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Successful Strategy of Pre-implantation Genetic Testing for Beta-Thalassemia (c.17A>T Mutation)-Hb E Disease Using Multiplex Fluorescent PCR and Mini-Sequencing

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Abstract

Objectives: Hemoglobin E disease, c.26G>A variant of beta-globin gene, is the most common hemoglobinopathy in Asia. Compound heterozygotes inheriting Hb E disease and beta-thalassemia generate beta-thalassemia-Hb E disease with severe anemia. This study aimed to develop a pre-implantation genetic testing for monogenic disorders (PGT-M) protocol for beta–thalassemia (c.17A>T mutation)-Hb E disease (c.26G>A mutation) using multiplex fluorescent polymerase chain reaction (PCR) and mini-sequencing.

Materials and Methods: bthalw1 primers were used to amplify a beta-globin gene fragment covering both mutations, i.e. beta–thalassemia (c.17A>T) and Hb E disease. D21S11 microsatellite marker was included for contamination detection. Novel mini-sequencing primers were designed and tested for detection of both mutations.

Results: Pre-clinical work up of the optimized PGT-M protocol using 20 single buccal cells of a heterozygous subject showed 100% amplification efficiency and 0% allele drop out (ADO) rate for both primers. In clinical PGT-M cycle, 15 embryos were subjected to biopsy. The results showed two normal, one heterozygous for beta-thalassemia, six heterozygous for Hb E disease, one affected for beta-thalassemia-Hb E disease and five with ambiguous results. Two normally diagnosed embryos were chosen for transfer, one singleton pregnancy was obtained. A healthy baby boy was resulted. Postnatal testing confirmed PGT results.

Conclusions: Novel PGT-M protocols for beta-thalassemia-Hb E disease using multiplex fluorescent PCR and mini-sequencing were developed and described here. The protocol was applied in a clinical PGT-M cycle and gave rise to one successful pregnancy and consequently a healthy baby boy. Mini-sequencing was proved to be rapid, accurate and cost-effective protocol for PGT-M.

Keywords: Beta-thalassemia-Hb E disease, Embryo selection, Mini-sequencing, Multiplex fluorescent polymerase chain reaction, Pre-implantation genetic testing for monogenic disorders

Introduction

Hemoglobin E disease, c.26G>A variant of beta-globin gene, is the commonest hemoglobinopathy in Asia (1). Compound heterozygotes inheriting Hb E disease and beta-thalassemia gene generate beta-thalassemia-Hb E disease with severe symptoms. Beta-thalassemia-Hb E disease contributes to approximately half of all severe beta-thalassemia syndrome worldwide (2). In Thailand, about 30%-50% of the population are thalassemia carriers, i.e. around 24 million, in addition to the 600 000 living patients (3). Clinical characteristics of thalassemia syndrome vary ranging from mild or asymptomatic anemia to life-threatening conditions requiring blood transfusions. One factor influencing severity of the symptoms includes type of mutations which possesses almost 200 different mutations resulting in either absent or reduced synthesis of beta-globin chains. In severe cases, clinical pictures are the results of marked ineffective erythropoiesis leading to erythroid marrow expansion, osteopenia and bone deformities. Continuous blood transfusion is needed in severe cases and causes iron overload damaging multiple organs and reduces quality of life and life expectancy. Iron chelator therapy can prolong life expectancy with some side effects. Bone marrow transplantation is the present ultimate option with high expenses and risk (4).

The present standard strategy to prevent new cases of severe thalassemia syndrome includes population screening, genetic counselling, offering prenatal diagnosis (PND) to couples at risk and option for termination of affected pregnancy (5). Pre-implantation genetic testing for monogenic disorders (PGT-M) or embryo selection is an alternative to traditional PND giving couples at risk a chance to start pregnancy ensuring that their baby will be healthy. In other word, PGT-M is an option for abortion free fetal diagnostic process. During PGT-M process, the couples undergo in vitro fertilization treatment with ovarian stimulation and intracytoplasmic sperm injection (ICSI). Embryos are then biopsied at polar body stage, cleavage stage or blastocyst stage using a micromanipulator. Single cell polymerase chain reaction

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Key Messages

- This study demonstrates how to develop, test and validate novel PCR protocol for PGT-M.
- This study exhibits mini-sequencing techniques for PGT-M of beta-thalassemia-Hb E disease and a successful clinical PGT.
- Mini-sequencing can be used as a universal protocol for PGT of monogenic disorders with single nucleotide mutation.

(PCR) is used for monogenic disorders diagnosis (6). Major problems concerning PCR protocols for PGT-M are PCR amplification efficiency of the templates with very low copy number, allele drop out (ADO) and contamination (7). PGT-M allows selecting 'unaffected' embryos for transfer to the uterus.

The aim of this study was to develop novel PCR protocol using integration of fluorescent multiplex PCR and mini-sequencing to detect particular mutations of beta-thalassemia-Hb E disease within the embryos, i.e. beta-thalassemia (c.17A>T) mutation and hemoglobin E disease (c.26G>A) mutation for PGT-M. The protocol would then be validated and clinically applied. This will offer a new therapeutic perspective for couples at risk of having thalassemia offspring as well as for those with other monogenic disorders with single nucleotide change.

Materials and Methods

Patient Details

The wife and husband of family at risk for beta-thalassemia-Hb E disease offspring were 38 and 40 years old respectively. She carried beta–thalassemia (c.17A>T) and he carried Hb E disease (c.26G>A). She experienced one termination of pregnancy due to positive PND of beta-thalassemia-Hb E disease. The couples were counselled regarding the project and consent were obtained. The patient went through routine ovarian stimulation. Oocytes were collected and fertilized using ICSI to eliminate risk of sperm DNA contamination in subsequent PCR amplification.

Preclinical Work up

Sixty single buccal cells of a heterozygous subject were isolated, washed, lysed and amplified using primers for beta-thalassemia (c.17A>T) mutation and Hb E disease (c.26G>A) mutation with different Taq DNA polymerase kits including Vivantis Taq DNA Polymerase (Bang Trading 1992 Co., Ltd., Bangkok, Thailand), FastStart™ Taq DNA Polymerase (Roche Diagnostics (Thailand) Ltd., Bangkok, Thailand) and QIAGEN Multiplex PCR Kit (S.M. Chemical Supplies Co., Ltd., Chiang Mai, Thailand), 20 single cells each. Reagents, primers concentration and thermal cycler protocols were tested, optimised and compared. The composition of PCR mixtures and thermal cycler conditions were shown in Table 1.

Single Cell Isolation and Cell Lysis

Blastocyst stage embryo biopsy was performed on Day 5 using micromanipulator (8). Biopsied trophectoderms were washed with a minimum of four fresh PBS (GibcoBRL , GibThai Co., Ltd., Chiang Mai, Thailand) droplets and 4% bovine serum albumin (Sigma , S.M. Chemical Supplies Co., Ltd.) on a 10 cm Petri dish in a laminar flow cabinet under a dissecting microscope. The cells were then transferred to 200 µL micro-centrifuge tubes preloaded with lysis buffer, i.e., 1 mL of 17 mmol/L sodium dodecyl sulfate (SDS, Sigma) and 2 mL of 125 mg/mL proteinase K (PK, Roche Diagnostics (Thailand) Ltd.). A 2 µL aliquot of the last washing drop PBS was also taken as a control. Samples were then incubated at 37°C for 1 hour, proteinase K was then inactivated by heating at 99°C for 15 minutes on a thermal cycler (Roche Diagnostics Ltd., Thailand) (9).

Multiplex Fluorescent PCR for PGT-M

Extracted DNA from trophectoderms was amplified by using combination of bthalw1 (5'-CCT GAG GAG AAG TCT GCC GTT AC-3' and 5'-GTG CAG CTC ACT CAG TGT GGC-3') (10) covering c.17A>T and c.26G>A mutations and D21S11 primers (6'FAM-5'-TAT GTG AGT CAA TTC CCC AAG TGA-3' and 5'-GTT GTA TTA GTC AAT GTT CTC CAG-3') (11) for contamination detection. PCR mixture consisted of 200 mM of each primer, 0.2 mM deoxynucleoside triphosphates (dNTPs) (Roche Diagnostics Ltd., Thailand), 7.5 µL of 2X QIAGEN Multiplex PCR Master Mix and was made up to a total volume of 15 µL with distilled deionized water. Amplifications were performed with the conditions: denaturation 94°C for 30 seconds, annealing at 60°C 90 seconds and extension at 72°C for 90 seconds for 37 cycles. These were preceded by denaturation at 95°C for 4 minutes and followed with final extension at 72°C for 10 minutes. Multiplex amplified products were tagged with two different fluorochromes using labelled primers. This allowed analysis to be performed on an automated laser fluorescent sequencer ABI Prism 3130 (GenePlus Co., Ltd., Bangkok, Thailand) (12). bthalw1 and D21S11 fragments were labelled with the fluorescent dye VIC (green) and NED (yellow/black), respectively.

Fragment Analysis on ABI Prism 3130

A mixture of 1 µL fluorescent PCR products, 10 µL deionized formamide (GenePlus Co., Ltd.) and 1 µL GenescanTM-500 LIZ size standard (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 seconds injection time, 15000 V, 60°C, 20 minutes) on an automated laser fluorescent sequencer ABI Prism 3130. The data was analysed by GeneMapper software version 4.0 (GenePlus Co., Ltd.).

Table 1. Pre-clinical workup comparing Efficiency of Multiplex Fluorescent PCR Using Different Taq Polymerase Enzymes Including Vivantis Taq DNA Polymerase, FastStart™ Taq DNA Polymerase and QIAGEN® Multiplex PCR Kit

Taq Polymerase	Vivantis Taq DNA Polymerase (n = 20)	FastStart™ Taq DNA Polymerase (n = 20)	QIAGEN® Multiplex PCR Kit (n = 20)	Note
PCR Mixtures	• 1 µL of 10X ViBuffer S with 17.5mM $MgCl_2$ • 0.2 mM dNTPs • 0.5 U Taq DNA Polymerase • 200 mM of each primer • Water	• 1 µL of 10X PCR Reaction Buffer with 20 mM $MgCl_2$ • 0.2 mM dNTPs • 0.5 U FastStart™ Taq DNA Polymerase • 200 mM of each primer • Water	• 7.5 µL of 2X QIAGEN Multiplex PCR Master Mix • 200 mM of each primer • Water	
Total volume	10 µL	10 µL	15 µL	

Thermal cycles	• 95°C 12 min
	• 35 cycles of
	  • 95°C 30 s
	  • 60°C 30 s
	  • 72°C 45 s
	72°C 10 min
		• 95°C 4 min
		• 40 cycles of
		  • 95°C 45 s
		  • 60°C 45 s
		  • 72°C 60 s
		72°C 10 min
			• 95°C 4 min
			• 37 cycles of
			  • 94°C 30 s
			  • 60°C 90 s
			  • 72°C 90 s
			• 72°C 10 min	

Results				***P* Value***
• bthalw1				
- AA	20/20 (100%)	19/20 (95%)	20/20 (100%)	0.368
- ADO	2/20 (10%)	2/18 (11.1%)	0/20 (0%)	0.264
- AF	0/20 (0%)	1/20 (5%)	0/20 (0%)	0.368
Chi-square = 3.94, degrees of freedom = 4, *P* value = 0.41				
• D21S11				
- AA	20/20 (100%)	20/20 (100%)	20/20 (100%)	1.000
- ADO	2/20 (10%)	0/20 (0%)	0/20 (0%)	0.135
- AF	0/20 (0%)	0/20 (0%)	0/20 (0%)	1.000

*Cochran Q test.

A total of 60 single buccal cells of a heterozygous subject were employed for testing, 20 single cells each. The efficiency of different protocols was compared in term of appropriate amplification (AA), allele drop out (ADO) and amplification failure (AF) rates.

Mini-Sequencing Assay

For Hb E disease (c.26G>A) and beta-thalassemia (c.17A>T) analysis, the amplified PCR products were treated with Exonuclease I/Alkaline Phosphatase using ExoProStar™ 1-Step (Bang Trading 1992 Co., Ltd.) to remove unincorporated primers and dNTPs from PCR reactions prior to DNA mini-sequencing. 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 30 minutes and then 80°C for 15 minutes. Mini-sequencing reaction mixture was set up on ice and comprised 5.0 µL of SNaPshot Multiplex Kit (GenePlus Co., Ltd.), 0.5 µL of primer (0.2 µM stock) (anti-sense beta-thalassemia (c.17A>T) 5'-CCG TTA CTG CCC TGT GGG GC-3' or hemoglobin E disease (c.26G>A) 5'-ACG TGG ATG AAG TTG GTG GT-3'), 3.0 µL of purified templates and distilled deionized water in a total volume of 10 µL. The thermal cycles were performed with the conditions 96°C for 10 seconds , 50°C for 5 seconds and 60°C for 30 seconds for 25 cycles.

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 deionised formamide (GenePlus Co., Ltd.) was prepared and heated to 95°C for 5 minutes. Denatured samples were subjected to capillary electrophoresis using POP-7 (5 seconds injection time, 15,000 V, 60°C, 24 minutes). Data was analysed 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) (13).

Statistical Analysis

All analyses were performed using 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. To compare the efficiency among the three protocols, Cochran Q test was used (comparing three proportions among the related data). In cases of significant difference was found, post-hoc test to compare between each pair would be used. A *P* values of less than 0.05 was considered significant.

Results

Multiplex fluorescent PCR protocol for PGT-M of beta-thalassemia-Hb E disease was developed, optimised and tested specifically for the couples. Following multiplex fluorescent PCR, mini-sequencing was employed for c.17A>T and c.26G>A mutations identification. D21S11 polymorphic marker was used for contamination detection in the process of embryo biopsy and PCR reaction.

Preclinical Assessment of Methodology

Sixty single buccal cells of a heterozygous subject were

subjected to multiplex PCR using primers for beta-thalassemia-Hb E disease with 3 different Taq polymerase kits including Vivantis Taq DNA Polymerase, FastStart™ Taq DNA Polymerase and QIAGEN Multiplex PCR Kit, 20 for each platform. Reagents, primers concentration and thermal cycler protocols were tested, optimised and compared for the efficiency in term of appropriate amplification (AA), ADO and amplification failure (AF) rates. The optimised protocols of each platform were shown in Table 1. Vivantis Taq DNA Polymerase gave 100% (20/20) AA and 10% (2/20) ADO; FastStart™ Taq DNA Polymerase gave 95% (19/20) AA, 11.1% (2/18) ADO and 5% (1/20) AF; while QIAGEN Multiplex PCR Kit gave 100% (20/20) AA and 0% (0/20) ADO for bthalw1 primers, Cochran Q test p value of 0.368, 0.264 and 0.368, respectively. Vivantis Taq DNA Polymerase gave 100% (20/20) AA and 10% (2/20) ADO; FastStart™ Taq DNA Polymerase gave 95% (19/20) AA and 0% (0/20) ADO; while QIAGEN Multiplex PCR Kit gave 100% (20/20) AA and 0% (0/20) ADO for D21S11 primers, Cochran Q test p value of 1.000, 0.135 and 1.000, respectively. QIAGEN Multiplex PCR Kit showed the highest AA and lowest ADO rates for both sets of primers, consequently, it was chosen for clinical PGT-M in this study. No DNA contamination was detected in all PBS control, PK/SDS control and no template control PCR mixtures samples.

Clinical PGT-M results

During clinical PGT-M cycle, 22 oocytes were obtained, 20 were sperm-injected and 15 blastocyst stage embryos were subjected to biopsy on day 5 after fertilisation. Molecular analyses using the optimised multiplex fluorescent PCR with QIAGEN Multiplex PCR Kit and mini-sequencing showed two normal (embryos No. 9 and 13), one heterozygous for c.17A>T mutation (embryo No. 5), six heterozygous for Hb E disease (embryos No. 2, 4, 6, 7, 11 and 12), one affected for beta-thalassemia-Hb E disease (embryo No. 10) and five with unclear results (embryos No. 1, 3, 8, 14 and 15). Examples of mini-sequencing results of anti-sense c.17A>T mutation and hemoglobin E disease were demonstrated in Figures 1 and 2. No DNA contamination was detected in all PBS control, PK/SDS

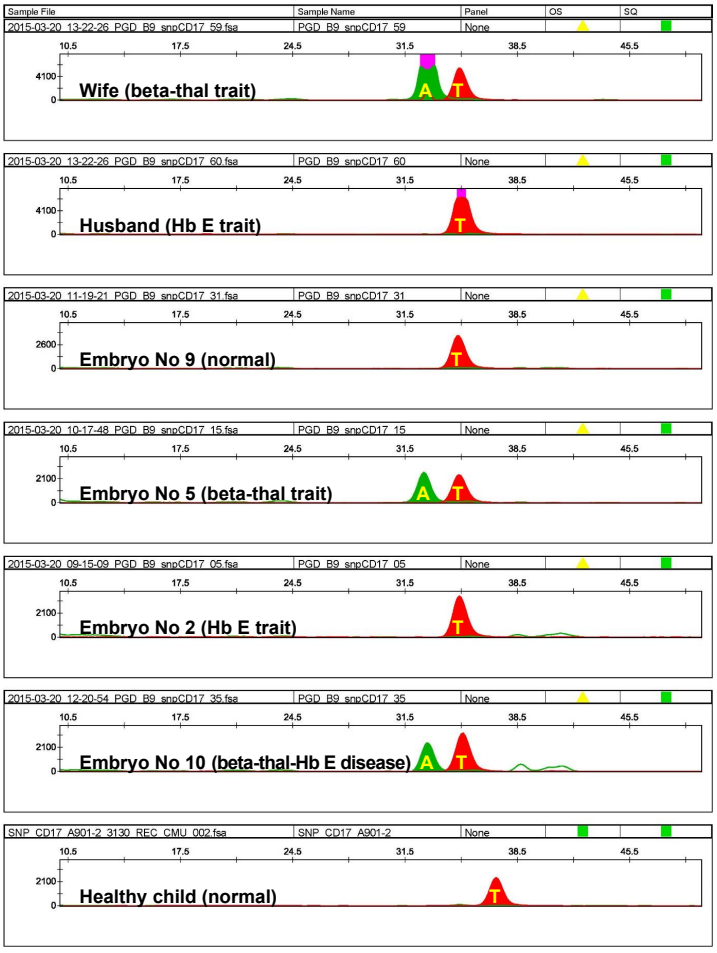

Figure 1. Examples of mini-sequencing results of anti-sense beta-thalassemia (c.17A>T), Showing as T(red)>A (green) from the wife (heterozygous beta–thalassemia (c.17A>T)), the husband (heterozygous Hb E disease (c.26G>A)) and embryo No 9 (normal), embryo No 5 (heterozygous beta–thalassemia (c.17A>T)), embryo No 2 (heterozygous Hb E disease (c.26G>A)), embryo No 10 (beta-thalassemia (c.17A>T)-Hb E disease (c.26G>A)) and prenatal diagnosis confirmation of the healthy baby (normal), respectively.

control and no template control PCR mixtures samples. Two normally diagnosed embryos (embryos No. 9 and 13) were chosen for transfer, one clinical singleton pregnancy was obtained. A healthy baby boy was resulted. PND confirmed homozygous normal genotype of beta-globin gene. The rest of the un-affected embryos were thawed and transferred after delivery of the successful pregnancy, unfortunately that the embryos either died in culture medium or failed to implant.

Discussion

Beta-thalassemia-Hb E disease is one of severe thalassemia syndromes. PGT-M is an alternative to traditional invasive PND. Difficulty of PGT-M for thalassemias is due to wide variety of mutations among population. Therefore, different families with different mutations need different molecular analysis protocols. Moreover, beta-thalassemia-Hb E disease is a kind of compound heterozygous needing more than one molecular analysis protocols for one family. In this study, we developed novel multiplex fluorescent PCR protocol using mini-sequencing to detect both beta-thalassemia (c.17A>T) and hemoglobin E disease (c.26G>A). We demonstrated that mini-sequencing can be used as a universal protocol for PGT-M of conditions with point mutations.

Former PGT-M protocols for beta-thalassemias employed various analysis methods. Restriction fragment length polymorphism (RFLP) need nested PCR which is labor intensive and susceptible to ADO and contamination (14,15). The combination of RFLP and reverse dot-blot is even more complicated and labor intensive without significant advantage (16). Denaturing gradient gel electrophoresis tends to be a scanning or screening technique to detect various mutations at a time (17), however, it is not a diagnostic test. Single cell sequencing provides a comprehensive various mutations detection in one go (18), however, it is labor intensive, expensive and the analysis can be problematic. Whole genome amplification is even more expensive and labour intensive (19).

PGT-M protocol using single step multiplex fluorescent PCR provides the advantage of an increased sensitivity and specificity (12). Nested PCR is not needed. It has been used for PGT-M of beta- and alpha-thalassemias with

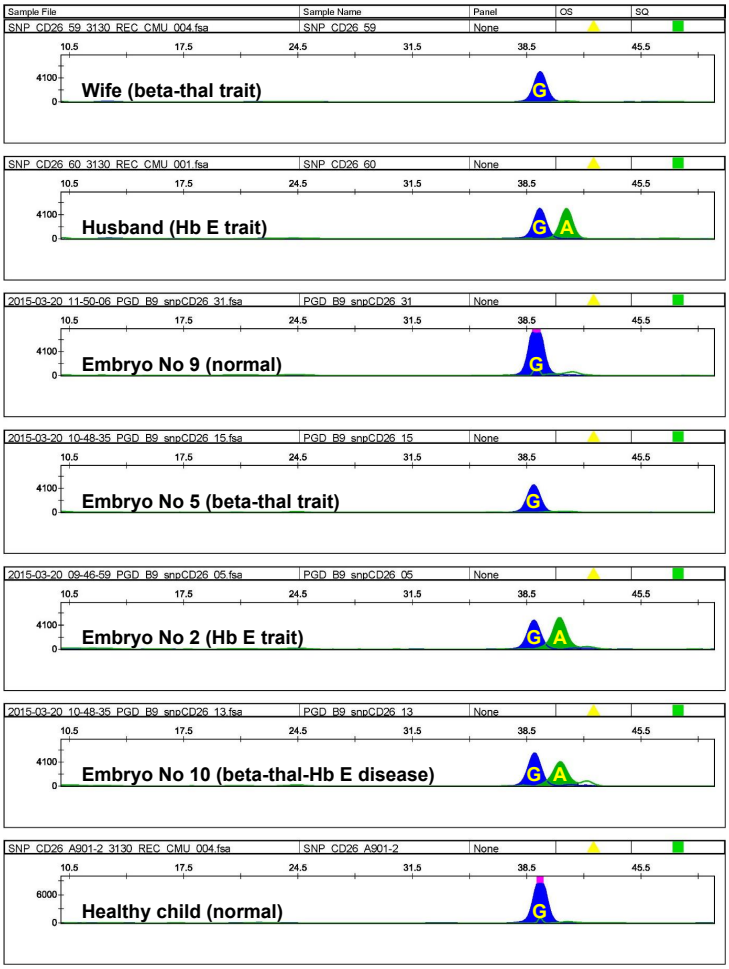

Figure 2. Examples of mini-sequencing results of hemoglobin E disease (c.26G>A), showing as G(blue)>A(green). from the wife (heterozygous beta–thalassemia (c.17A>T)), the husband (heterozygous Hb E disease (c.26G>A)) and embryo No 9 (normal), embryo No 5 (heterozygous beta–thalassemia (c.17A>T)), embryo No 2 (heterozygous Hb E disease (c.26G>A)), embryo No 10 (beta-thalassemia (c.17A>T)-Hb E disease (c.26G>A)) and prenatal diagnosis confirmation of the healthy baby (normal), respectively.

deletions (9,12). However, for single base substitution mutations, subsequent mini-sequencing is added for specific nucleotide detection. Mini-sequencing protocol was adapted from traditional Sanger's sequencing protocol, which is labour-intensive, time consuming, expensive and sometimes unnecessary. Mini-sequencing is performed by extending only one nucleotide after the mini-sequencing primer. By changing the PCR primers and the mini-sequencing primers, the protocol can be employed as an all-purpose PGT-M protocol reducing time and expenses for developing PGT-M protocols for new mutations.

In addition to AF, ADO is also major problem encountering PGT-M, situation when one of the alleles of a heterozygous sample fails to amplify (13). PGT-M protocol in this study employed two mini-sequencing reactions in order to detect both beta-thalassemia (c.17A>T) and hemoglobin E disease (c.26G>A) lying within the same amplified fragment. The present of both mutations in the same sample is interpreted as affected, i.e., beta-thalassemia-Hb E disease. ADO in a compound heterozygous sample leads to misinterpretation as a heterozygous conclusion, consequently, the transfer of an affected embryo. Preclinical study using 60 heterozygous single cells was carried out to compare efficiency of three different PCR protocols. QIAGEN Multiplex PCR Master Mix showed the best efficiency, i.e., 100% AA and 0% ADO rates for both sets of primers.

The limitations of this study include the number of embryos available for PGT-M and the number and quality of biopsied cells for PCR analysis. These limitations are sometimes unpredictable. If there are a lot of embryos and the number and quality of biopsied cells are great, PCR analysis should be smooth. However, if there are only a few embryos and the number and quality of the biopsied cells are poor, PCR results may be challenging. Fortunately that there were enough number of embryos for PGT-M in this study.

In conclusion, novel PGT-M protocol for detecting two different mutations within beta-globin gene using multiplex fluorescent PCR incorporating with mini-sequencing was developed and optimised. Novel mini-sequencing primers specifically for detecting beta-thalassemia (c.17A>T mutation) and Hb E disease (c.26G>A mutation) were designed and tested for effective mutation detection. D21S11informative microsatellite marker was included to detect contamination. Clinical PGT-M cycle was performed and successful pregnancy was resulted, resulting in a healthy baby boy. The embryo's genotypes were confirmed by PND. The newly developed PGT-M protocol for beta-thalassemia (c.17A>T)-Hb E disease was proved to be rapid, accurate and cost-effective. In addition, this protocol can simply be modified for any other monogenic disorders with single base change. Future studies in this area are to clinically applied mini-sequencing protocol to PGT-M of beta-thalassaemia with different mutations and PGT-M of other monogenic diseases.

Authors' Contribution

WL, NJ, and WP: concept and design. WL, NJ, SP, TP, TT, WS, and WP conducted the research. WL, NJ, and WP: data collection and interpretation of the data. All authors approved the final manuscript and took responsibility for the integrity of the data.

Conflict of Interests

Authors declare that they have no conflict of interests.

Ethical Issues

The project was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University, Thailand (study code: OBG-2561-05938).

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