

First Successful Preimplantation Genetic Diagnosis in Singapore – Avoidance of β -Thalassaemia Major

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Abstract

Introduction: We report on the first successful preimplantation genetic diagnosis (PGD) in Singapore. **Clinical Picture:** A couple who are β -thalassaemia carriers and have an affected daughter requested for PGD. **Treatment:** Two cycles of PGD were performed on the couple. β -thalassaemia mutations were detected using a nested PCR and minisequencing strategy, and unaffected embryos were selected for transfer. **Outcome:** A singleton pregnancy was achieved in the second PGD cycle, resulting in the birth of a healthy baby boy with carrier genotype. **Conclusions:** This case report documents the first successful PGD in Singapore, involving a couple at-risk of transmitting β -thalassaemia major.

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Key words: Beta-thalassaemia, Multiplex minisequencing, Polymerase chain reaction, Preimplantation genetic diagnosis

Introduction

As one of the most common genetic disorders worldwide, β -thalassaemia represents a significant healthcare burden in many countries in the malaria belt. In Singapore, the carrier frequency for β -thalassaemia mutations is 2.7% in the Chinese, 6.3% in the Malays and 0.7% in the Indians.¹ Couples who are both heterozygous for the β -thalassaemia gene have a 25% risk of conceiving a child with β -thalassaemia major, a debilitating blood disorder requiring lifelong blood transfusions and associated with reduced life expectancy (~40 to 50 years). Genetic counselling and prenatal diagnosis enable at-risk couples to avoid having an affected child. Although these programmes have significantly decreased the number of births of β -thalassaemia major children in Singapore over the last 10 years,² couples with an affected fetus are invariably faced with the difficult decision to terminate the pregnancy. Preimplantation genetic diagnosis (PGD) presents an alternative option to prenatal diagnosis. It involves in vitro fertilisation (IVF) followed by determination of the genetic status of embryos. As only the unaffected embryos are

transferred to establish pregnancy, PGD may be an attractive option for at-risk couples who have had multiple affected pregnancies and resultant terminations, who have religious or cultural objections to pregnancy terminations, or who are secondarily sub-fertile and require assisted reproduction.

We report here on the first successful application of PGD in Singapore, for a couple at risk of transmitting β -thalassaemia major (IVSII,654 (C→T) / -28TATA (A→G)), resulting in the birth of a healthy baby boy.

Case Report

The patient is a 36-year-old Chinese woman heterozygous for the common Southeast Asian β -thalassaemia mutation IVSII,654 (C→T) and her husband who carries the -28TATA (A→G) mutation. They have an affected 6-year-old daughter (compound heterozygous for both mutations) who requires regular blood transfusions. The PGD procedure was performed under a study protocol approved by the Domain-Specific Review Board of the National Healthcare Group (C/00/549) and the Ethics Review Board of the Singapore General Hospital (155/2002).

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Table 1. Primer Sequences for Nested Multiplex PCR Analysis of the β -globin Gene and the D7S486 Micro-satellite Locus

Name	5' →3' sequence	GenBank ID: Nucleotides	Concentration	Amplicon (size)
First round PCR primers				
<i>β-globin locus</i>				
β -F*	ACGGCTGTCATCACTTAGAC	HUMHBB: 62010-62029	0.2 μ M	1457bp
β -R1*	AAGAGGTATGAACATGATTAGC	HUMHBB: 63466-63445	0.2 μ M	
<i>D7S486 micro-satellite locus</i>				
D7S486-1F	GCTGGAATCTGTTCTGGC	Z16567: 2-19	0.2 μ M	197-229bp
D7S486-1R	GCAATGAGCCGAGATCCTG	Z16567: 230-212	0.2 μ M	
Second round PCR primers				
<i>β-globin locus</i>				
BGLO-2F	GTCATCACTTAGACCTCACC	HUMHBB: 62016-62035	0.2 μ M	1409bp
BGLO-2R	CAGAATAATCCAGCCTTATCC	HUMHBB: 63424-63404	0.2 μ M	
<i>D7S486 micro-satellite locus</i>				
D7S486-2F	AAAGGCCAATGGTATATCCC	Z16567: 52-71	0.2 μ M	114-146bp
D7S486-2R-FAM	FAM-GCCCAGGTGATTGATAGTGC	Z16567: 197-178	0.2 μ M	
Cycle-minisequencing primers				
SNPIVSH654	TGATAATTTCTGGGTTAAGG	HUMHBB: 63265-63284	0.2 μ M	
SNP-28TATA*	(gact) ⁷ GATGGCTCTGCCCTGACTT	HUMHBB: 62128-62110	0.2 μ M	

*Refer to reference 3

After ovarian stimulation, oocytes were retrieved and fertilised by intra-cytoplasmic sperm injection (ICSI). Embryos were cultured for 3 days and biopsied after reaching the 6- to 10-cell stage. An acidic tyrodes solution was used to dissolve an opening in the zona pellucida, and up to 2 blastomeres were removed from each embryo. Blastomeres were transferred into a wash buffer (0.4% bovine serum albumin/1X phosphate buffered saline), then transferred individually into 0.2 mL reaction tubes containing 5 μ L of lysis buffer (0.2 M potassium hydroxide, pH 14.0). Reaction tubes were incubated at 65°C for 10 minutes, after which 5 μ L of neutralisation buffer (0.2 M Tricine, pH 5.7) was added.

Lysed and neutralised blastomere DNA was subjected to a nested polymerase chain reaction (PCR) amplification protocol. In the first PCR round, both the β -globin gene and the D7S486 microsatellite marker were simultaneously amplified in a volume of 50 μ L containing the lysed and neutralised blastomere, 0.2 μ M of each first round PCR primer (Table 1), 0.2 mM of each deoxyribonucleotide triphosphate (dNTP) (Roche Biochemicals), and 2.5 units of HotStarTaq DNA Polymerase (Qiagen) in 1X PCR buffer containing 1.5 mM MgCl₂. Thermal cycling was performed in a Biometra T3 thermal cycler (Biometra), with an initial 15 minutes enzyme activation at 95°C, followed by 30 cycles of 98°C denaturation for 45 seconds, 55°C annealing for 45

seconds, and 72°C extension for 2 minutes, culminating in a final 5 minute extension at 72°C.

In the second PCR round, secondary amplifications of the β -globin gene and D7S486 marker were performed separately using 3 μ L aliquots of first round PCR product. PCR reaction conditions were similar to the first PCR round, except that the primers were replaced with second round PCR primers for β -globin or D7S486 (Table 1). In addition, the amount of DNA polymerase was reduced to 1 unit. Thermal cycling conditions were similar to the first round PCR, except that the cycle number was 30 for β -globin and 15 for D7S486. To monitor for allele drop-out and/or exogenous DNA contamination of sample tubes, D7S486 alleles were resolved on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems) and analysed using GeneScan 3.7 software.

For β -globin mutation detection, a 2.5 μ L aliquot of the second round PCR product for β -globin was incubated with 1.5 μ L of an enzyme mix, containing 5 units of exonuclease I (ExoI) (USB Corporation) and 1 unit of shrimp alkaline phosphatase (SAP) (USB Corporation), at 37°C for 15 minutes followed by 80°C for 15 minutes. A multiplex minisequencing reaction was performed on the treated PCR product using the SNaPshot™ multiplex ready reaction mix (Applied Biosystems) and 0.2 μ M of each minisequencing primer (Table 1), in accordance with the manufacturer's

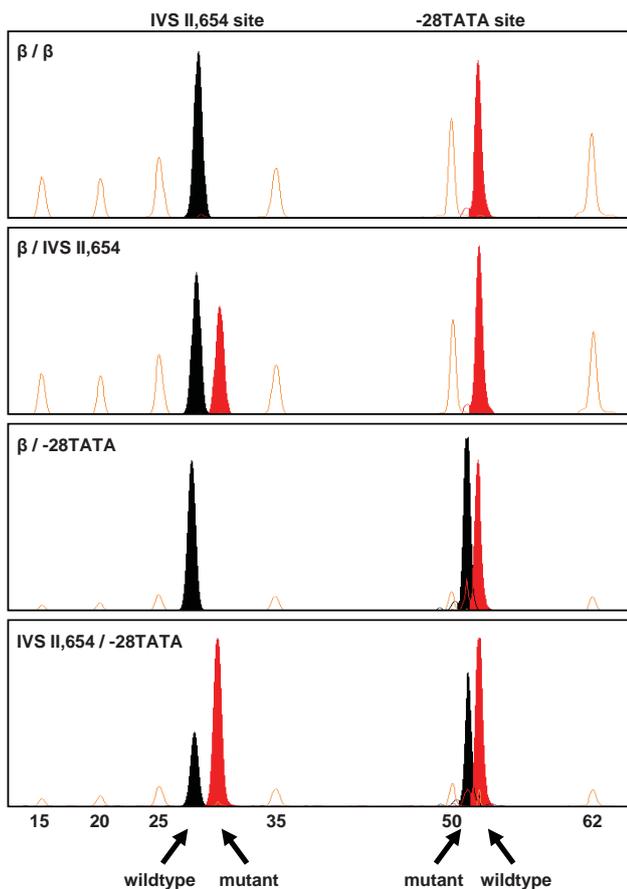


Fig. 1. GeneScan™ electropherograms of minisequencing results at the IVSII,654 (C→T) and -28TATA (A→G) mutation sites. Shown from top to bottom are electropherograms indicating normal (β/β), carrier ($\beta/IVSII,654$ and $\beta/-28TATA$), and affected ($IVSII,654/-28TATA$) genotypes. Unfilled peaks indicate LIZ-120™ molecular size marker fragments.

protocol. Minisequencing products were resolved on the ABI PRISM® 3100 Genetic Analyser (Applied Biosystems) and analysed using GeneScan 3.7 software. β -globin alleles were determined based on the colour and position of fluorescent peaks in the electropherogram, examples of which are shown in Figure 1.

Two PGD cycles were performed on the couple and the results are summarised in Table 2. In the first cycle, 10 oocytes were recovered, of which 6 were fertilised after ICSI. On day 3 post-fertilisation, 4 embryos developed to at least the 6-cell stage while a fifth embryo reached the 5-cell stage, and these 5 embryos were biopsied. One blastomere was removed from the 5-cell stage embryo, while 2 blastomeres were removed from the more developed embryos. Of the 5 embryos tested, only 3 produced analysable results. Two embryos were diagnosed as normal (β/β) and were transferred, but no pregnancy resulted.

A second PGD cycle was initiated 10 months later. In this cycle, 13 oocytes were recovered, of which 5 fertilised. On day 3 post-fertilisation, 4 embryos developed to the minimum 6-cell stage. Two blastomeres were removed from each of these 4 embryos and all gave analysable results. One carrier ($\beta/-28TATA$) and 2 normal (β/β) embryos were transferred back to the patient. A singleton pregnancy resulted, and a healthy baby boy was delivered at term in February 2006. Postnatal DNA analysis indicated that the child has a carrier genotype ($\beta/-28TATA$), thus confirming his clinically unaffected status.

Discussion

This report documents the first successful performance of preimplantation genetic diagnosis in Singapore. In this

Table 2. Summary of PGD Cycles

	PGD Cycle 1	PGD Cycle 2
Oocytes retrieved	10	13
Oocytes fertilised after ICSI	6	5
Embryos biopsied	5	4
Embryos diagnosed as:		
Normal (β/β)	2	2
Carrier ($\beta/IVSII,654$ or $-28TATA$)	0	1
Affected ($IVSII,654/-28TATA$)	1	1
Embryos without a diagnosis	2	0
Embryos transferred (Genotype)	2 (both β/β)	3 (β/β x2, $\beta/-28TATA$ x1)
Outcome	No pregnancy	Singleton pregnancy; ($\beta/-28TATA$)

instance, PGD was employed to help a β -thalassaemia carrier couple to have a healthy child. The thalassaemias (α and β) are the most common genetic disorders in Singapore.² For β -thalassaemia, more than 200 mutations that have been identified, most of these being point mutations or small deletions within the β -globin gene.⁴ Homozygotes and compound heterozygotes for β -thalassaemia mutations suffer from β -thalassaemia major, which presents with severe anaemia requiring blood transfusions and expensive iron chelation therapy.⁴ Although PGD is intended to enable at-risk couples to embark on pregnancies with unaffected fetuses, prenatal genetic testing is still strongly recommended once a PGD pregnancy is achieved to confirm the diagnostic results. In the case, however, the couple had declined confirmatory prenatal diagnosis due to the associated risk of miscarriage.

PGD has been an option for β -thalassaemia prevention for several years.⁵⁻¹⁰ It is currently the only option for at-risk couples to begin with a pregnancy knowing in advance the unaffected genetic status of their offspring. PGD may thus be an attractive option for certain at-risk couples such as those for whom pregnancy termination is religiously or culturally unacceptable. Additionally, couples who have had previous affected children and/or pregnancies and undergone previous pregnancy terminations, but do not wish to face the associated emotional disappointments and anguish again, may find PGD an acceptable route to having more (unaffected) children. Finally, some at-risk couples may be sub-fertile and require assisted reproduction to conceive. Given their difficulty in conceiving, PGD becomes an all the more compelling option to ensure that a successful pregnancy is also an unaffected pregnancy.

In Singapore, the PGD programme began as an Institutional Review Board approved research protocol in 2003 funded by the Biomedical Research Council (BMRC) of the Agency for Science, Technology and Research (A*STAR), which covered IVF and PGD costs for study participants. The first participants enrolled in the programme in March 2004. Four couples including the couple in this report, 2 couples

for α -thalassaemia and 2 for β -thalassaemia, underwent a total of 5 PGD cycles before the sixth cycle resulted in this successful unaffected birth.

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