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SPECIAL ARTICLE

Pre-implantation Genetic Testing for Monogenic Disorders at Chiang Mai University : 20 Years Experience

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ABSTRACT

Objectives: The prevention and control program for severe thalassemias in Thailand has been very successful. However, at present, some families at risk of having an offspring with severe thalassemias are looking for a better option other than termination of pregnancy. Pre-implantation genetic testing of monogenic disorders (PGT-M) or embryo selection would be the correct answer. This study presents the PGT-M with 20 years experience at Chiang Mai University.

Materials and Methods: The couples at risk of having the offspring with Hemoglobin Bart's (Hb Bart's) disease, beta-thalassemia major, and beta-thalassemia-hemoglobin (Hb) E disease came in for genetic counselling and PGT-M treatment. PGT-M protocols are based on multiplex fluorescent polymerase chain reaction (PCR) and mini-sequencing. PGT-M protocols for Hb Bart's disease, beta-thalassemia major, and beta-thalassemia-Hb E disease have been developed, tested, and clinically applied.

Results: Since 2003, a total of 168 PGT-M cycles in 125 families have been performed, giving rise to a total of 75 pregnancies (85 healthy babies). A total of 132 clinical PGT-M cycles were performed for 111 couples at risk of having the offspring with Hb Bart's disease, beta-thalassemia major, and beta-thalassemia-Hb E disease giving rise to 66 pregnancies with 76 babies. No misdiagnosis has been detected. Notably, three families were at risk of having the offspring with both Hb Bart's disease and beta-thalassemia-Hb E disease. Two families had already had a affected child with beta-thalassemia-Hb E disease and came in for PGT-M of beta-thalassemia-Hb E disease and human leukocyte antigen (HLA) matching.

Conclusion: The pregnancy rates were 44.6%, however, some PGT-M cycles are still on-going and the embryos are kept frozen. More pregnancies should be obtained when the patients return for embryo transfer. In addition to severe thalassemias, PGT-M for other rare diseases have been done. During the past 20 years, over 20 PGT-M protocols have been developed, tested, and clinically applied. All protocols were novel and home grown.

Keywords: embryo selection, multiplex fluorescent polymerase chain reaction (F-PCR), pre-implantation genetic diagnosis (PGD), pre-implantation genetic testing for monogenic disorders (PGT-M), thalassemias

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Introduction

Pre-implantation Genetic Testing (PGT)

Pre-implantation genetic testing (PGT) for embryo selection was first performed for sex determination using PCR to avoid X-linked disorders by Alan Handyside at Hammersmith Hospital, London[1]. For PGT, single blastomeres for PCR analysis were biopsied[2] from embryos generated using in vitro fertilization (IVF) techniques[3]. Since then until 2018, over 100,000 clinical PGT cycles have been performed worldwide resulting in over 20,000 clinical pregnancies and more than 13,000 PGT babies were born according to European Society of Human Reproduction and Embryology (ESHRE) PGT Consortium[4]. Indications for PGT include PGT for monogenic disorders (PGT-M), PGT for aneuploidies (PGT-A) and PGT for structural rearrangements (PGT-SR). Blastocyst biopsy[5] has become the most popular sampling methods instead of cleavage stage embryo biopsy in the early age. Blastocyst biopsy provided more cells, consequently, eases molecular analysis, however, the embryos need to be kept frozen after the biopsy waiting for transfer. Therefore, molecular procedures do not need to rush.

Molecular Genetic Techniques

Due to misdiagnosis of embryo gender in the very first cases from allele drop out (ADO)[6], fluorescent in situ hybridization (FISH) was recommended for sexing[7], numerical chromosomal abnormalities and chromosome translocations[8]. The most popular platform, AneuVysis, limited to 5 chromosomes probes, i.e. chromosomes 21, 18, 13, X and Y. Comparative genome hybridization array (aCGH) replaced FISH in 2011 when BlueGnome could overcome the 72-hour incubation period into 16-hour incubation period. 24Sure could reveal copy number variation (CNV) of 22 chromosomes, X and Y[9]. Since 2015 Next Generation Sequencing (NGS) CGH has become the main platform for PGT-A due to its higher sensitivity and lower cost[10].

- Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR)[11] has been the main tool for PGT-M including Myotonic Dystrophy[12], beta-thalassemia[13], alpha-thalassemia (-SEA)[14], beta-thalassemia-Hb E disease[15], Duchenne muscular dystrophy[16], Marfan's syndrome[17], Oculocutaneous Albinism (OCA)[18], etc. The present standard of blastocyst biopsy provides 5-10 trophectoderm for PCR analysis which is better than the formally cleavage stage embryo biopsy which provides only 1-2 blastomeres for the analysis. Embryo freezing after blastocyst biopsy also reduces the time pressure of the analysis. Prior to embryo freezing techniques, cleavage stage embryo biopsy on day 3 with embryo transfer on day 4, the time frame for PGT was restricted to only less than 24 hours. However, amplification failure, ADO[19] and contamination[20] are still crucial obstacles in PCR procedures. The details of techniques and obstacles for PGT-M were discussed previously[21].

- Fluorescent PCR (F-PCR)

The use of fluorescent PCR (F-PCR)[22] by labelling the primers with fluorochromes helps in increasing the sensitivity and the specificity of PGT-M. The combination of several sets of primers using different fluorescent dyes facilitates multiplex F-PCR[14]. This enables the analysis of more than one locus/gene at a time. PGT-M protocols at Chiang Mai University are based on multiplex F-PCR. The addition

of whole genome amplification (WGA) can increase the DNA templates of low copy number, i.e. samples from embryo biopsy, enormously, as a result, PCR can be performed as many times as required[23].

- Real-time PCR (RT-PCR)

Although real-time PCR are usually employed for quantitative assay, it can also be used as qualitative function. Allele discrimination assay can distinguish between homozygous normal, heterozygous and homozygous affected of single nucleotide substation mutation. This has been demonstrated as an simple, quick and accurate assay for the identification of Hb E disease (c.26G>A)[24].

Thalassemias

Thalassemias and hemoglobinopathy, autosomal recessive conditions, are the most common monogenic disorder. alpha-Thalassemia, beta-thalassemia, and Hb E disease are common in Thailand. Hb Bart's disease (homozygous alpha-thalassemia-1), beta-thalassemia major, and beta-thalassemia-Hb E disease are the most severe forms of thalassemia syndrome[25]. beta-Thalassemia major and beta-thalassemia-Hb E disease patients start having anemia and need blood transfusion since 6 months after birth. The patients also need iron chelators and bone marrow transplantation as indicated. There are more than 200 mutations of beta-thalassemia reported. Families with different mutations need different primers/protocols for PGT-M. It is usual that one family may carry more than one mutation and the affected members are compound heterozygous. This makes molecular diagnosis even more sophisticated. The most severe form of alpha-thalassemia causes homozygous alpha-thalassemia-1 or Hb Bart's hydrop fetalis. Fetuses with Hb Bart's die in utero or soon after birth, however, cause significant maternal morbidity and mortality. Therefore, the aim of Hb Bart's control is to save the mothers.

Prevention and Control of Thalassemias

At present, most of the thalassemias management focuses on supportive measures. Countries with the prevalence of thalassemias develop prevention and control program by genetic counselling, population screening, prenatal diagnosis and the option for termination of affected pregnancy in order to reduce the number of new cases[25]. However, invasive prenatal diagnosis possesses some risk of miscarriage and the choice of termination of pregnancy is unpleasant. Embryo selection gives the couples at risk the chance to start the pregnancy with assuring that the baby is unaffected and the option for termination of pregnancy is omitted.

This study presents the PGT-M protocols for thalassemias with 20 years experience at Chiang Mai University.

Materials and Methods

The couples at risk of having the offspring with Hb Bart's disease, beta-thalassemia major, and beta-thalassemia-Hb E disease came in for genetic counselling and PGT-M treatment. Standard in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) procedures, embryo culture and embryo biopsy were carried out. The biopsied cells were then delivered to the Department of Obstetrics and Gynaecology, Faculty of Medicine, Chiang Mai University, Thailand for PGT-M. DNA extraction was performed using proteinase K/sodium dodecyl sulfate (PK/SDS) protocol as previously described[15]. One µl of 17 mmol/l sodium dodecyl sulfate (SDS, Sigma®) and 2 µl of 125 mg/ml proteinase K (PK, Roche Diagnostics (Thailand) Ltd.) were added into 2 µl of phosphate-buffered saline (PBS, Cell Signaling Technology) with 0.1% polyvinyl alcohol (PVA, Sigma-Aldrich) containing the biopsied cells. The mixtures were incubated at 37°C for 1 h, and at 99°C for 15 min on a thermal cycler (Roche Diagnostics (Thailand) Ltd.). PGT-M protocols for Hb Bart's disease, beta-thalassemia major, and beta-thalassemia-Hb E disease were developed, tested and clinically applied. Informed consent was obtained. The study was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University, Thailand (study code: OBG-

2564-08660).

PGT-M protocols for alpha-Thalassemia(-SEA) using multiplex F-PCR (PAF protocol)

The extracted DNA was amplified using PAF protocol (Table 1) as gap F-PCR for alpha-thalassemia(SEA) deletion determination(14). D16S475, an short tandemly repeat (STR) linking to alpha-globin gene (HBA), was included for contamination detection and linkage analysis. The primers were labelled with the fluorescent dye 6'FAM® (blue), VIC® (green), PET® (red) and NED® (yellow/black). Primers details, PCR mixtures and thermal cycler programs are demonstrated in Table 1. The PCR products were then analyzed by Fragment Analysis on an automated laser fluorescent sequencer ABI Prism® 3130 (GenePlus Co., Ltd.). Three amplified fragments (288bp 6'FAM® (blue), 130bp VIC® (green), and 110bp PET® (red)) of the normal allele and one (217bp 6'FAM® (blue) & VIC® (green)) of the mutant allele can be identified by F-PCR

Table 1. Pre-implantation genetic testing for monogenic disorders (PGT-M) protocols for thalassemias. PAF is PGT-M protocol for alpha-thalassemia(-SEA). PBF is PGT-M protocol for beta-thalassemia with deletion and insertion using F-PCR i.e. CD41/42 (-TTCT). PEF is PGT-M protocol for beta-thalassemia with single base substitution using mini-sequencing i.e. Hb E disease (c.26G>A), c.17A>T.

Protocols	Primers	Sequences	Labeling Dyes	Mixtures	Thermal Cycles	Interpretations	References
PAF	W1	5'-GAA GGA GGG GAG AAG CTG AG-3'	6'FAM®	• 1 µl of 10X PCR Reaction Buffer with 20 mM MgCl2 • 2 µl of 5X GC-RICH solution • 0.2 mM dNTPs • 0.5 U FastStart™ Taq DNA Polymerase • 200 mM of each primers • Water • 10 µl total volume	• 95°C 4 min • 40 cycles of • 95°C 45 s • 60°C 45 s • 72°C 60 s • 72°C 10 min	6'FAM® (blue) (288bp) normal	(14)
	W2	5'-TGT GGA AAA GTT CCC TGA GC-3'	-			VIC® (green) (130bp) normal	
	W3	5'-TGC ACA CCT ATG TCC CAG TT-3'	-			PET® (red) (110bp) normal	
	W4	5'-TTG AGA CGA TGC TTG CTT TG-3'	VIC®			6'FAM® (blue) & VIC® (green) (217bp) mutant	
	W5	5'-GCC ACT GCC TGC TGG TG-3'	PET®				
	W6	5'-AGG TCA GCA CGG TGC TCA C-3'	-				
	D16S475F	5'-AGG GGT TGA CAG AGT GAG ACT CC-3'	NED®			HBA STR linked marker	
	D16S475R	5'-CAG GAA CAG AAA ATA CTG CAC GG-3'	-				
PBF	bthalw1f	5'-CCT GAG GAG AAG TCT GCC GTT AC-3'	VIC®	• 5 µl of 2X QIAGEN® Multiplex PCR Master Mix • 200 mM of each primers • Water • 10 µl total volume	• 95°C 15 min • 37 cycles of • 94°C 30 s • 60°C 90 s • 72°C 90 s • 72°C 10 min	c.41/42 (-TTCT) VIC® (green) (388bp) normal VIC® (green) (384bp) mutant	(13)
	bthalw1r	5'-GTG CAG CTC ACT CAG TGT GGC-3'	-				
	HUMTH01f	5'-AGG GTA TCT GGG CTC TGG-3'	NED®			HBB STR linked marker	
	HUMTH01r	5'-CTT CCG AGT GCA GGT CAC-3'	-				
PEF				• 0.86 µl of ExoProStar™ 1-Step • 2.14 µL of the PCR products • 3 µl total volume	• 37°C 30 min • 80°C 15 min	(purification step)	(15)
	c.26G>A	5'-ACG TGG ATG AAG TTG GTG GT-3'	-	• 5 µL of SNaPshot® Multiplex Kit • 200 mM of mini-sequencing primer • 3 µL of purified templates • Water • 10 µl total volume	• 25 cycles of • 96°C 10 s • 50°C 5 s • 60°C 30 s	G(blue)>A(green)	(15)
	c.17A>T	5'-CAA CTT CAT CCA CGT TCA CCT-3'	-			Complimentary T(red)>A(green)	

HBA = alpha-globin gene, HBB = beta-globin gene, STR = short tandemly repeat

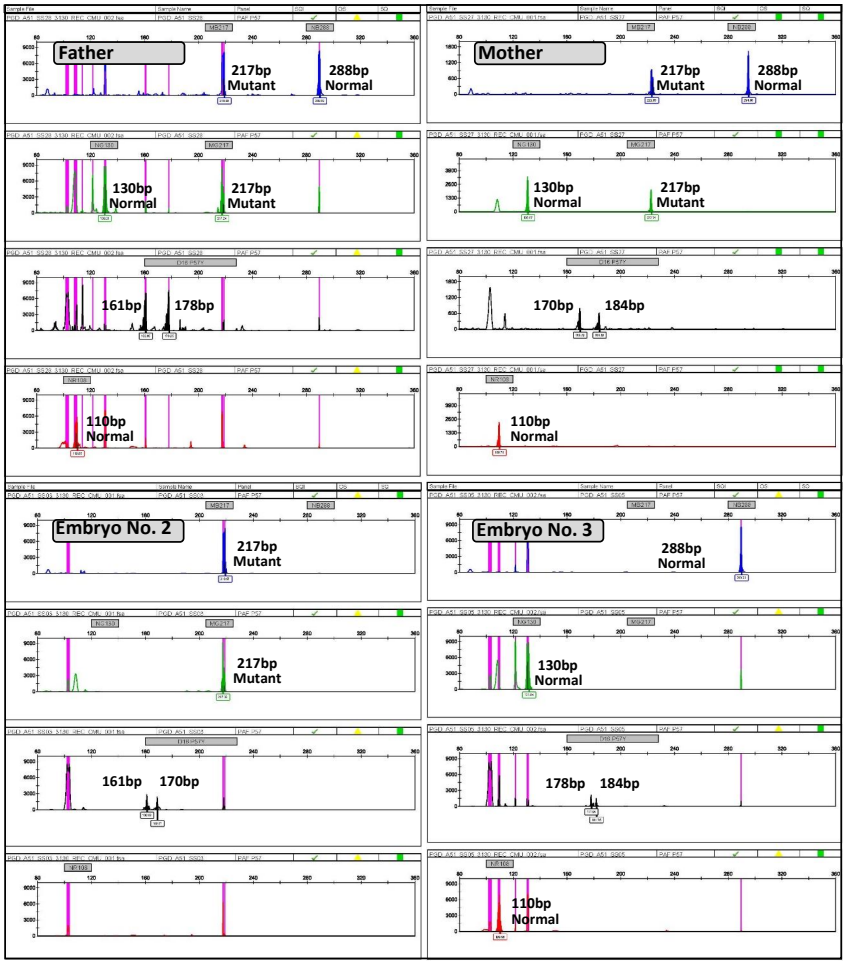

Fig. 1. Pre-implantation genetic testing for monogenic disorders (PGT-M) of Hb Bart's disease (alpha-thalassemia^{-SEA}) using PAF multiplex fluorescent PCR protocol (Table 1) for family A. The fluorograms show PAF analysis of heterozygous genotype of alpha-thalassemia^{-SEA} of the father and the mother (normal 288bp, 6'FAM®, blue; normal 130bp, VIC®, green; normal 110bp, PET®, red; and mutant 217bp 6'FAM®, blue & VIC®, green), Hb Bart's genotype of embryo No. 2 (mutant 217bp 6'FAM®, blue & VIC®, green), and normal genotype of embryo No. 3 (normal 288bp, 6'FAM®, blue; normal 130bp, VIC®, green; and normal 110bp, PET®, red). Short tandemly repeat markers, D16S475, was labeled with NED® (yellow/black).

(Fig. 1).

PGT-M protocols for beta-Thalassemia using multiplex F-PCR (PBF protocol)

The extracted DNA was amplified using PBF protocol (Table 1) as multiplex F-PCR[13]. HUMTH01, an short tandemly repeat (STR) linking to beta-globin gene (HBB), was included for contamination detection and linkage analysis. The primers were labelled with the fluorescent dye 6'FAM® (blue) and NED® (yellow/black). Primers details, PCR mixtures and thermal cycler programs are demonstrated in Table 1. The PCR products were then analyzed by Fragment Analysis on an automated laser fluorescent sequencer ABI Prism® 3130 (GenePlus Co., Ltd.). Mutations with deletions and insertions i.e. c.41/42 (-TTCT) (388bp 6'FAM® (blue) fragment as normal allele and 384bp 6'FAM® (blue) fragment as mutant allele (4bp deleted)) can be identified by F-PCR

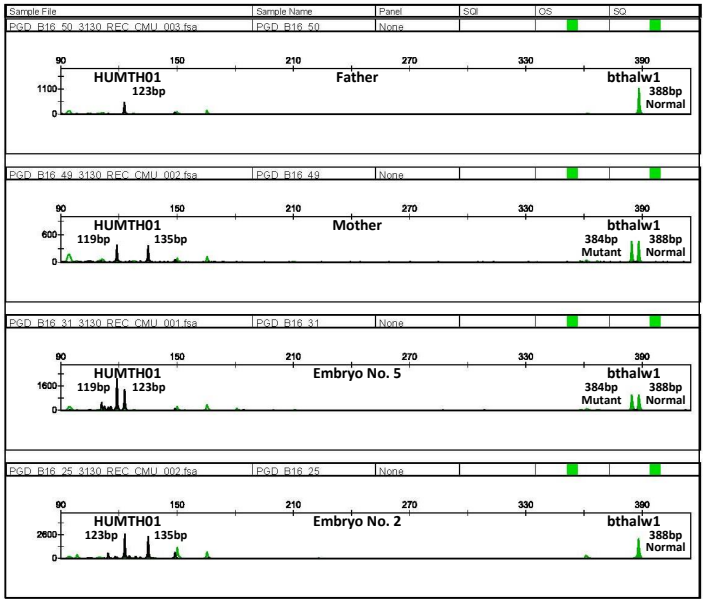

Fig. 2. Pre-implantation genetic testing for monogenic disorders (PGT-M) of beta-thalassemia c.41/42(-TTCT) using PBF multiplex fluorescent PCR protocol (Table 1) for family B. The fluorograms show PBF analysis of normal genotype of beta-thalassemia c.41/42(-TTCT) of the father and embryo No. 2 (388bp, VIC®, green) and heterozygous genotype of the mother and embryo No. 5 (384bp and 388bp, VIC®, green). Short tandemly repeat markers, HUMTH01, was labeled with NED® (yellow/black).

(Fig. 2).

PGT-M protocols for Hb E disease using mini-sequencing (PEF protocol)

The PCR products from PBF protocol was amplified using PEF protocol (Table 1) as mini-sequencing reaction. The amplified products were purified with Exonuclease I/Alkaline Phosphatase using ExoProStar™ 1-Step (Bang Trading 1992 Co., Ltd.) to remove unincorporated primers and dNTPs from previous PCR reactions. 2.14 µL of PCR products were added into 0.2-mL centrifuge tubes containing 0.86 µl of ExoProStar™ 1-Step. The mixtures were incubated at 37°C for 30min and 80°C for 15 min. Mini-sequencing reaction mixture comprised 5.0 µL of SNaPshot® Multiplex Kit (GenePlus Co., Ltd.), 200 mM of the mini-sequencing primers (Table 1), 3.0 µL of the purified templates and distilled deionized water in a total volume of 10 µL. The thermal cycles program was 96°C for 10 s, 50°C for 5 s and 60°C for 30 s for 25 cycles. The mini-seqeuncing products were then analyzed by Fragment Analysis on the automated laser fluorescent sequencer ABI Prism® 3130 (Fig. 3).

Fragment analysis on ABI Prism® 3130

A mixture of 1 µl of fluorescent PCR products, 1 µl Genescan™-500 LIZ® size standard (GenePlus Co., Ltd.), and 10 µl of deionized formamide (GenePlus Co., Ltd.) was prepared and denatured at 95°C for 5 minutes. Denatured samples were subjected to capillary electrophoresis using Performance Optimized Polymer 7 (POP-7®, GenePlus Co., Ltd.; 5 s injection time, 15,000 V, 60°C, 20 min) on the automated laser fluorescent sequencer ABI Prism® 3130. The data were analyzed by GeneMapper® software; version 4.0 (GenePlus Co., Ltd.)[14].

Mini-sequencing analysis on ABI Prism® 3130

A mixture of 1 µL of purified mini-sequencing product, 1 µL of GeneScan™-120 LIZ® (GenePlus Co., Ltd.) size standard, and 10 µL of deionized formamide

was prepared and denatured to 95°C for 5 min. The denatured samples were subjected to capillary electrophoresis using POP-7® (5 s injection time, 15,000 V, 60°C, 24 min) on the automated laser fluorescent sequencer ABI Prism® 3130. The data were analyzed by GeneMapper® software; version 4.0. The color of individual peaks was interpreted as A (Green, dR6G™ dye), C (Yellow/Black, dTAMRA™ dye), G (Blue, dR110™ dye) and T (Red, dROX™ dye)[15].

Statistical Analysis

Analysis was performed by SPSS version 21.0 (IBM Corp. Released 2012; IBM SPSS Statistics for Windows, Armonk, NY). Descriptive data were presented as means or percentage as appropriate. P-values of less than 0.05 was considered significant.

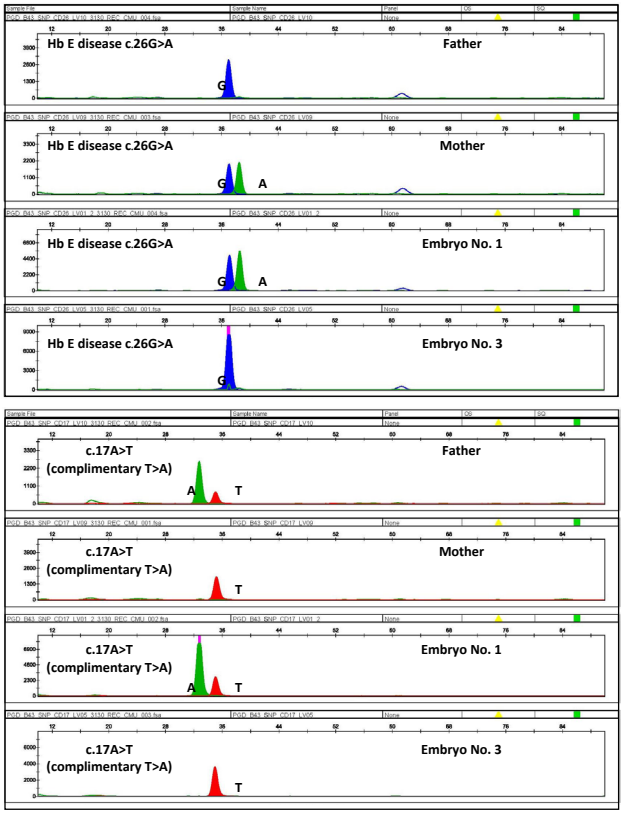

Fig. 3. Pre-implantation genetic testing for monogenic disorders (PGT-M) of beta-thalassemia-Hb E disease (c.26G>A & c.17A>T) using PEF mini-sequencing protocol (Table 1) for family C. The fluorograms of Hb E disease c.26G>A show normal genotypes (G, blue) of the father and embryo No. 3 and heterozygous genotypes (G, blue and A, green) of the mother and embryo No. 1. The fluorograms of beta-thalassemia c.17A>T (complimentary analysis as T>A in this figure) show of normal genotypes (T, red) of the mother and embryo No. 3 and heterozygous genotypes (T, red and A, green) of the father and embryo No. 1. Embryo No. 1 possesses the mutant alleles of both Hb E disease c.26G>A and beta-thalassemia c.17A>T. Therefore, embryo No. 1 is compound heterozygous for beta-thalassemia-Hb E disease.

Results

PGT-M at Chiang Mai University

Since 2003, a total of 168 clinical PGT-M cycles have been done for 125 families. In addition to alpha-thalassemia, beta-thalassemia major and beta-thalassemia-Hb E disease, other monogenic disorders including rare conditions were also included for the PGT-M. Families with affected beta-

thalassemia-Hb E disease offspring who sought for PGT-M in order to having a second healthy child decided to have HLA-matched un-affected embryos as well. Our 20 years experience gives rise to 75 pregnancies with 85 healthy babies. However, the pregnancy outcomes are expected to be higher as some PGT-M cycles are still ongoing and the embryos are kept frozen waiting for transfer. No contamination or misdiagnosis was detected.

PGT-M for Thalassemias

A total of 111 couples at risk of having the offspring with Hb Bart's disease, beta-thalassemia major, and beta-thalassemia-Hb E disease (c.26G>A) came in for genetic counselling and PGT-M treatment. All PGT-M protocols were developed and tested. A total of 132 clinical PGT-M cycles were carried out giving rise to 66 pregnancies with 76 babies (Table 2).

Table 2. Pre-implantation genetic testing for monogenic disorders (PGT-M) at Chiang Mai University.

Conditions	Families (Cycles)	Pregnancies (Babies)	Families (Cycles)	Pregnancies (Babies)
Thalassemias	**111 (132)**	**66 (76)**		
- alpha-Thalassemia				
• Hb Bart's disease (alpha-Thalassemia$^{(-SEA)}$)[14]	58 (68)	32 (36)		
• Hb H-CS	1 (3)	1 (1)		
- beta-Thalassemia major	12 (12)	8 (9)		
• c.17A>T & -28A>G			1 (1)	0
• c.17A>T & c.17A>T			3 (3)	3 (3)
• c.17A>T & c.35C>A			1 (1)	0
• c.41/42(-TTCT) & -28A>G			1 (1)	1 (1)
• c.41/42(-TTCT) & c.41/42(-TTCT)[13]			4 (4)	2 (2)
• c.41/42(-TTCT) & IVS1-nt1G>T			1 (1)	1 (2)
• c.41/42(-TTCT) & c.71/72(+A)			1 (1)	1 (1)
- beta-Thalassemia-Hb E disease	35 (43)	22 (27)		
• Hm c.26G>A & c.17A>T			4 (5)	3 (4)
• Hm c.26G>A & c.41/42(-TTCT)			7 (10)	4 (5)
• c.26G>A & c.17A>T[15]			10 (13)	7 (8)
• c.26G>A & c.30G>C			1 (2)	2 (3)
• c.26G>A & c.41/42(-TTCT)			10 (10)	4 (5)
• c.26G>A & c.71/72(+A)			1 (1)	2 (2)
• c.26G>A & -3.5kb deletion			1 (1)	0
• c.26G>A & unknown deletion			1 (1)	0
- beta-Thalassemia-Hb E disease & HLA matching	2 (3)	1 (1)		
- Hb Bart's disease & beta-Thalassemia-Hb E disease	3 (3)	2 (2)		
Marfan syndrome[17]	**1 (2)**	**1 (1)**		
Karyomapping	**13 (34)**	**8 (8)**		
• Hb Bart's disease (alpha-Thalassemia)			2 (5)	0
• beta-Thalassemia-Hb E disease[22]			2 (10)	4 (4)
• Duchene muscular dystrophy (DMD)[22]			2 (4)	2 (2)
• Polycystic kidney type 1 (PKD1)			2 (4)	0
• Spinal muscular atrophy (SMA)			2 (5)	0
• Oculocutaneous albinism (OCA) type 1[18]			1 (1)	0
• Infantile neuroaxonal dystrophy type 1 (INAD1)			1 (3)	2 (2)
• Usher syndrome (Hearing loss)			1 (2)	0
Total	**125 (168)**	**75 (85)**		

CS = Constant Spring, Hm = homozygous

PGT-M for alpha-Thalassemia$^{(-SEA)}$

Fifty eight alpha-thalassemia families carried^{-SEA} deletion had 68 PGT-M cycles, giving rise to 32 pregnancies with 36 healthy babies. One family were at risk of having offspring with Hb H-Constant Spring. The patient went through 3 PGT-M cycles, and had one un-affected baby. Interestingly, three families were at risk of having offspring with both Hb Bart's disease and beta-thalassemia-Hb E disease, consequently, embryos with either conditions needed to be excluded. Each family had one PGT-M cycle so far, two families succeeded with one baby each (Table 2). Examples of PGT-M for alpha-thalassemia$^{(-SEA)}$ using PAF multiplex F-PCR is demonstrated in Fig. 1.

PGT-M for beta-Thalassemia major and beta-thalassemia-Hb E disease

Twelve families were at risk of having offspring with beta-thalassemia major and 35 families were at risk of beta-thalassemia-Hb E disease (c.26G>A). A total of 55 clinical PGT-M cycles were performed, resulting in 30 pregnancies with 36 babies. beta-Thalassemia mutations of the PGT-M families included -28A>G, c.17A>T, c.30G>C, c.35C>A, c.41/42 (-TTCT), IVS1-nt1G>T, c.71/72(+A), and -3.5kb deletion. Unfortunately, there were 11 families whose one of the spouse was homozygous for Hb E disease, therefore, half of their embryos would be affected. Moreover, beta-thalassemia mutation could not identified in one family at risk of have offspring with beta-thalassemia-Hb E disease. PGT-M was carried out by using STR-based linkage analysis incorporating with mutation analysis of c.26G>A and predictive analysis for the absent of normal c.26G>A allele (Table 2). Examples of PGT-M for beta-thalassemia c.41/42 (-TTCT) using PBF multiplex F-PCR is shown in Figure 2. Examples of PGT-M for beta-thalassemia c.17A>T and c.26G>A using PEF mini-sequencing is demonstrated in Fig. 3.

PGT-M for other monogenic disorders and karyomapping

In addition to PGT-M of thalassemias, one family with Marfan syndrome came through for PGT-M. Two PGT-M cycles were carried out resulting in one baby[17]. Additionally, 34 PGT-M cycles were performed using single nucleotide polymorphism microarray (aSNP) based karyomapping for 13 families resulting in 8 babies. PCR-based protocols were developed, clinically applied and confirmed haplotyping results in all embryos. The rest of the embryos are still kept frozen waiting for transfer. The conditions of the PGT-M karyomapping done included alpha-thalassemia, beta-thalassemia-Hb E disease[23], Duchene muscular dystrophy (DMD)[16], Polycystic kidney type 1 (PKD1), Spinal muscular atrophy (SMA), Oculocutaneous albinism (OCA) type 1[18], Infantile neuroaxonal dystrophy type 1 (INAD1), and Usher syndrome (hearing loss) (Table 2).

Discussion

PGT-M at Chiang Mai University, Thailand started in 2003. The first two cases were performed for beta-thalassemia major c.41/42(-TTCT) using PBF multiplex F-PCR protocol for mutation analysis of 4 bp deletion[13]. Both couples carried the same mutations. During that time, most IVF centers performed cleavage stage day 3 embryo biopsy and transferred the embryos on day 4. Therefore, the results needed to be reported within 24 hours. The laboratory works were carried out under time pressure. Another difficulty was that day 3 embryo biopsy provided only 1-2 cells for PGT-M. Consequently, amplification efficiency, ADO, and contamination could deteriorate the accuracy of the results. These problems became ease when the IVF laboratories switched to do day 5 blastocyst biopsy with embryo freezing. With these changes, more cells and time were available for PGT-M.

PGT-M for alpha-thalassemia^{-SEA} has been available since 2007 when the new set of primers, PAF protocol, for alpha-thalassemia^{-SEA} was developed[14]. The original published gap PCR primers for alpha-thalassemia^{-SEA} was effective on genomic DNA and prenatal samples. However, the efficiency reduced to lower than 50% at the single cell level. The

newly designed PAF primers were effective and accurate for genotyping on genomic DNA, prenatal samples and single cells. All of the PGT-M for alpha-thalassemia were done for $^{-SEA}$ deletion which is the most common mutation in Thailand. Hb Bart's disease has become the most frequent indication for PGT-M here due to its high prevalence in this region.

Novel PCR protocol was developed for genotyping of Hb Constant Spring (CS). PGT-M was successfully carried out. One healthy baby was obtained. Three families were at risk of have offspring with both alpha-thalassemia and beta-thalassemia-Hb E disease. This event was not unusual in Thailand. However, excluding both conditions, only 56.25% of the embryos would be unaffected. One clinical PGT-M cycle was performed for each family, two succeeded in having a pregnancy. The third family will return for their second PGT-M treatment.

PGT-M for beta-thalassemia major and beta-thalassemia-Hb E disease is challenging as there are a wide variety of mutations among the population i.e. there were 10 mutations in this study. Each mutation needed a particular PGT-M protocol for genotyping. As the families who requested for PGT-M already had the mutation reports, PGT-M focused on the particular mutations of each family. It is noticed that one of the spouse of 11 families was homozygous for Hb E disease. Consequently, all of their gametes would have Hb E disease, therefore, half of the embryos would be affected. Previous report on PGT-M of beta-thalassemia using nested PCR with mini-sequencing were in 23 couples with 42 clinical cycles. Four successful pregnancies were resulted[26].

Two families who already had an affected child with beta-thalassemia-Hb E disease came for PGT-M. Their affected children had transfusion dependent anemia and were waiting for donors in order to have bone marrow transplantation treatment. It would be great if their unaffected second child were HLA matched to the affected elder siblings. Therefore, in addition to excluding beta-thalassemia-Hb E disease, STR-based linkage analysis HLA matching was also performed. Three PGT-M cycled had been carried out, resulting in one unaffected baby. The second family will return for the second PGT-M treatment. Successful PGT-M for beta-thalassemia major and beta-thalassemia-Hb E disease with HLA matching has been reported[27]. From 8 families with affected children, one successful pregnancy was obtained. HLA-matched sibling bone marrow transplantation for beta-thalassemia major was then performed.

One family came through for IVF treatment. Family history revealed Marfan syndrome with aortic replacement surgery in the husband and his father. Exome sequencing demonstrated c.3373C>T mutation within the fibrillin-1 (FBN1) gene. Two clinical PGT-M cycles were performed giving rise to one healthy baby[17]. PGT-M for Marfan syndrome is also challenging because Marfan families have different mutations. However, with the present exome sequencing technology, identifying the mutation from the proband is much quicker.

We also experienced PGT-M using karyomapping for 34 cycles in 13 families. Karyomapping employs aSNP technology for SNP-based haplotyping and SNP-based chromosome balance or copy number variation (CNV) information. In addition to alpha-thalassemia and beta-thalassemia-Hb E disease, the additional 6 rare conditions were performed including DMD, PKD1, SMA, OCA1, INAD1, and Usher syndrome. The specific mutation of each family was revealed by exome sequencing. Novel PCR protocols for genotyping and STR-based linkage analysis were developed for each family. PCR-based genotyping confirmed haplotyping results in every embryo. The pregnancy rates reported in Table 2 seems low, however, due to the covid-19 outbreak the patients will return for embryo transfer in the future and there should be more pregnancy outcomes.

Notably, in the karyomapping analysis of one of the PKD1 families, the reference DNA who was the affected sister of the affected husband had identical snp information. Therefore, her DNA was an uninformative reference. However, the aSNP-based haplotyping was proceeded by using one of the affected embryos, which was identified by PCR-based

genotyping, as the reference. It was shown that even with the hardest effort the sophisticated aSNP could go wrong on the day of the analysis. Without PCR-based analysis results, karyomapping analysis would have failed. One pitfall of the karyomapping software was that, with the uninformative reference in this case, the software proceeded with providing the incorrect haplotyping reports. If the operators had not validated the haplotyping with the PCR-based results, there would have been the chance of transfer affected embryo. Even though, karyomapping can be a universal protocol that provides both haplotyping and CNV, PCR-based genotyping should be performed along side to confirm the results.

Conclusion

The prevention and control program for severe thalassemias in Thailand has been done for over 20 years. The program has been very successful. However, at present, some families at risk of having an offspring with severe thalassemias are looking for a better option other than termination of pregnancy. PGT-M or embryo selection would be the correct answer. During the past 20 years, a total of 168 PGT-M cycles in 125 families have been done at Chiang Mai University, resulting in a total of 75 pregnancies (85 healthy babies). The pregnancy rates were 44.6%, whereas, some PGT-M cycles are still on-going and the embryos are kept frozen. More pregnancies should be obtained when the patients return for embryo transfer. PGT-M protocols at Chiang Mai University are based on multiplex F-PCR and mini-sequencing. Over 20 protocols have been developed, tested, and clinically applied for PGT-M. All protocols were novel and home grown. Waiting time for the work up prior to clinical PGT-M is about 2 weeks to 2 months (in case of developing a new protocol). So far, no misdiagnosis has been found.

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Potential conflicts of interest

The authors declare no conflicts of interest.

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