH PCR reaction buffer, 1 M of GC-RICH resolution solution, 0.4 mM of dNTPs, 0.2 µM of each primers, 0.5 U of GC-RICH enzyme mix (Roche) and 1 µl of the whole genome amplified DNA. A highly polymorphic marker (I1CAHD-F: 5'- FAM-TAT GCC ACT ACA CTA CAA CCT G-3' and I1CAHD-R: 5'- AGC ATG TGG TAT TGT CAA AGT G-3')(16) located downstream of the trinucleotide repeats was used as a marker to determine contamination, if any, since the scientists shared no common allele with the couple. The primers were amplified in a 10 µl reaction mixture containing 1X High Fidelity PCR Buffer, 2 mM of MgSO₄ 0.2 mM of dNTPs, 0.2 µM of each primers, 10 % glycogen, 0.2 U of Platinum Taq High Fidelity DNA polymerase (Invitrogen, Carlsbad, CA) and 2 µl of whole genome amplified DNA.

Post-PCR analysis was performed by resolving 0.5 µl of the PCR products in a ABI 3730xl DNA Analyzer with the use of GeneScan 500 LIZ size standard (Applied Biosystems, Foster City, CA). Data were analyzed by the Peak Scanner Software (version 1, Applied Biosystems, Foster City, CA).

Results

Lymphocyte Testing

Before the PGD cycle, the reliability of the single cell assay was evaluated by single lymphocyte testing in couple. The amplification efficiencies of the primers used ranged from 90% to 100% while the allelic dropout (ADO) rate varied from zero% to 30%.

PGD Cycle

The couple underwent one treatment cycle. Fifteen oocytes were retrieved and 13 of them were at the metaphase II stage. Eleven oocytes were fertilized (85%) as evidenced by the formation of 2 pronuclei at 16 hours after ICSI. On Day 3, 10 good quality embryos underwent embryo biopsy and PGD. One blastomere was biopsied from each embryo except one (embryo 1); the first blastomere of embryo 1 lysed after biopsy and a second blastomere was obtained from the same embryo. Five embryos were diagnosed to be normal, 2 were abnormal and 3 had no conclusive diagnosis due to allelic dropout or amplification failure. Two blastocysts were replaced on Day 5 and another two good quality normal blastocysts were cryopreserved. No contamination was detected in all negative controls. Those embryos with no conclusive result were later confirmed to be genetically abnormal, i.e. carrying the expanded allele. An intrauterine singleton pregnancy was confirmed later. The result of the subsequent prenatal diagnosis was concordant with the PGD result. The woman gave birth to a normal baby girl by lower segment caesarian section.

Discussion

PGD of monogenetic disease usually employs nested multiplex PCR on DNA material from a single cell (14, 17, and 18). This approach involves tedious optimization of the multiplex PCR condition, and very often only a limited number of primers can be used simultaneously. Whole genome amplification by isothermal MDA offers a reliable method of amplifying the whole genomic DNA from single cells. MDA was catalyzed by phi 29 DNA

polymerase which has been reported to have 100 times lower error rate than *Taq* DNA polymerase (19). The fidelity of the reaction makes it suitable for monogenetic disease diagnosis at single cell level. Thus it can serve as a universal platform for PGD of different monogenetic disease without the need for optimization of multiplex PCR. Moreover, the MDA reaction takes only 1.5 hours to complete, and the PGD results can be obtained on the same day. This article reports the first clinical application of MDA on Huntington's disease.

In this case, MDA plus PCR provide a very promising amplification efficiency of 90%-100%. According to the result from single lymphocyte, the ADO rates vary among primers ranging from zero% to 30%. It is noteworthy that all the ADO events occurred in the husband's lymphocytes, probably due to the large size difference of the two alleles. A similar phenomenon was observed in clinical PGD case. All the embryos with no conclusive results on account of ADO or amplification failure were later found to carry the husband's abnormal allele. The relative high incident of ADO in the clinical PGD cycle may also be due to the high ADO rate observed in blastomere. Indeed it was reported that ADO rate in single cell PCR vary significantly among different cell types (20).

Whole genome amplification by MDA provides sufficient DNA for the analysis of additional microsatellite markers enabling more accurate diagnosis of the genotype of embryos. It has been reported that each additional linked marker analyzed would reduce the misdiagnosis rate by half (20). In this case, there was no pedigree information available from the couple, and therefore linkage analysis was not performed. In the future, MDA can be used to perform haplotype analysis on single spermatozoon of husband to provide linkage information in clinical PGD for the diagnosis and detection of contamination. We had included a highly polymorphic marker located in the intron 1 of *IT15* gene for PGD (20). This intragenic marker helped to determine possible contamination, either from the maternal cumulus cells, or from the scientists involved in PGD because the couple and the scientists

share no common allele for this marker. This marker thus serves as an important measure to prevent the false positive result in PGD.

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