Preimplantation Genetic Diagnosis for Alpha Thalassemia: Experience in Siriraj Hospital

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ABSTRACT

Objective: Detection of fetal thalassemia using preimplantation genetic diagnosis (PGD) can make a diagnosis before pregnancy so termination of pregnancy in that patient is eliminated. The objective of this study was to develop a single gene polymerase chain reaction (PCR) protocol for PGD of alpha thalassemia in Siriraj Hospital.

Methods: A couple with a history of repeated Bart's hemoglobinopathy in fetus underwent an artificial reproductive technique (ART) process using a standard ovarian stimulation protocol with intracytoplasmic sperm injection (ICSI) to reduce sperm DNA contamination. On day 3 post fertilization, laser biopsy was performed on the cleavage stage embryos to obtain a blastomere for PCR analysis of alpha thalassemia 1 SEA type.

Results: 11 embryos from a total of 15 oocytes were biopsied, 2 normal, 1 alpha thal 1 trait and 3 affected embryos were detected. No contamination and allele drop out were detected, but a high PCR failure rate of 5 from 11 total biopsied embryos.

Conclusion: PGD for alpha thalassemia was first established in Siriraj Hospital, but the result had a high failure rate so then optimized laboratory techniques were required.

Keywords: Alpha thalassemia, preimplantation genetic diagnosis (PGD), single PCR

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Thalassemia is the most common single gene disease especially in Thailand and South East Asia. The genetic defects affect the production of the globin chain that changes the proportion of hemoglobin. Two main types of thalassemia, alpha and beta thalassemia, have different clinical manifestations and the severity of diseases varies from asymptomatic to a severe form which need transfusion or bone marrow transplantation. In pregnant women who have a fetus with Bart's hydrops fetalis, the most severe form of alpha thalassemia, may develop preeclampsia that increased maternal morbidity. The strategies for prevention of thalassemia are detection of carriers and termination of pregnancy to avoid the birth of affected children.[1-4] The prevalence of the thalassemic trait in pregnant women varies from 10 to 60 percent.[5-7] The prenatal diagnosis for high risk couples includes cordocentesis and chorionic villi sampling for polymerase chain reaction (PCR) which are in worldwide use. However, disadvantages are the risk for fetal loss and the need of termination of pregnancy that may cause stress and others psychological problems.

Preimplantation genetic diagnosis (PGD) is a recent technique that makes diagnosis before pregnancy possible. The high risk couples widely accept this technique because there is no affected fetus transfer to the uterus, so no need for termination.[8-10] The process of PGD involves a standard in vitro fertilization (IVF) procedure followed with embryonic cell biopsy and testing of a single cell of a blastomere. For testing of single gene disorders especially in case of single cell testing, DNA amplification with PCR technique is required. Many techniques of PCR such as nested PCR, multiplex PCR, PCR with use of denaturing gradient gel electrophoresis (DGGE) or fluorescent PCR have been used to increase the accuracy.[11-16] However, amplification failure, allele drop out and contamination are still major problems of PCR at a single cell level. For the contami-

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nation problem, the use of intracytoplasmic sperm injection (ICSI) was performed to avoid the contamination of paternal DNA from the standard insemination of IVF. The source of fetal DNA from biopsy also varies from polar body, blastomere or trophectoderm.[17-19] Polar body biopsy removes useless products of meiosis that is probably safe, although it contains only maternal DNA and cannot detect the paternal contribution. Trophectoderm from blastocyst biopsy tends to have a better outcome because more cells are available. However, the disadvantage is this technique needs very a quick diagnostic time because of very little suitable time for uterine transfer. Then we prefer using a blastomere from a cleavage stage embryo to prevent unpredictable results because this is our first experience in Siriraj Hospital.

The objective of this study was to develop a single gene PCR protocol for the PGD of alpha thalassemia in Siriraj Hospital.

MATERIALS AND METHODS

Patient history

The first case of PGD for alpha thalassemia was a Thai woman 36 years old. She came to the infertility unit of Siriraj Hospital for PGD because of her bad obstetric history. Her first conception was six years ago and she underwent miscarriage with unknown causes. Five months later, she came to the clinic for antenatal care. Unfortunately, she did not test for thalassemic screening. At 7 months of gestation, she developed preeclampsia and underwent cesarean section for termination of pregnancy, but she found that her baby had developed hydrops fetalis from Bart's hemoglobinopathy. Two years ago, she came to the antenatal clinic of Siriraj Hospital to check her pregnancy status. The blood testing result revealed that she and her husband had alpha thal 1 trait (Southeast Asia type). After counseling for her 25% risk to have Bart's hydrops in fetus and risks of prenatal diagnosis, she decided to undergo prenatal diagnosis from chorionic villi sampling. However, the result suffered her again, because she had repeated Bart's hemoglobinopathy. Two years after termination of pregnancy, she wanted to have a non-thalassemic baby and she knew that PGD could eliminate the chance of induced abortion. After counseling for failure rate, accuracy and treatment detail, she decided to take artificial reproductive technique (ART) for PGD. This study was approved by Ethics Committee at Siriraj Hospital Mahidol University.

Ovarian stimulation, oocyte retrieval, insemination and embryo biopsy

The patient underwent routine ovarian stimulation with a standard long protocol of GnRH analog start on day 21 of the pre-stimulation cycle followed with 300 units of recombinant FSH for 10 days. Oocyte retrieval was performed at 36 hours after triggering with hCG. A total of 15 oocytes were inseminated by mean of ICSI to eliminate contamination from DNA sperm. (Figure 1) Eleven from fifteen oocytes were fertilized, and underwent cleavage stage embryo biopsy. On day 3 post fertilization, laser biopsy was performed (Figure 2) and one or two blastomeres were removed for PCR, depending on the number of blastomeres contained in the embryo; one blastomere for 4-5 cells stage and two blastomeres for more than 5 cells stage.

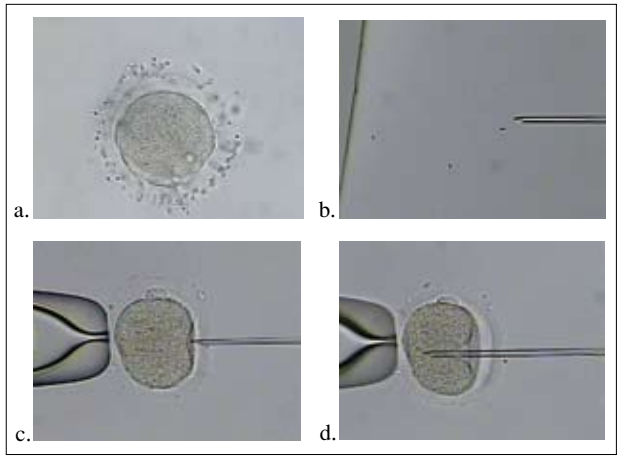

Fig 1. Numerous sperm attached around oocyte in standard in vitro fertilization (IVF) might result in contamination during PCR amplification. (a) For reducing contamination from paternal DNA, intracytoplasmic sperm injection (ICSI) was used by selected only one sperm (b) to inject into M2 oocyte (c), (d).

Cell lysis procedure

Biopsied single blastomeres were collected individually in 0.2 mL PCR tubes containing 2.5 µL of cell-lysis buffer comprising sterile deinoized water and 50 µg/mL proteinase K (Invitrogen, USA). The samples were incubated at 37°C for 60 minutes followed by 65°C for 10 minutes and the proteinase K was then inactivated by heating to 95°C for 10 minutes.[16]

PCR procedure for alpha thalassemia 1 SEA deletion type

After cell lysis, PCR fragments of either normal (194 bp) or alpha thalassemia 1 SEA type (550 bp) were amplified by using three previously reported primers; A4 (5'-GGGGCGCCTTGGGGAGGTTC-3'), A1B (5'-GTTCCCTGAGCCCCGACACG-3') and A9 (5'-ATATATGGGTCTGGAAGTATC-3').[20]

The 22.5 µL of PCR reaction comprised 2.5 µL of 10x PCR buffer, 3.5 mM $MgCl_2$ (Bioline, USA), 200 µM dNTPs (Promega, USA) 5 pmole of each primer,

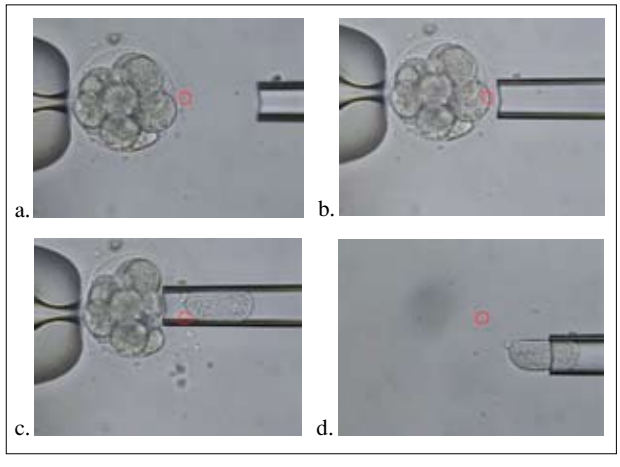

Fig 2. Cleavage stage embryo biopsy was performed by laser drilling of zona pellucid (a) then negative pressure was applied gently through biopsy pipette (b), (c) to remove blastomere for PCR process (d).

0.5 U of *Taq* DNA polymerase (Bioline, USA), and filled up sterile deionized water to 22.5 µL were added into each extracted DNA tube. The PCR profile was started with initial denaturation at 95°C for 7 minutes and followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 63°C for 30 seconds and elongation at 72°C for 30 seconds, with the additional extension at 72°C for 10 minutes. PCR products were electrophoresed on 1.5% LE agarose gel, stained with ethidium bromide, and photographed.

To avoid any risk of contamination, most stringent precautions were taken in all experiments. Manipulation of cells and PCR set-up were carried out in separate UV-treated laminar flow hoods. PCR set-up employed dedicated PCR pipette tips with filters. PCR negative (blank) controls included tubes containing cell lysis mixture only were included through the PCR set-up. Genomic DNAs from normal individuals, relevant carriers as well as the embryos' parents served as positive controls in respective experiments.

RESULTS

From a total of 15 oocytes retrieved, all fifteen oocytes were inseminated by ICSI. On day 1 post fertilization, 8 oocytes were 2PN stage and 3 oocytes became abnormal fertilization (1 oocyte with 3PN and 2 oocytes with 1PN stage). All of eleven embryos were cultured and then underwent biopsy on day 3 post fertilization. The stage of the embryo on the day of biopsy was shown in Table 1. The results of PCR showed that 2 embryos were normal (embryo 3 and 11), 1 embryo was alpha thal 1 trait (embryo 6), 3 embryos were Bart's (embryo 1, 4, 10) and 5 embryos were PCR failure (embryo 2, 5, 7, 8, 9). The gap-PCR diagram for detection of α-thalassemia 1 SEA deletion and three different genotypes from representative blastomeres were showed together with their parental DNA analysis. (Figure 3) Notice that 3 in 5 embryos of PCR failure were abnormally fertilized (embryo 2, 7, 8) that were not routinely checked because they were unsafe for transfer. No contamination or allele drop out were detected. Normal and trait (embryo 3, 6, 11) were planned for transfer on day 5 post fertilization, but on day 4, all planned transferred embryos were in the grade 3 compact stage then transfer was performed on that day. One affected embryo (embryo 1) and one PCR failure embryo (embryo 5) became blastocysts then embryo 5 underwent verification for further study in the next cycle. Others embryos were arrested on day 5 post fertilization. Routine luteal support with progesterone was prescribed. Unfortunately, 12 days after transferral, the patient complained with vaginal bleeding and passed small tissue from her vagina. The result of beta hCG level was only 0.7 mIU/ml, so the drugs were discontinued.

DISCUSSION

Thalassemia is prevalent in South East Asia and the Mediterranean and causes a burden from multiple transfusion or bone marrow transplantation. Pregnant women with Bart's hemoglobinopathy in their fetus are at an increased risk of preeclmpsia and morbidity. The effective program for prevention of thalassemia is screening and termination of an affected fetus. Although compli-

TABLE 1. Results of preimplantation genetic diagnosis for alpha thalassemia.

Embryo No.	Day 1 stage	No. of total cell on biopsy	No. of cell removed	PCR result
1	2PN*	10	2	Affected
2	1PN	6	2	Failed
3	2PN	4	1	Normal
4	2PN	5	1	Affected
5	2PN	4	1	Failed
6	2PN	8	2	Heterozygous
7	3PN	4	1	Failed
8	1PN	4	1	Failed
9	2PN	9	2	Failed
10	2PN	4	1	Affected
11	2PN	4	1	Normal

* PN = pronucleus, 1PN = haploid, 2PN = diploid, 3PN= triploid.

cations of termination were significantly decreased, many patients suffered from the procedure not only from pain, but also stress and guilt especially in patients with repeated affected fetus. Many studies asked about the attitude of this topic and concluded that the patients accepted preimplantation genetic diagnosis to eliminate pregnancy termination.[8-10]

Preimplantation genetic disease is a recent technique for couples who have risk for some of the known genetic diseases and they know the result before pregnancy that reduces the chance of pregnancy termination. The process of PGD involves assisted reproductive technology is collaborated with molecular genetic diagnosis techniques, so interdepartmental communication and laboratory techniques are keys to success.

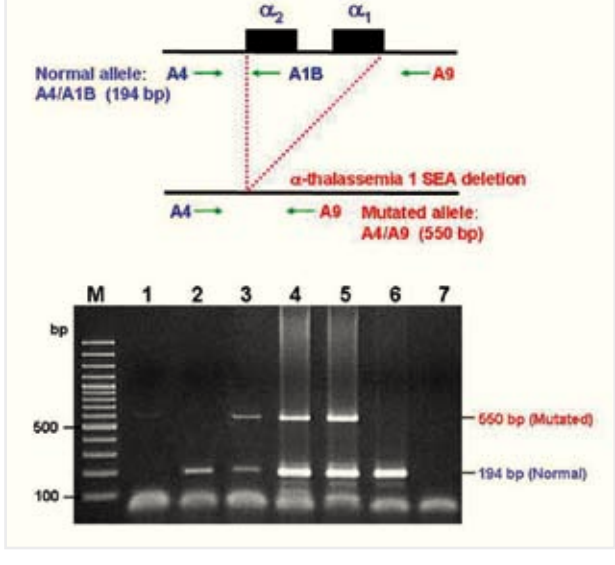

Fig 3. Diagram of gap-PCR for detection of α-thalassemia 1 SEA deletion and agarose-gel electrophoresis showing 3 different genotypes from representative blastomeres in the present of relevant controls.

Lane M = 100 bp DNA marker, 1 = blastomere having α-thal-1 (SEA) only, 2 = blastomere having the normal allele only, 3 = blastomere having heterozygous α-thal-1 (SEA), 4 and 5 = maternal and paternal DNA having heterozygous α-thal-1 (SEA), 6 = Normal control DNA, and 7 = negative control (blank).

Because this was the first time of PGD for thalassemia in Siriraj Hospital, about 2 months before the procedure started, blastomeres from abnormal fertilized or arrested embryos were sent for molecular genetic division for test of amplification. After the laboratory setting was prepared, the process of ovarian stimulation and IVF/ICSI was performed. We used a blastomere instead of trophectoderm because the process of single cell PCR may need time for evaluation. If trophectoderm were used, the time of the PCR process may cause embryonic problems because there would be a little time for transfer. For the future, when all processes are well established, we may use blastocyst biopsy because many studies have shown better outcomes from more cells removed for PCR testing.[19,21]

The result of PCR did not surprise us because the chance to have Bart's hemoglobinopathy in the embryo was 25 percent and we found 3 from a total of 11 embryos (27.27%) were affected. Although alleles drop out was not found in this study, PCR failure was an important problem because 5 from a total of 11 embryos (45.45%) had an inconclusive result from PCR amplification failure. Fortunately, 3 in 5 inconclusive embryos had abnormal fertilization and would not have been routinely transferred. To solve this problem, blastocyst biopsy may reduce amplification failure rate because we can remove more cells for PCR. However, this process requires quick and accurate diagnostic technique because the blastocyst has little time that is suitable for transfer. Other diagnostic techniques may be needed for improved outcome. In this study, we used PCR with DGGE analysis that is widely accepted and provides more information than standard PCR. However, in many studies, more advanced techniques such as fluorescent multiplex PCR had better results and used less time.[15,22] Additionally, fluorescent multiplex PCR can use multiple probes for simultaneous detection of abnormal genetic defects such as trisomy 21 (Down's syndrome) that many patients including our patient had an increased risk of because of their age.

In conclusion, PGD for alpha thalassemia was first established in Siriraj Hospital. However, the outcome result desired is not yet satisfied because of a high PCR failure rate. For better results, optimized laboratory techniques and instruments are required. We will establish an optimal process using fluorescent multiplex PCR within the year 2008. Not only alpha thalassemia, but also known genetic defects such as beta thalassemia or trisomy 21 can be detected before pregnancy using the PGD program.

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