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SPECIAL ARTICLE

Pre-implantation Genetic Diagnosis of Thalassemias

Wirawit Piyamongkol, M.D., PhD*.

** Department of Obstetrics and Gynaecology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand*

ABSTRACT

Beta-Thalassemia major, beta-thalassemia-Hb E disease and Hb Bart's disease are severe hereditary anemia which are prevalent in Thailand and neighborhood countries. Thalassemia syndromes and hemoglobinopathy cost significant health and economic burden and sometimes maternal morbidity and mortality. The present strategy to reduce new cases is population screening, prenatal genetic diagnosis (PND) and the option for termination of affected pregnancy (TOP) following thoroughly genetic counselling. The advances of reproductive technology and molecular genetics facilitate genetic testing of the embryos prior to transfer into the womb, therefore, embryo selection is possible. Pre-implantation genetic diagnosis (PGD) consists of sampling techniques from the embryos and molecular genetic analysis techniques. Polar body biopsy, cleavage stage embryo biopsy or blastocyst biopsy can be used for sampling DNA material from the embryos. Polymerase chain reaction (PCR) is employed for the analysis of thalassemia mutations. PGD is an alternative to the traditional PND, providing the couples at risk of having severe thalassemia babies an opportunity to get pregnant with a healthy one without the need for TOP. Since 2004, a total of 64 PGD cycles have been performed at the Department of Obstetrics and Gynaecology, Faculty of Medicine, Chiang Mai University, including 37 alpha-thalassemia, 5 beta-thalassemia and 22 beta-thalassemia-Hb E disease, giving rise to 24 healthy pregnancies (27 babies).

Keywords: Embryo selection, hemoglobinopathy, multiplex fluorescent polymerase chain reaction (PCR), pre-implantation genetic diagnosis (PGD), thalassemias.

Correspondence to: *Wirawit Piyamongkol, M,D, PhD., Department of Obstetrics and Gynaecology, Faculty of Medicine, Chiang Mai University, 110 Intawaroros Road, Sripoom, Mueang, Chiang Mai 50200, Thailand, E-mail address: wirawit.p@cmu.ac.th*

Thalassemias

Globin genes mutations causing thalassemia syndrome and hemoglobinopathy are the commonest single gene disorder and cost significant health and economic burden worldwide. Beta-Thalassemia, Hb E disease and alpha-thalassemia are prevalent in Thai and neighborhood population. Homozygous beta-thalassemia (beta-thalassemia major), compound heterozygous beta-thalassemia-Hb E disease and homozygous alpha-thalassemia-1 (Hb Bart's disease) are accounted as the most severe forms[1].

Beta-Thalassemia, an autosomal recessive disorder, is caused by over 200 mutations within the beta-globin gene, characterized by anemia, spleenomegaly, and bone marrow expansion with skeleton deformities. The patients with beta-thalassemia

major or beta-thalassemia-Hb E disease suffer from severe anemia and need regular blood transfusion since the first year of life. Despite adequate blood transfusion, most patients die from complications of iron loading with an average age of 20 years old, unless iron chelators are provided regularly[2]. The access to bone marrow transplantation treatment is still limited and risky option.

Alpha-Thalassemia is more common than beta-thalassemia. However, the patients are infrequently seen in the hospital, as the most severe form (homozygous affected or Hb Bart's hydrop fetalis) leads to still birth and heterozygotes have no symptom and do not need health care. Homozygous alpha-thalassemia leads to the absence of alpha-globin chain synthesis and causes Hb Bart's hydrop fetalis syndrome. Mothers who carry Hb Bart's babies are likely to develop life-threatening complications, i.e. eclampsia, dystocia and hemorrhage. Therefore, the aim of detecting homozygous alpha-thalassemia is to prevent maternal morbidity and mortality.

Prevention and Control Strategy

Since the effective treatment of thalassemias is still limited, most countries who face thalassemias acquire prevention and control programs. Pre-conception or prenatal genetic screening is employed to all couples in order to identify couples at risk of having severe thalassemia offspring. Thoroughly genetic counselling is given to the couples at risk and choices of invasive prenatal diagnosis are offered. Either chrionic villous sampling at 11-13 weeks, amniocentesis at 16-20 weeks or fetal blood sampling at 19-24 weeks can be done for fetal genetic diagnosis. For the diagnosis of Hb Bart's hydrop fetalis, ultrasonography measuring peak systolic velocity of the middle cerebral artery (PSV-MCA) and fetal hydropic signs surveillance can be additional non-invasive options[3]. Negative results reassure the parents that their babies will be healthy, however, in case of affected babies termination of pregnancy is offered for parental decision[1].

Pre-implantation Genetic Diagnosis (PGD)

The first PGD attempt was demonstrated by sexing rabbit blastocysts[4]. However, in that experiment there was no surviving embryo. In human, in vitro fertilization was first successful in 1969[5]. This knowledge has made it possible to study preimplantation human embryos. The preliminary PGD model was performed in mouse embryos using biochemical testing for hypoxanthine phosphoribosyl transferase (HPRT) enzyme deficiency, which is the cause of Lesch-Nyhan syndrome[6]. DNA based PGD model for beta-Globin gene testing in mouse embryos was also successfully carried out[7]. It was exhibited that the development of Day 3 8-cell stage preimplantation human embryo was not compromised following the biopsy[8].

The first successful PGD pregnancies were performed for sex identification using PCR in order to avoid X-linked disorders[9]. IVF procedures was used to generate several embryos and then single blastomeres were biopsied at the cleavage stage[10]. Most of PGD cycles were performed for single gene disorders, some were for chromosomal abnormalities and sexing in families at risk of X-linked diseases. The patients decided to performed PGD because of moral or religious objection to pregnancy termination, a third possessed fertility problems as well as carrying a genetic condition, while a quarter already had termination of pregnancy and do not want to go through another[11].

Sampling Techniques

Sampling of DNA materials for genetic analysis can be carried out using polar body biopsy, cleavage stage Day 3 embryo biopsy or Day 5 blastocyst biopsy. The obtained samples can then by analyzed for the particular disease and healthy embryos can be chosen for transfer.

- ***Polar Body Biopsy***

Polar body biopsy is a form of preconception diagnosis. The procedure is supposed to be non-invasive to the embryos and allows longer analysis time comparing with cleavage stage embryo biopsy. The first polar body which is extruded from primary oocyte is used for genetic analysis[12]. The genetic results of the first polar body are complementary to that of the

oocyte. For example, if the first polar body of a heterozygous subject shows genetic mutation, it means that the oocyte is normal and can be chosen for fertilization. However, meiotic recombination (crossing-over) can turn the predicted results inaccurate. The chance of recombination of telomeric genes can be up to 50% and that of centromeric genes can be less than 1%. Therefore, there is a need to analyzed both first and second polar bodies in order to have comprehensive predictive results[13]. Therefore, the techniques are labor-intensive. In addition, paternal mutation cannot be analyzed by this technique, therefore, sex identification for X linked diseases and the diagnosis of mutations carried by male partners, especially autosomal dominant disorders, cannot be done. In the analysis of autosomal recessive conditions in which both parents are carriers, the oocytes with a mutation will be discarded, even they may generate a heterozygous embryo when fertilized with a sperm with normal allele. Moreover, post-zygotic events, i.e. mosaicism, are not diagnosed by polar body biopsy.

- Cleavage Stage Day 3 Embryo Biopsy

Following fertilization, the zygote undergoes cleavage cell division every 24 hours. On day 3 when the embryo is at 8 cells stage, 1-2 blastomeres can be biopsied without affecting the development of the embryo. More than 90% of the embryos can survive the biopsy procedure[8]. Blastomeres at this stage are totipotent, have not been assigned to be any specific tissues or organs yet. Cleavage stage embryo biopsy on day 3 used to be the most popular technique for PGD[11].

The procedure is performed by zona drilling and blastomere aspiration. The embryo is held using a holding pipette on a micromanipulator. Acid Tyrodes solution is applied to drill a hole in the zona pellucida[8]. The zona can be cut mechanically in a V shape by partial zona dissection (PZD)[14]. Drilling the zona using a laser gives more control of the size of the hole[15]. The blastomere is then gently aspirated through the hole using a biopsy pipette.

Cleavage stage biopsy possesses some disadvantages. Using this technique, only 1-2 cells can be obtained and only 24 hours is available for genetic analysis as most IVF centers prefer to transfer the embryos on day 4. Therefore, the analysis techniques need to be fast, sensitive and accurate. Because of the chance of chromosomal mosaicism in preimplantation embryos[16] and allele specific amplification failure or allele drop out (ADO) of the PCR assays from single cells, the analysis of two blastomeres (separately) from each embryo is recommended to reduce the chance of misdiagnosis.

- Blastocyst Biopsy

Blastocysts can be obtained from extended culture of IVF embryos. Blastocyst biopsy can be done on day 5 to day 6 post-fertilization when the blastocyst consists of about 120 cells. A blastocyst contains two cell types; inner cell mass which will develop into the embryo and trophectoderm which will develop into the placental membranes. A hole is drilled in the zona pellucida and the embryo is put back in the culture medium. When the trophectoderm cells herniate from the embryo[17], 10-30 trophectoderm cells can be biopsied for genetic diagnosis. The disadvantage of this technique is that the analysis of extraembryonic cells may not represent the inner cell mass or the embryo. With the longer in vitro development, the number of the surviving embryos reduces. Moreover, adding up with the genetic analysis time the endometrium is not appropriate for implantation. Recently, due to the improvement of extended embryo culture medium and techniques, there are more surviving embryos to blastocysts stage. Incorporating with embryo freezing techniques, blastocyst biopsy has gained in popularity worldwide. Additional advantages of this technique include more number of biopsied cells and more time for the analysis.

Molecular Genetic Diagnosis

Fluorescent in situ hybridization (FISH) was used for sexing[18], and detecting numerical chromosomal abnormalities and chromosome translocations[19]. Recently comparative genome hybridization array (aCGH) and NextGen Sequencing (NGS) have gained popularity for comprehensive chromosome analysis.

Polymerase chain reaction (PCR) is employed for diagnosis of single gene disorders, including Duchenne muscular dystrophy[20], Fragile X syndrome[21], Tay Sachs disease[22], Marfan's syndrome[23], Myotonic Dystrophy[24], Charcot Marie Tooth type 1A[25], familial adenomatous polyposis coli (FAPC)[26], Huntington's chorea[27], severe inherited skin diseases[28], sickle cell anaemia[29], spinal muscular atrophy[30], beta-thalassaemia[31], congenital adrenal hyperplasia[32], Lesch Nyhan syndrome[33], medium chain acyl CoA dehydrogenase (MCAD) deficiency[34] and alpha-thalassemia[35]. Data demonstates no significant difference in pregnancy rates between those from PGD and routine IVF cycles[11].

- Single Cell Polymerase Chain Reaction (PCR)

PCR is a powerful molecular technique for rapidly multiplying a specific DNA fragment to a level that can be further analyzed by other methods[36]. Various modified techniques have been developed for various purposes, including forensic assay, evolutionary biology, genetic screening, mutation analysis, PND and PGD of single gene disorders. More sophisticated and modern advanced techniques have been applied. However, amplification failure, ADO and contamination are essential problems during PCR at the single cell level. Due to the wide variety of mutations within the same and among different genes, particular analysis methods are needed for particular mutations.

- Amplification Failure (AF)

Amplification failure of single cell PCR was realized when the first series of PGD for X-linked disorders was misdiagnosed[37]. Y chromosome specific sequences were amplified from the biopsied blastomeres in order to avoid transfer of male embryos. The absence of amplification indicates a female embryo. However, amplification failure leads to the same diagnosis result. Amplification failure is not an unusual phenomenon during single cell PCR and can be found about 10%[38].

Possible causes of amplification failure include the isolated cell may be lost during transfer to the PCR tube or the cell may be anucleate or in the process of degeneration. For this reason, current protocols are designed not to interpret a missing result as normal, but as an affected genotype. This design will not lead to serious misdiagnosis in cases of amplification failure, but may reduce the number of un-affected embryos for transfer. Amplification failure may also be caused by the suboptimal conditions of the lysis protocol, primers combinations, PCR protocol or poor cell quality. Therefore, single cell PCR protocols need be thoroughly optimized before clinical apply.

- Allele Drop Out (ADO)

One common problem of single cell PCR is ADO or allele specific amplification failure when one of the two alleles in a heterozygous sample randomly fails to amplify[39]. Consequently only one allele is shown after PCR, giving the interpretation as a homozygote. This problem is particular to PCR with low copy number of DNA templates and can give rise to misinterpretation. Misdiagnoses that might have caused from ADO have been reported. ADO is especially crucial in the analysis of dominant disorders or recessive disorders with two different mutations (compound heterozygous). However, in PGD of a recessive disease, ADO would not lead to the transfer of an affected embryo, but will reduce the number of heterozygous (unaffected carrier) embryos for transfer.

ADO occurs about 2-20% of single cell PCR[40]. ADO may causes from several theories, the foremost of which are: DNA degradation causing PCR-refractory breaks in both DNA strands; and inaccessibility of the DNA templates because of imperfect PCR conditions or incomplete cell lysis. Possible methods in order to improve amplification efficiency and minimize ADO include using highly sensitive fluorescent PCR (F-PCR) techniques[41], increasing PCR denaturation temperature[40], and the use of different cell lysis buffers[42]. However, none of these methods seems to consistently eliminate ADO. Most experienced PGD laboratories can generally reduce ADO rates to 5-10%. Strategy to avoid misdiagnosis caused from ADO involves the addition of a polymorphic linked marker analysis together with the mutation analysis reaction as

a multiplex PCR[43] or in separate reactions after whole genome amplification (WGA)[26] for back up linkage analysis results. In addition, it is suggested to interpret the results from two cells of each embryo to reduce the risk of misdiagnosis from ADO[38].

- Contamination

Contamination is another crucial problem encountering single cell PCR. ICSI is recommended for fertilization in order to eliminate the chance of paternal (sperm) DNA contamination. All maternal cumulus cells need to be removed before insemination in order to reduce the risk of maternal DNA contamination. The biopsied blastomeres need to be washed several times in clean medium in order to get rid of any remaining cumulus cells or DNA that may remain in the culture medium prior to transferring into the PCR tubes. PCR set up in a DNA-free environment separating from the analysis area can reduce the chance of getting 'carry over' PCR products from previous experiments. All media and reagents need to be tested before use. Nested PCR by amplifying the first amplified products using the second set of primers situated internally to those used in the first reaction was recommended to prevent carry over contamination[44]. Some PGD centers employ a restriction enzyme to digest extraneous DNA in PCR mixture prior to adding target DNA[45].

Despite all efforts, contamination may still occur and lead to misdiagnosis. Misdiagnosis from maternal DNA was documented[27]. In case of mother carrying the mutation gene of a dominant disorder, maternal DNA contamination will not lead to misdiagnosis, but a reduced number of embryos for transfer. In case of recessive disorders, contamination of maternal heterozygous DNA in a homozygote affected cell will lead to a heterozygous interpretation, and the transfer of affected embryos. To reduce the chance of misdiagnosis from contamination, DNA fingerprinting is used to trace down the presence of contamination by co-amplifying a highly polymorphic marker with the test gene as a multiplex PCR. The genotype in an embryo that deviates from the 4 possible combinations of parental alleles indicates the presence of contamination.

- Nested PCR

PCR templates with very low copy number, especially a single copy, need more PCR cycles in order to produce enough PCR products for further analysis on traditional gel electrophoresis. However, the very last PCR cycles will be less efficient due to the reduced substrates, i.e. primers, dNTPs, polymerase enzyme, etc. For this reason, a second amplification with a fresh identical PCR mixture was employed in order to produce enough PCR products to a detectable level[46]. Nested PCR involving two successive amplification reactions is an improved technique for single cell PCR. The second reaction tube consists of fresh PCR mixture with a different set of primers. The second set of primers is designed to situate within the first amplicon and generates a shorter DNA fragment. This technique is useful not only in increasing sensitivity, but also increasing specificity and reducing risk of contamination[47].

- Fluorescent PCR (F-PCR)

Conventional techniques of analyzing PCR products using electrophoresis include ethidium bromide or silver staining or radioactive labelled primers, which are either less sensitive or time consuming. The use of F-PCR with an improved sensitivity and specificity is useful for single cell PCR[48]. Oligonucleotide primers are attached with fluorescent molecules generating amplified products labelled with fluorescent dye. When these F-PCR products migrate under electrophoresis to the point where laser intersects, fluorescent molecules will be triggered and generate a signal with a specific wavelength which can be detected by a CCD (charged couple device) sensor and analyzed by computer software. F-PCR products from a single cell can be identified after only 35 cycles of amplification. This excludes the need for nested PCR and accelerates the analysis. Using F-PCR, size standards can be run in the same lane, consequently, size determination is as accurate as a single base pair difference.

- Multiplex PCR

Molecular analysis of single cell PCR for PGD can be done only once. Multiplex PCR permits more than one locus to be analyzed by combining unrelated sets of primers in a PCR reaction(35). Each combination of primer sets in multiplex PCR needs to be optimized for the relative primer concentrations, annealing temperatures and reaction buffers in order to minimize interaction between unrelated primers or PCR products. The PCR products of different loci can be differentiated by designing the primers to generate different amplified fragments sizes. The analysis of multiplex PCR products is easier on F-PCR. By labelling the primers with different dyes, the fragments from different sets of primers can be simply identified, even those with the same fragment size. This facilitates the analysis of multiple loci from a single cell, i.e. the analyses of more than one disease or different mutations and polymorphic markers. The additional polymorphic marker can be useful for a backup linkage analysis results and contamination detection[35].

- PGD of Thalassemias

PGD for thalassemias at the Department of Obstetrics and Gynaecology, Faculty of Medicine, Chiang Mai University started in 2004. Day 3 cleavage stage embryo biopsy was employed during the early years and then Day 5 blastocyst biopsy has been routinely performed during the last few years. Multiplex F-PCR protocols were developed for PGD of beta-thalassemia[31], and alpha-thalassemia[35]. Multiplex F-PCR protocol incorporating with mini-sequencing was also developed for more beta-thalassemia mutations and beta-thalassemia-Hb E disease. A total of 64 PGD cycles have been performed, including 37 alpha-thalassemia, 5 beta-thalassemia and 22 beta-thalassemia-Hb E disease, giving rise to 24 healthy pregnancies (27 babies). No contamination or misdiagnosis was detected. More PGD protocols for more mutations of thalassemias and other single gene disorders using advanced techniques are underdeveloped.

Conclusions

Severe thalassemias and hemoglobinopathy are prevalent and cause significant health and economic problems in Thailand and neighbor countries. Effective treatment is still limited. The present strategy to reduce new cases is population screening, prenatal diagnosis and termination of affected pregnancy. PGD is an alternative to the traditional PND, providing the couples at risk of having affected babies an opportunity to start a pregnancy with a healthy one without the need for TOP. PGD center at Chiang Mai University was established in 2004 and performed 64 PGD cycles, giving rise to 24 pregnancies (27 babies).

Potential conflicts of interest

The author declare no conflict of interest.

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