Preimplantation genetic diagnosis of alpha-thalassemia^{-SEA} using novel multiplex fluorescent PCR

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Abstract

Purpose Preimplantation genetic diagnosis (PGD) is an alternative to prenatal diagnosis (PND) giving couples at risk a chance to start a pregnancy with a disease-free baby. This study aimed to develop a new PGD protocol for alpha-thalassemia^{-SEA} mutation, the commonest Mendelian disorder.

Patients and methods Multiplex fluorescent PCR was employed for mutation, contamination and linkage analysis. A couple experienced termination of pregnancy following positive PND decided to join the project.

Results Novel primers for alpha-thalassemia^{-SEA} mutation amplifying 5 DNA fragments were developed. Two PGD cycles were performed, resulting in an un-affected baby. PND confirmed the heterozygous result. From 24 embryos, 87.5% of affected genotype were of best quality compared to 0% and 18.2% of those with normal and heterozygous, respectively.

Conclusions A novel PCR protocol for the common alpha-thalassemia^{-SEA} mutation is reported. This test should be

widely applicable. Interestingly, a potential effect of alpha-thalassemia^{-SEA} mutation on preimplantation embryonic development was noticed.

Keywords Embryo selection · Multiplex fluorescent single cell polymerase chain reaction (PCR) · Preimplantation genetic diagnosis (PGD) · Alpha-thalassemia

Introduction

alpha-Thalassemia is the most common single gene disorder in the world and causes significant maternal mortality in endemic areas [1]. alpha-Thalassemia is more common than beta-thalassemia but is less frequently found in the hospital, as the most severe form (homozygous affected) leads to intrauterine death and carriers are generally asymptomatic and do not need much health care attention. Homozygosity for an alpha-globin gene defect leads to absence of alpha-globin chain synthesis and causes Hb Bart's hydrops fetalis syndrome. Mothers carrying these fetuses are likely to develop potentially life-threatening obstetric complications, i.e. eclampsia, dystocia and obstetric hemorrhage. Therefore, the aim of prenatal diagnosis (PND) for homozygous alpha-thalassemia is to prevent serious maternal mortality and morbidity.

Preimplantation genetic diagnosis (PGD) is the genetic testing of preimplantation stage embryos, generated using in vitro fertilization (IVF) technology, for inheritable single gene defects, allowing selection of unaffected embryos prior to establishment of pregnancy [2]. This gives couples the chance to start a pregnancy with a disease free baby. Consequently, the need for termination of an affected pregnancy can be eliminated. For this reason, many patients find PGD preferable to conventional PND.

Capsule Novel PGD protocol for alpha-thalassemia^{-SEA} mutation was developed, tested and clinically applied in two PGD cycles resulting in the birth of an un-affected baby.

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The aim of this study was to develop a single cell PCR protocol for PGD of alpha-thalassemia^{SEA} mutation, which is the most common alpha-thalassemia-1 mutation in the region, and perform clinical PGD for couples at risk.

Patients and methods

Patient details

The mother and father of family ‘A’ were 28 and 49 years old respectively. Both carried the alpha-thalassemia^{SEA} mutation and experienced one termination of pregnancy following positive PND of alpha-thalassemia. The couple were counseled regarding the project and consent were obtained. This project was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University, Thailand.

ICSI procedure and cleavage stage embryo biopsy

The patients underwent routine superovulation using recombinant FSH (Puregon® Pen, MSD (Thailand) Ltd., Bangkok, Thailand) and synthetic decapeptide ganirelix (Orgalutran®, MSD (Thailand) Ltd., Bangkok, Thailand) and oocytes were fertilized using intracytoplasmic sperm injection (ICSI). ICSI was used as a precautionary measure to reduce the risk of sperm DNA contamination in subsequent PCR amplification. Laser biopsy was performed on Day 3 post-fertilization (4–9 cell stage), allowing two blastomeres to be removed from embryos consisting of 6⁺ cells and one blastomere from embryos with 4–5 cells. Cleavage stage embryos were graded 1, 2–, 2, 2+ and 3 where grade 1 had the best morphology and grade 3 was a highly fragmented, poor quality embryo [3].

Single cell isolation

Buccal cells, isolated by micromanipulation, and biopsied blastomeres were transferred into droplets of phosphate-

buffered saline (PBS) (GibcoBRL®, GibThai Co., Ltd., Chiang Mai, Thailand) with 4% bovine serum albumin (BSA) (Sigma®, S.M. Chemical Supplies Co., Ltd., Chiang Mai, Thailand) on a 5 cm Petri dish in a laminar flow cabinet. Cells were washed in a minimum of four fresh PBS droplets, while visualizing under a dissecting microscope, and were then transferred to thin-wall microcentrifuge tubes. A 2 µl aliquot of the last washing drop was also taken as a blank for each single blastomere. Cell lysis was carried out as described previously [4].

Multiplex fluorescent PCR

Extracted DNA from single cells was amplified using a combination of W1, W2, W3 and W4 primers as gap PCR for alpha-thalassemia^{SEA} mutation detection, W5 and W6 as internal control fragment of alpha-globin gene family and a microsatellite D16S475 flanking to alpha-globin gene for contamination detection and linkage analysis (Table 1) [5]. Multiplex fluorescent PCR was performed as previously described [6]. Multiplex amplified products from single cells were each tagged with different fluorochromes using labeled primers. This allowed analysis to be performed on an automated laser fluorescent sequencer ABI Prism® 3130xl (Gene Systems Co., Ltd., Bangkok, Thailand) [7].

Fragment analysis

A mixture of 1 µl fluorescent PCR products, 10 µl deionized formamide and 0.1 µl size standard (Genescan™-500 LIZ®; Gene Systems Co., Ltd., Bangkok, Thailand) was prepared and denatured at 95°C for 5 min. The denatured sample was subjected to capillary electrophoresis using Performance Optimized Polymer 7 (POP-7®, Gene Systems Co., Ltd., Bangkok, Thailand; 50 s injection time, 15,000 V, 60°C, 20 min) on an automated DNA sequencer ABI Prism® 3130xl (Gene Systems Co., Ltd., Bangkok, Thailand). The data was analyzed by GeneMapper® software version 4.0 (Gene Systems Co., Ltd., Bangkok, Thailand).

Table 1 Details of primers for alpha-thalassemia^{SEA} mutation analysis

Primers	Sequences	Labeling dye	References
W1	5'-GAA GGA GGG GAG AAG CTG AG-3'	FAM-B	NCBI: NG_000006
W2	5'-TGT GGA AAA GTT CCC TGA GC-3'	–	
W3	5'-TGC ACA CCT ATG TCC CAG TT-3'	–	
W4	5'-TTG AGA CGA TGC TTG CTT TG-3'	VIC-G	
W5	5'-GCC ACT GCC TGC TGG TG-3'	PET-R	NCBI: NG_000006
W6	5'-AGG TCA GCA CGG TGC TCA C-3'	–	
D16S475F	5'-AGG GGT TGA CAG AGT GAG ACT CC-3'	NED-Y	GDB: 198327
D16S475R	5'-CAG GAA CAG AAA ATA CTG CAC GG-3'	–	

Results

Design of novel primers for alpha-thalassemia^{-SEA} analysis

A new set of primers for alpha-thalassemia^{-SEA} mutation analysis, including W1, W2, W3 and W4 primers was designed as gap PCR, W5 and W6 primers were employed as an internal control fragment of alpha1 exon3 within the alpha-globin gene (NCBI: NG_000006) and the D16S475 (GDB: 198327) microsatellite in close proximity to the alpha-globin gene was used to assist with contamination detection and linkage analysis. In a sample of normal genotype, the W1-W2 primer pair amplifies the 5' end breakpoint of alpha-thalassemia^{-SEA} mutation and W3-W4 primer pair amplifies the 3' end breakpoint. In a homozygous mutant genotype sample, the W1-W4 primer pair amplifies the deleted breakpoint of the alpha-thalassemia^{-SEA} mutation (Fig. 1). W1, W4, W5 and D16S475 forward primers were labeled with blue (6-FAM®), green (VIC®), red (PET®) and yellow (NED®) fluorescent dyes, respectively. In a normal sample, amplification of W1–W2, W3–W4 and W5–W6 primer pairs gives rise to 288 bp blue N1, 130 bp green N2 and 110 bp red N3 fragments of normal alpha-globin gene. In a homozygous alpha-thalassemia^{-SEA} deletion sample, W1–W4 primer pair gives rise to 217 bp blue&green M1 fragment indicating the presence of the deletion (Fig. 1).

Preclinical assessment of methodology

From 55 single heterozygous buccal cells derived from the couple, multiplex fluorescent PCR showed an amplification efficiency of 78.2% for N1, 96.4% for N2, 96.4% for N3 fragments giving the overall diagnostic efficiency of 100% for the normal alpha-thalassemia^{-SEA} allele (i.e. all cells

with a normal allele produced one or more PCR fragments indicative of normality). The amplification of the M1 fragment corresponding to the alpha-thalassemia^{-SEA} deletion allele was 85.5%. The linked microsatellite, D16S475, showed an amplification efficiency of 90.9% with 8% allele drop out rate. The application of the protocol on 25 spare single human blastomeres donated for research showed an overall amplification efficiency of 84% for both normal alpha-thalassemia^{-SEA} allele and D16S475 fragment.

Preimplantation diagnosis results

PGD cycle 1 for family 'A' gave 14 oocytes, all were sperm injected and all 14 embryos were good quality for biopsy on day 3 post-fertilization. Mutation analysis of alpha-thalassemia^{-SEA} from single biopsied blastomeres revealed four embryos to be normal (A2, A8, A13, A14), two heterozygous (A4, A9), six affected (A5, A6, A7, A10, A11, A12) and two without result (A1, A3) (Table 2). Analysis of D16S475 fragment flanking to alpha-globin gene showed no evidence of contamination in all biopsied blastomeres. Linkage analysis of D16S475 fragment confirmed mutation analysis of two normal (A8, A13), one heterozygous (A4) and four affected embryos (A6, A7, A11, A12). Linkage analysis was not concordant with direct mutation detection in three embryos (A2, A5, A14). Heterozygous results were obtained for two apparently normal embryos (A2, A14) and one apparently affected embryo (A5), revealing the possibility of ADO. One normal (A14) and two heterozygous (A4, A8) embryos were chosen for transfer on day 4, unfortunately, no pregnancy was resulted.

Ten oocytes were collected and sperm injected in PGD cycle 2 of family 'A'. Eight embryos were of sufficient quality for biopsy on day 3. Mutation analysis of alpha-

Fig. 1 Diagram showing alpha-globin gene family and alpha-thalassemia^{-SEA} deletion with primers map

Table 2 Preimplantation genetic diagnosis of alpha-thalassaemia^{-SEA} mutation results of family ‘A’, cycle 1 (embryos A1–A14) and cycle 2 (embryos B1–B10)

Embryo no.	Embryo grade ^a (no. of cells) before biopsy (Day 3)	Cells taken (<i>n</i>)	Cell no.	alpha-Thal ^{-SEA} results	Microsatellite linked marker (D16S475) results	Diagnosis	Outcomes	Confirmation results
A1	2 ⁺ [8]	2	A1.1 A1.2	No result Lysed	No result	No result	Untransferred	Ht ^b
A2	3 [6]	2	A2.1 A2.2	Normal Lysed	NC ^c , suggestive of Ht	Normal or Ht	Untransferred (arrested)	Ht
A3	3 [8]	2	A3.1 A3.2	No result No result	No result No result	No result	Untransferred	Ht
A4	2 ⁺ [9]	2	A4.1 A4.2	Ht Ht	NC, suggestive of Ht NC, suggestive of Ht	Ht	ET ^d (early blastocyst)	-
A5	2 [7]	2	A5.1 A5.2	Affected No result	NC, suggestive of Ht No result	Affected or Ht	Untransferred	Ht
A6	1 [8]	2	A6.1 A6.2	Affected Affected	NC, suggestive of Affected NC, suggestive of Affected	Affected	Untransferred	Affected
A7	1 [8]	2	A7.1 A7.2	Affected Affected	No result NC, suggestive of Affected	Affected	Untransferred	Affected
A8	3 [8]	2	A8.1 A8.2	Normal Normal	NC, suggestive of Normal NC, suggestive of Normal	Normal	ET (morula)	-
A9	1 [7]	2	A9.1 A9.2	Ht Ht	No result Allele drop-out	Ht	Untransferred	Ht
A10	1 [8]	2	A10.1 A10.1	Affected No result	No result Allele drop-out	Affected	Untransferred	Affected
A11	1 [8]	2	A11.1 A11.2	Affected No result	NC, suggestive of Affected Allele drop-out	Affected	Untransferred	Affected
A12	2 ⁺ [7]	2	A12.1 A12.2	Affected Affected	NC, suggestive of Affected NC, suggestive of Affected	Affected	Untransferred	Affected
A13	2 ⁺ [7]	2	A13.1 A13.2	Normal Normal	Allele drop-out NC, suggestive of Normal	Normal	Untransferred (arrested)	Normal
A14	2 ⁺ [9]	2	A14.1 A14.2	Normal Normal	No result NC, suggestive of Ht	Normal or Ht	ET (early blastocyst)	–
B1	2 ⁺ [8]	2	B1.1 B1.2	No result No result	No result No result	No result	Untransferred	Normal
B2	1 [8]	2	B2.1 B2.2	Ht Ht	Allele drop-out Allele drop-out	Ht	ET (blastocyst)	Ht (PND)
B3	2 ⁺ [7]	2	B3.1 B3.2	No result Lysed	No result	No result	Untransferred	Normal
B4	1[7]	2	B4.1 B4.2	Affected Lysed	NC, suggestive of Affected	Affected	Untransferred	Affected
B5	1[7]	2	B5.1 B5.2	Affected Lysed	Allele drop-out	Affected	Untransferred	Affected
B6	2 ⁺ [5]	1	B6.1	No result	No result	No result	Untransferred	Normal
B7	1[6]	1	B7.1	Affected	No result	Affected	Untransferred	Affected
B8	1[5]	1	B8.1	Normal	No result	Normal	Untransferred	Ht
B9	1[4]	0	–	–	–	–	Untransferred	Ht
B10	1[4]	0	–	–	–	–	Untransferred	Ht

^a Embryos were graded 1, 2⁺, 2, 2⁺ and 3 where grade 1 had the best morphology and grade 3 was a highly fragmented, poor quality embryo^b Ht Heterozygous^c NC No contamination detected^d ET Embryo transferred

thalassemia^{-SEA} showed one embryo to be normal (B8), one heterozygous (B2), three affected (B4, B5, B7) and three without result (B1, B3, B6). No contamination was detected. Linkage analysis results confirmed affected result in one embryo (B4). One heterozygous embryo (B2) was chosen for transfer on day 6 at blastocyst stage, one pregnancy resulted. PND by fetal blood sampling confirmed heterozygous genotype of the fetus. A disease-free baby boy, 3,280 g, was born on 2nd October 2008.

Preclinical work-up results for family 'A' confirmed the heterozygous genotype of both of the parents showing 288 bp blue N1, 130 bp green N2 and 110 bp red N3 fragments of normal allele and 217 bp blue&green M1 fragment of mutant allele of alpha-thalassemia^{-SEA} mutation (Fig. 2a and b). The homozygous affected genotype of their first fetus was confirmed by detection of only the 217 bp blue&green M1 fragment, corresponding to the alpha-thalassemia^{-SEA} mutation. The D16S475 linkage locus exhibited 164 bp/170 bp and 166 bp/180 bp alleles for the mother and the father, respectively (Fig. 2a and b). Their Hb Bart's fetus acquired 170 bp and 180 bp mutant linked

alleles from the mother and the father, respectively, therefore, the maternal 164 bp allele and the paternal 166 bp allele are predicted to be linked to the normal allele. Multiplex fluorescent PCR on normal control sample from an unrelated subject gave only 288 bp blue N1, 130 bp green N2 and 110 bp red N3 of normal allele. Subsequent PGD analysis of blastomere A4.1 demonstrated an heterozygous genotype with 164 bp normal linked allele and 180 bp mutant linked allele from the mother and the father, respectively (Fig. 3a). Normal genotype blastomere A8.2 showed 164 bp and 166 bp normal linked allele from both of the parents (Fig. 3b).

Summarizing the data of a total of 36 single biopsied blastomeres from 2 clinical PGD cycles gave amplification efficiency of 72.2% (26/36) and 63.8% (23/36), ADO rates of 19.2% (5/26) and 30.4% (7/23) for alpha-globin gene and D16S475 locus, respectively. Follow-up analyses were performed on the untransferred embryos. For this purpose whole embryos were transferred to PCR tubes and subjected to the same protocol as used for the actual PGD. The initial diagnosis was confirmed in all cases. No

Fig. 2 Multiplex fluorescent PCR results from GeneMapper® on an automated DNA sequencer ABI Prism® 3130xl for alpha-thalassemia^{-SEA} mutation. **a** and **b** show results from heterozygous samples of mother

and father of family 'A' with positive normal (blue-N1, green-N2, red-N3) and mutant (blue&green-M1) fragments. Yellow/black-LA1 fragment is D16S475 microsatellite marker flanking to alpha-globin gene

Fig. 3 Multiplex fluorescent PCR results from GeneMapper® on an automated DNA sequencer ABI Prism® 3130xl for alpha-thalassemia^{-SEA} mutation. **a** shows results from heterozygous single biopsied blastomere (A4.1) from clinical PGD for family 'A' with positive normal (blue-N1, green-N2, red-N3) and mutant (blue&green-M1) fragments. **b** shows results from normal single biopsied blastomere (A8.2) from clinical PGD for family 'A' with positive only normal (blue-N1, green-N2, red-N3) fragments. Yellow/black-LA1 fragment is D16S475 microsatellite marker flanking to alpha-globin gene

contamination was detected. A total of 24 embryos, including untransferred embryos and two arrested embryos (B9 and B10), in this study (Table 2) revealed 5 normal, 11 heterozygous and 8 affected embryos.

Discussion

Homozygous genotype of alpha-thalassemia causes severe anemia which leads to high output heart failure and hydrops fetalis syndrome in utero. The fetal hydropic phenotype always causes serious maternal complications. Prior to the era of heterozygotes screening and prenatal diagnosis of alpha-thalassemia, several mothers to such homozygous alpha-thalassemia fetuses died from eclampsia or hemorrhage every year. The introduction of prenatal control of severe thalassemia: Chiang Mai strategy [8] helps in early detection of homozygous alpha-thalassemia fetuses and termination of pregnancy before maternal complications begin, eliminating maternal death. Application of PGD provides the advantage over traditional PND in avoiding termination of affected pregnancy and its complications. Given its high prevalence in many parts of the world, PGD of alpha-thalassemia should be in high demand, but to date there have been relatively few reports on it [9].

In this study, a new set of primers was developed and tested and clinically applied employing a gap PCR strategy for alpha-thalassemia^{-SEA}. In addition, primers amplifying an internal control for the normal fragment, lying within alpha-thalassemia^{-SEA} deletion, was included in the protocol. A microsatellite marker flanking to alpha-globin gene family

was also included to assist in the detection of contamination and to provide a linkage-based back-up for direct mutation detection. The optimized PGD protocol, multiplex fluorescent PCR using 4 sets of primers amplifying 5 distinct DNA fragments, was successfully performed on heterozygote single cells. Preclinical workup of this novel PGD protocol on single buccal cells and single spared blastomeres gave acceptable amplification efficiency (>85%) and ADO. The detection of a normal allele was successful in 100% of single buccal cells from mutation carriers, while the mutant allele was successfully detected in 85.5%.

Comparing with previously designed primers for gap PCR [10], which amplify across the 5' end breakpoint of the normal allele and mutant allele, the new protocol in this

Table 3 Analysis the relationship of genotypes and quality of preimplantation embryos in this study ($n=24$) shows that most embryos with affected genotype were of better quality than those with normal and heterozygous genotypes i.e. 87.5% vs 0% and 18.2% with statistically significance (Yates' correction Chi square=9.17, p -value=0.01)

Genotypes	Embryo quality		Sum
	Grade I	Grade>I	
Normal	0 (0%)	5 (100%)	5 (20.8%)
Heterozygous	2 (18.2%)	9 (81.8%)	11 (45.8%)
Affected	7 (87.5%)	1 (12.5%)	8 (33.4%)
Sum	9 (37.5%)	15 (62.5%)	24

Chi square=13.28, degrees of freedom=2, p -value=0.00

Yates' correction Chi square=9.17, p -value=0.01

study amplifies across both the 5' and 3' breakpoints with different annealing sites. In addition, an internal control fragment within the breakpoint is also amplified. Therefore, novel protocol in this study amplifies three fragments corresponding to the normal allele. Traditional diagnostic strategies based upon amplification of a single fragment for the normal and mutant alleles, are statistically at higher risk of amplification failure due to polymorphisms at the sites of primer annealing and other problems related to single cell PCR. In the case of polymorphism of alpha-globin gene sequences failure of either normal or mutant alleles to anneal could lead to misdiagnosis. The redundant testing used in the strategy described here can rescue the diagnosis [11]. As demonstrated during the preclinical workup, amplification efficiency for the normal alpha-thalassemia^{SEA} allele using the new combination of primers reached 100%. Detecting normal allele is crucial for diagnosis of normal and heterozygous genotypes in autosomal recessive disorders. Moreover, a polymorphic marker next to the breakpoint is also included to provide an even more robust diagnosis.

From 22 embryos in two clinical PGD cycles (Table 2), mutation analysis demonstrated 5 normal, 3 heterozygous, 9 affected and 5 without result. In addition to showing no contamination in all single blastomeres, linkage analysis results using D16S475 locus confirmed mutation analysis results in two normal, one heterozygous and 5 affected embryos, while suggested two normal and one affected results to be heterozygous embryos indicating ADO of those blastomeres. This demonstrated the benefit of the incorporating D16S475 analysis for back up linkage analysis results in addition to contamination detection. Confirmation analysis of untransferred embryos revealed the overall genotyping distribution to be 5 (20.8%) normal, 11 (45.8%) heterozygous and 8 (33.4%) affected. As expected, ADO made the heterozygote ratio lower than actual prevalence while normal and homozygous affected ratio higher than expected. ADO would not lead an affected embryo to be diagnosed normal in this case, but could lead to a reduced number of heterozygous (unaffected) embryos for available transfer, leading to a lower pregnancy rate. It is worth noting that the problem of ADO did not affect the accuracy of diagnosis in this study because of the failsafe design of PCR protocol.

Analysis the relationship of genotypes and quality of preimplantation embryos in this study ($n=24$) shows that most embryos with affected genotype were of better quality than those with normal and heterozygous genotypes i.e. 87.5% vs 0% and 18.2% with statistically significance (Yates' correction Chi square=9.17, p -value=0.01) (Table 3). This observation indicated that homozygous affected genotype of alpha-thalassemia^{SEA} mutation may benefit development of preimplantation human embryos. This may be the result of the presence of alpha-thalassemia^{SEA} mutation.

There is a possibility that embryos of this family inherited a defective gene which deteriorates embryonic development during preimplantation stage and un-affected embryos developed poorly, while homozygous alpha-thalassemia^{SEA} genotype rescues those critical effects.

Conclusions

In conclusion, a novel PGD protocol for alpha-thalassemia^{SEA} using multiplex fluorescent PCR was developed, tested and clinically applied in two PGD cycles. New sets of primers for the alpha-thalassemia^{SEA} mutation were designed for detecting three fragments of the normal allele and one fragment of the mutant allele, providing a test with built in redundancy and high accuracy. In addition, a microsatellite next to alpha-globin gene was included for contamination detection and backup linkage analysis. This multiplex gap PCR protocol can be useful for PGD as well as PND. This represents the first reported birth following PGD of alpha-thalassemia^{SEA} in Thailand. Interesting data regarding an apparent beneficial effect of alpha-thalassemia^{SEA} mutation to preimplantation embryonic development was also noticed.

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