Reproductive BioMedicine Online (2010) 21, 642–648

www.sciencedirect.com
www.rbmonline.com

ARTICLE

Simplified PGD of common determinants of haemoglobin Bart's hydrops fetalis syndrome using multiplex-microsatellite PCR

Wen Wang [a,1], Christine HA Yap [b], Seong Feei Loh [c], Arnold SC Tan [a], Mui Nee Lim [b], Ethiraj B Prasath [c], Melinda LH Chan [c], Wei Chin Tan [a], Boran Jiang [a], Gare Hoon Yeo [a], Joyce Mathew [d], Angela Ho [e], Sherry SY Ho [d], Peng Cheang Wong [d], Mahesh A Choolani [d], Samuel S Chong [a,*]

[a] *Preimplantation Genetic Diagnosis Center, University Children's Medical Institute, Department of Pediatrics, Yong Loo Lin School of Medicine, National University Health System, Singapore 119074, Singapore;* [b] *Center for Assisted Reproduction, Department of Obstetrics and Gynecology, Singapore General Hospital, Singapore 169608, Singapore;* [c] *KK IVF, Department of Reproductive Medicine, KK Women's and Children's Hospital, Singapore 229899, Singapore;* [d] *Department of Obstetrics and Gynecology, Yong Loo Lin School of Medicine, National University Health System, Singapore 119074, Singapore;* [e] *Center for Assisted Reproduction Private Limited, Paragon Medical Center, Singapore 238859, Singapore*

[*] Corresponding author. *E-mail address:* paecs@nus.edu.sg (S.S. Chong). [1] Present address: Department of Obstetrics and Gynecology, University of California, Irvine, USA.

Wen Wang received her PhD in Molecular Genetics from the National University of Singapore in 2005. Her studies focused on molecular diagnosis and mutation characterization in alpha- and beta-thalassaemia. After her graduation, she undertook post-doctoral work in the Preimplantation Genetic Diagnosis Center at the National University Hospital, Singapore. Her research interests include developing novel and reliable single-cell assays for different genetic disorders.

Abstract The high incidence of double-gene deletions in α-thalassaemia increases the risk of having pregnancies with homozygous α^0-thalassaemia, the cause of the lethal haemoglobin (Hb) Bart's hydrops fetalis syndrome. Preimplantation genetic diagnosis (PGD) has played an important role in preventing such cases. However, the current gap-PCR based PGD protocol for deletional α-thalassaemia requires specific primer design for each specific deletion. A universal PGD assay applicable to all common deletional determinants of Hb Bart's hydrops fetalis syndrome has been developed. Microsatellite markers *16PTEL05* and *16PTEL06* within the α-globin gene cluster were co-amplified with a third microsatellite marker outside the affected region in a multiplex-PCR reaction and analysed by capillary electrophoresis. Eight informed couples at risk of having Hb Bart's hydrops fetalis were recruited in this study and all patients underwent standard procedures associated with IVF. A total of 47 embryos were analysed. Three pregnancies

1472-6483/$ - see front matter
doi:10.1016/j.rbmo.2010.06.021

were achieved from three couples, with the births of two healthy babies and one ongoing pregnancy. This work has successfully adapted an earlier protocol and developed a simple and reliable single-cell assay applicable to PGD of Hb Bart's hydrops fetalis syndrome regardless of type of deletion.

KEYWORDS: α-thalassaemia, Hb Bart's hydrops fetalis syndrome, microsatellite marker, polymerase chain reaction, preimplantation genetic diagnosis

Introduction

As one of the most common genetic disorders worldwide, α-thalassaemia constitutes a significant healthcare burden in many countries. Homozygosity for double α-globin gene deletions *in cis*, which results in complete lack of the α-globin peptide, accounts for most of the severe lethal cases (Weatherall and Clegg, 2001). Among the double-gene deletions *in cis*, $--^{SEA}$, $--^{FIL}$ and $--^{THAI}$ are prevalent in Southeast Asia, while $--^{MED}$ and $-(\alpha)^{20.5}$ are common in the Mediterranean region (Ng and Law, 2003; Weatherall, 2001). In Southern China, the double-gene deletion carrier frequency is 11.9% (Cai et al., 2002). This high incidence of double-gene deletions increases the risk of having pregnancies with homozygous α^0-thalassaemia, the cause of the lethal Hb Bart's hydrops fetalis syndrome. For example, the $--^{SEA}$ and $--^{FIL}$ deletions account for 98.8% of Hb Bart's hydrops fetalis syndrome cases in Taiwan (Higgs et al., 1999; Ko et al., 1991). This disorder also carries risks to the mother, including eclampsia and other post-partum complications (Chui and Waye, 1998; Guy et al., 1985; Ko et al., 1991; Liang et al., 1985). Prenatal diagnosis and preimplantation genetic diagnosis (PGD) play an important role in preventing such cases. PGD involves IVF followed by determination of the genetic status of embryos so that only the unaffected embryos are transferred to establish pregnancy. Therefore, 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.

Previously reported PGD tests for Hb Bart's hydrops fetalis syndrome utilize gap polymerase chain reaction (gap-PCR) to amplify across the specific deletion junction (Chan et al., 2006; Deng et al., 2006; Kuliev et al., 2005; Xu et al., 2009; Yap et al., 2009b). To detect the double-gene deletions in the α-globin gene cluster, specific gap-PCR primer sets have to be employed. In cases where the at-risk partners each carry a different α-thalassaemia double-gene deletion, multiplex gap-PCR involving two primer sets are necessary to detect the two different deletions. As a result, depending on the types of deletions present in the at-risk couple, different combinations of gap-PCR primer sets may need to be optimized. Moreover, if repetitive *Alu* sequences reside close to a deletion breakpoint junction, this may place constraints on the ability to design close proximity primers that generate easily amplifiable small gap-PCR fragments. As a result, pre-amplification of larger fragments (>500 bp) prior to nested PCR of smaller fragments may be necessary to achieve specific amplification and detection of the deletion junction from single cells.

A fluorescent multiplex PCR assay has been recently developed for prenatal diagnosis of Hb Bart's hydrops fetalis syndrome, based on the amplification of two polymorphic microsatellite markers located in the α-globin gene cluster and lying within the deletion breakpoints of several common double-gene deletions ($--^{SEA}$, $--^{FIL}$, $--^{THAI}$, $--^{MED}$ and $-(\alpha)^{20.5}$) (Ho et al., 2007). This strategy has now been adapted to enable simplified PGD of Hb Bart's hydrops fetalis syndrome. This study describes the first eight cases of PGD for Hb Bart's that have been performed using this new strategy, with two unaffected births and one ongoing pregnancy.

Materials and methods

Patients

Eight couples were recruited for this study. In six couples, both partners carried the common Southeast Asian α-globin double-gene deletion ($\alpha\alpha/--^{SEA}$). In the other two couples, one partner was heterozygous for the $--^{SEA}$ deletion while the other partner carried the $--^{FIL}$ double-gene deletion. The PGD procedures were performed under a study protocol approved by the Domain-Specific Review Board of the National Healthcare Group (C/00/549) and under the Health Services Development Program of the Singapore Ministry of Health. Oocytes were obtained from female partners after ovarian stimulation with recombinant FSH and were fertilized by intra-cytoplasmic sperm injection resulting in embryos. On day 3, embryos at the 6–10 cell stage were biopsied by zona drilling, using either an acidic Tyrodes solution (Yap et al., 2009a) or a 1.44 μm diode laser (Research Instruments, UK) after a 5-min incubation in Ca^{2+}/Mg^{2+} free biopsy medium (Irvine Scientific, USA) (Dumoulin et al., 1998). Up to two blastomeres were removed through the zona opening. Blastomeres were washed by sequential transfer into five droplets containing 1× phosphate buffered saline (PBS) containing 0.4% bovine serum albumin (BSA) before being transferred in a 0.5 μl volume into 0.2 ml tubes. From the last wash droplet, a 0.5 μl volume of wash buffer was transferred into a 0.2 ml tube to serve as a media blank.

Single-cell processing

For preclinical validation of the *16PTEL* markers, lymphoblasts were isolated from a normal cell line that was heterozygous at the *16PTEL05* and *16PTEL06* microsatellite loci. For each case, genomic DNA from the couple was tested to select an informative unlinked marker for use in the PGD, after which validation was performed on their single lymphocytes. Cells were washed three times in 15 ml of wash

buffer (1× PBS containing 0.4% BSA) by centrifugation at 300 *g* for 5 min each time. The final cell pellet was re-suspended in 1–2 ml of wash buffer and individual lymphocytes were isolated under a compound microscope (Olympus, BHT) at ×100 magnification.

Single-cell multiplex-PCR assay

Each cell/blastomere was transferred into a 0.2 ml reaction tube containing 5 μl of lysis buffer (0.2 mol/l potassium hydroxide, pH 14.0). Reaction tubes were incubated at 65 °C for 10 min, after which 5 μl of neutralization buffer (0.2 mol/l Tricine, pH 5.7) was added. A single round of multiplex-PCR amplification was employed, involving previously published primers for the *16PTEL05* and *16PTEL06* microsatellite markers (Ho et al., 2007) that reside within the α-globin gene cluster (**Figure 1**) and a third informative microsatellite marker that is unlinked to this region. The unlinked markers were *HUMTHO1* (Kuliev et al., 1998), *D7S2847* (Goh et al., 2007) *D16S539* (Ho et al., 2007), and *D18S51*. Amplifications were performed in a volume of 50 μl containing the three pairs of fluorescently-labelled PCR primers at different concentrations (**Table 1**), 0.2 mmol/l of each deoxyribonucleotide triphosphate (dNTP) (Roche Biochemicals) and 2 units of HotStarTaq DNA polymerase (Qiagen) in 1× PCR buffer containing 1.5 mmol/l $MgCl_2$. Thermal cycling was performed in a GeneAmp PCR System 9700 (Applied Biosystems), with an initial 15 min enzyme activation at 95 °C, followed by 45 cycles of 98 °C denaturation for 45 sec, 60 °C annealing for 1 min and 72 °C extension for 1 min, and a final 5 min extension at 72 °C. PCR products were resolved in an ABI PRISM 3130XL Genetic Analyzer with GeneScan −500 ROX size standard and analysed using GeneMapper software v4.0 (Applied Biosystems).

Results

Both the *16PTEL05* and *16PTEL06* microsatellites lie within the deletion intervals of the three most common Southeast Asian double-gene deletions (- -^{SEA}, - -^{FIL} and - -^{THAI}) (**Figure 1**). The multiplex PCR amplifies the *16PTEL05* and *16PTEL06* microsatellites together with a third microsatellite locus (*D7S2847, D16S539, D18S51 or HUMTH01*), which serves as a control for PCR success while simultaneously monitoring for exogenous DNA contamination and bi-allelic inheritance of parental alleles. When a carrier couple is fully informative at the *16PTEL05* and *16PTEL06* loci, an unaffected embryo should carry both the maternal and paternal alleles at both loci, while a carrier embryo will carry either the maternal or paternal alleles at both loci. In marked contrast, complete absence of any allele in both *16PTEL05* and *16PTEL06* indicates inheritance of the chromosome 16 with the double-gene deletion from both parents and the embryo is expected to be affected with Hb Bart's hydrops fetalis syndrome.

Prior to clinical application, the assay was validated on single lymphoblasts from a normal cell line that was heterozygous at the *16PTEL05* and *16PTEL06* loci and the results are summarized in **Table 2**. Of 96 cells tested, 92 cells (95.8%) generated an amplification signal from at least one *16PTEL* locus, of which 88 cells (95.7%) successfully amplified both loci. The allele dropout (ADO) rates for *16PTEL05* and *16PTEL06* were 4.5% and 6.6%, respectively. However, among the cells where both loci amplified, the incidence of concurrent ADO of both *16PTEL05* and *16PTEL06* was only 2.3% (2/88). These results illustrate the importance of multiplexing the *16PTEL05* and *16PTEL06* loci to increase the genotyping accuracy of this PGD assay.

Prior to the start of each PGD case, lymphocytes were isolated from the peripheral blood of each couple, and a trial assay was performed to check for successful amplification of the expected alleles at each locus. Amplification success of lymphocytes tested in triplex PCR assays using the different unlinked third markers ranged from 83.3% (case 4) to 97.7% (case 7), with ADO ranging from 0% (*D16S539*) to 12.5% (*D18S51*) depending on marker as well as allele size differences. A total of nine PGD cycles were performed on eight couples that were recruited for this protocol. Seven cycles involved couples who were both carriers for the - -^{SEA} double-gene deletion, while two cycles involved couples who were carriers for different double-gene deletions (- -^{SEA} and - -^{FIL}). The details of each cycle are summarized in **Table 3**. Three pregnancies were achieved from three couples, one arising from a frozen–thawed embryo transfer and the other two from fresh embryo transfers. The pregnancy from the frozen–thawed embryo transfer resulted in the birth of a healthy baby girl, whose α-globin genotype was subsequently confirmed to be αα/- -^{SEA}. The second pregnancy arose from a fresh transfer cycle, culminating in the birth of a healthy baby boy. The parents' consent for genotyping of the boy to confirm the PGD result could

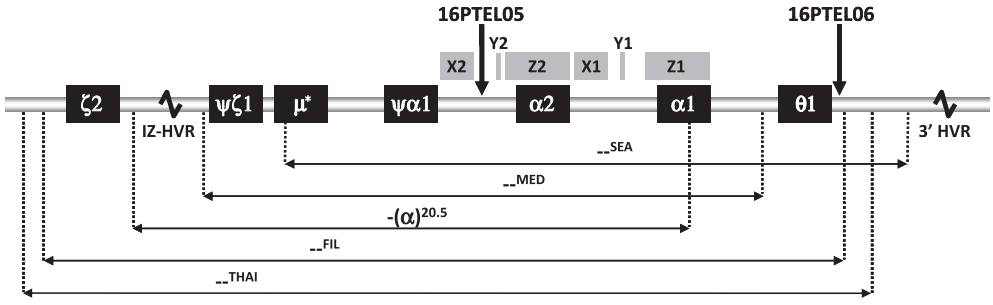

Figure 1 Schematic illustration of the α-globin gene cluster, indicating the extents of five common deletional determinants of Hb Bart's hydrops fetalis syndrome. The locations of the microsatellite markers *16PTEL05* and *16PTEL06* are indicated. Locations of the X, Y and Z sequence homology boxes and hypervariable regions (HVR) are also shown. *Also known as α^D (previously named $\varphi\alpha 2$).

Table 1 Primers used in single-cell multiplex PCR amplification of the α-globin locus and other microsatellite markers.

Name	*5′—3′ sequence*	*GenBank ID: Nucleotides*	*Concentration (μmol/l)*	*Amplicon size range (bp)*
16PTEL05-F (Fam)	Fam-ACTAAAATCTATCCATGCTTTCACACA	AE006462: 160810—160836	0.3	77—89
16PTEL05-R	GACACTCTCCTACTTTAAGTAACACAAAAAA	AE006462: 160894—160864	0.3	
16PTEL06-F (Hex)	Hex-AAACAAGAACAGGACATGGCTGT	AE006462: 171925—171947	0.2	100—142
16PTEL06-R	GTGTGGATGAGGCCTGCC	AE006462: 172030—172013	0.2	
D16S539-F (Fam)	Fam-GATCCCAAGCTCTTCCTCTT	G07925: 224—243	0.2	140—162
D16S539-R	ACGTTTGTGTGTGCATCTGT	G07925: 380—361	0.2	
D7S2847-F	TCACCTTCAGAAAGTATTGCC	G08621: 66—86	0.2	174—201
D7S2847-R (Fam)	Fam-TGAGGTGTTTCTCCAAGCTC	G08621: 261—242	0.2	
D18S51-F (Fam)	Fam-TGAGTGACAAATTGAGACCTGTCTC	X91253: 31—55	0.2	163—223
D18S51-R	CTAAGGTGGACATGTTGGCTTC	X91253: 253—232	0.2	
HUMTH01-F (Fam)	Fam-AGGGTATCTGGGCTCTGG	AF536811: 11613—11630	0.2	115—131
HUMTH01-R	CTTCCGAGTGCAGGTCAC	AF536811: 11735—11718	0.2	

Table 2 Summary of single-cell validation of the *16PTEL05* and *16PTEL06* loci.

Test	n *(%)*
Cells with successful amplification of either or both *16PTEL* loci	92/96 (95.8)
16PTEL05 locus	89/92 (96.7)
ADO at *16PTEL05* locus[a]	4/89 (4.5)
16PTEL06 locus	91/92 (98.9)
ADO at *16PTEL06* locus[b]	6/91 (6.6)
Both *16PTEL* loci	88/92 (95.7)
ADO at either or both *16PTEL* loci	6/88 (6.8)
ADO at *16PTEL05* locus only	2/88 (2.3)
ADO at *16PTEL06* locus only	2/88 (2.3)
ADO at both *16PTEL* loci	2/88 (2.3)
Media blanks with any amplification product	0/48 (0)
Reagent blanks with any amplification product	0/8 (0)

[a]ADO of allele 1 (79 bp) in three cells and allele 2 (83 bp) in one cell.
[b]ADO of allele 2 (123 bp) only; ADO of allele 1 (100 bp) was not observed.
ADO = allele dropout.

not be obtained. However, his healthy phenotype is confirmation of his clinically unaffected status. The third pregnancy has just entered into the third trimester and a confirmatory prenatal diagnosis was not performed.

Genotyping results from two PGD cases following single-round multiplex PCR are shown in **Figure 2**. The GeneScan electropherograms from cycle one of case 1 (**Table 3**) are shown in Panel A. Both partners are carriers for the $--^{SEA}$ double-gene deletion ($\alpha\alpha/--^{SEA}$). Eleven embryos were biopsied and analysed and embryos with the three possible genotypes of $\alpha\alpha/\alpha\alpha$, $\alpha\alpha/--^{SEA}$ and $--^{SEA}/--^{SEA}$ were observed. Panel B shows the results for case 4, where one partner is a carrier for $--^{SEA}$ and the other the $--^{FIL}$ double-gene deletion ($\alpha\alpha/--^{SEA}$ and $\alpha\alpha/--^{FIL}$). Only two embryos developed sufficiently for biopsy and analysis. One embryo was genotyped as normal ($\alpha\alpha/\alpha\alpha$) while the other embryo was genotyped as a carrier of the $--^{FIL}$ deletion ($\alpha\alpha/--^{FIL}$).

Discussion

Gap-PCR amplification across the $--^{SEA}$ double-gene deletion junction is currently the only method available for the preimplantation prevention of Hb Bart's hydrops fetalis syndrome (Chan et al., 2006; Deng et al., 2006; Kuliev et al., 2005; Xu et al., 2009; Yap et al., 2009b). However, there are many other deletional determinants of this disorder and all published protocols thus far are only applicable for detection of the $--^{SEA}$ double-gene deletion. For detection of other double-gene deletions using the gap-PCR strategy, specific primers and amplification protocols for each deletion need to be designed and optimized.

The multiplex microsatellite PCR strategy involves the amplification of two α-globin gene cluster microsatellite markers *16PTEL05* and *16PTEL06* and a third unlinked microsatellite marker (Ho et al., 2007). *16PTEL05* is located in between the ψα1 and α2 globin genes and lies within the deletion intervals of the three most common Southeast Asian double-gene deletions $--^{SEA}$, $--^{FIL}$ and $--^{THAI}$ and the two common Mediterranean double-gene deletions $--^{MED}$ and $-(\alpha)^{20.5}$ (Figure 1). *16PTEL06* is located downstream of the θ1 globin gene and also lies within the deletion intervals of $--^{SEA}$, $--^{FIL}$ and $--^{THAI}$ but not $--^{MED}$ and $-(\alpha)^{20.5}$. Thus *16PTEL06* is only useful for PGD of Hb Bart's hydrops fetalis syndrome arising from homozygosity or compound heterozygosity of the Southeast Asian double-gene deletions.

The small amplicon sizes of both *16PTEL05* and *16PTEL06* (<150 bp) ensure highly efficient amplification, which is ideal for single-cell PGD applications. As a result, only a single round of PCR is sufficient to generate highly specific products that can be detected on a fluorescent fragment analyser. The elimination of a nested PCR protocol, as is used in some gap-PCR methods, saves labour and time and reduces opportunities for exogenous DNA cross contamination. Furthermore, unlike gap-PCR strategies, this method represents a 'one-protocol-fits-all strategy' that can be

Table 3 Summary of the nine α-thalassaemia PGD cycles performed using the α-globin locus *16PTEL05* and *16PTEL06* markers.

Case/ cycle	*Maternal/ paternal deletions*	*No. of oocytes recovered*	*No. of oocytes fertilized*	*No. of embryos biopsied*	*Embryo genotypes (n)*	*Embryos transferred (n)*	*Unlinked microsatellite marker used*	*Outcome (baby's genotype)*
1/1	$--^{SEA}/--^{SEA}$	15	13	11	αα/αα (1) αα/$--^{SEA}$ (3) $--^{SEA}/--^{SEA}$ (6) No result (1)[a]	αα/αα (1) αα/$--^{SEA}$ (1)	D7S2847	No pregnancy
1/2[b]		NA	NA	NA	NA	αα/$--^{SEA}$ (1)[b]		Baby girl (αα/ $--^{SEA}$)
2/1	$--^{FIL}/--^{SEA}$	1	1	1	αα/αα or αα/$--^{SEA}$ (1)	αα/αα or αα/$--^{SEA}$ (1)	D7S2847	Baby boy[c]
3/1	$--^{SEA}/--^{SEA}$	12	4	2	αα/$--^{SEA}$ (1) No result (1)[a]	αα/$--^{SEA}$ (1)	HUMTH01	No pregnancy
4/1	$--^{SEA}/--^{FIL}$	4	2	2	αα/αα (1) αα/$--^{FIL}$ (1)	αα/αα (1) αα/$--^{FIL}$ (1)	D7S2847	No pregnancy
5/1	$--^{SEA}/--^{SEA}$	15	12	10	αα/$--^{SEA}$ (4) $--^{SEA}/--^{SEA}$ (2) No result (4)[a]	αα/$--^{SEA}$ (2)	D7S2847	No pregnancy
6/1	$--^{SEA}/--^{SEA}$	18	8	4	αα/αα or αα/$--^{SEA}$ (1) $--^{SEA}/--^{SEA}$ (1) No result (2)[a]	αα/αα or αα/$--^{SEA}$ (1)	D16S539	No pregnancy
6/2		14	10	4	αα/αα or αα/$--^{SEA}$ (1) No result (3)[a]	αα/αα or αα/$--^{SEA}$ (1)		No pregnancy
7/1	$--^{SEA}/--^{SEA}$	16	10	8	αα/αα (2) αα/$--^{SEA}$ (1) $--^{SEA}/--^{SEA}$ (3) No result (2)[a]	αα/αα (2)	HUMTH01	On-going pregnancy
8/1	$--^{SEA}/--^{SEA}$	18	9	5	αα/αα (2) αα/$--^{SEA}$ (2) $--^{SEA}/--^{SEA}$ (1)	αα/$--^{SEA}$ (1)	D18S51	No pregnancy

[a]No genotype could be determined due to amplification failure at the 16PTEL05 and/or 16PTEL06 loci.
[b]Freeze-thaw cycle. Two of the three αα/$--^{SEA}$ embryos from cycle 1 were frozen away and thawed out in this cycle, of which one embryo developed.
[c]Clinically unaffected; however, patient declined to have baby's carrier genotype confirmed.
NA = not applicable.

applied to PGD for any combination of the common Southeast Asian double-gene deletions using either or both *16PTEL05* and *16PTEL06* or to any combination of the common Mediterranean and Southeast Asian double-gene deletions when using *16PTEL05* only.

One minor drawback of this strategy is that when the parental *16PTEL05* and *16PTEL06* alleles are identical, it is not possible to differentiate between a carrier (αα/—) and a normal (αα/αα) embryo, since both genotypes will have essentially identical electropherogram profiles. Nevertheless, the presence of *16PTEL* product itself indicates that the embryo is not affected with Hb Bart's while absence of both markers indicates homozygous or compound heterozygous affected status. However, given the high heterozygosity index of 0.82 and 0.87 for *16PTEL05* and *16PTEL06*, respectively (Ho et al., 2007), the frequency of such a situation arising is expected to be low.

When the double-gene deletions of the partners are different, it is possible to infer the origin and even type of double-gene deletion a carrier embryo inherits if the *16PTEL05* and *16PTEL06* loci are informative. This is achieved through observing the amplicon sizes of *16PTEL05* and *16PTEL06* alleles present in the embryo, which inform on the parental origin of the intact chromosome 16. By inference, therefore, the parental origin and thus type of double-gene deletion of the deleted chromosome 16 can be determined, as illustrated in case 4 (**Figure 2B**).

Of the 47 embryos analysed in this series, 34 yielded clear diagnostic results while seven completely failed to generate any amplification product. Results for the remaining six embryos were insufficient to produce a clear diagnosis. The 85.1% of embryos (40/47) that generated an amplification signal is at the lower end of the percentage range observed preclinically (83.3—97.7%). Thirteen of the 47 embryos (27.7%) that were tested were diagnosed as Hb Bart's affected embryos, which is close to the expected 25%. However, because there were no results from certain embryos, it is not possible to determine the true number

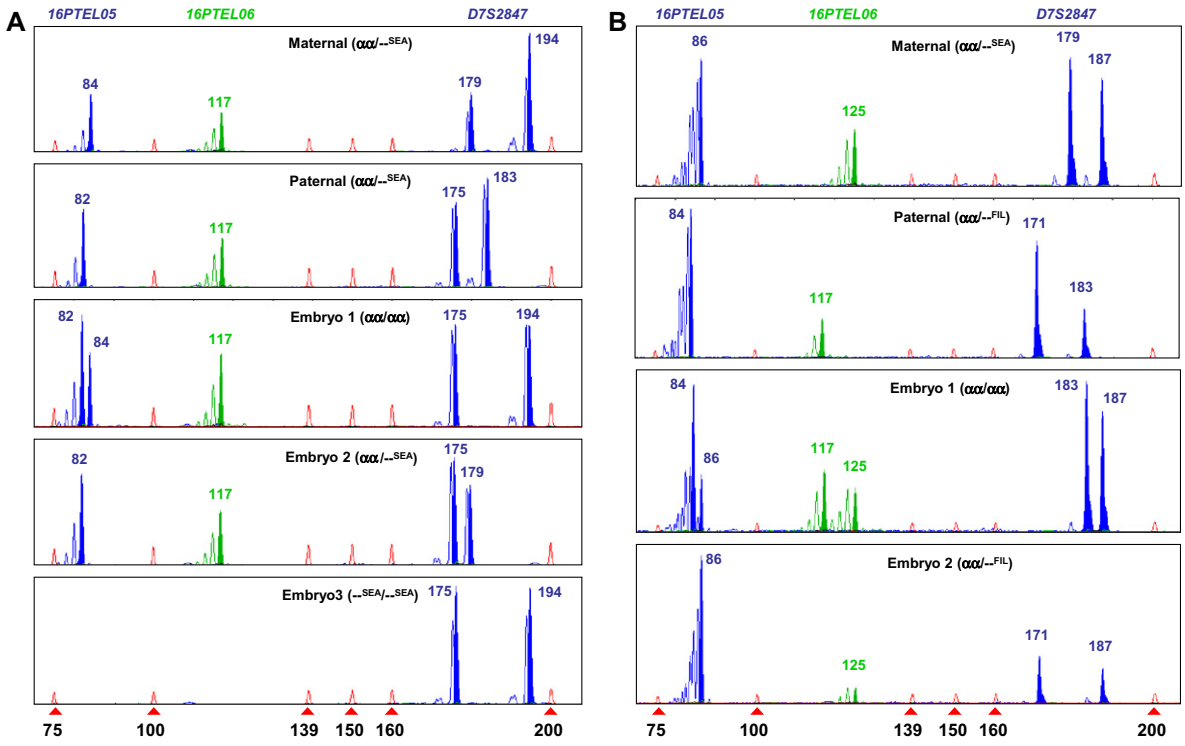

Figure 2 Microsatellite electropherograms from (**A**) PGD case 1 and (**B**) case 4 after PCR amplification, showing the peak patterns for *16PTEL05*, *16PTEL06* and *D7S2847*. The maternal and paternal electropherograms were generated from PCR-amplified single lymphocytes. Examples of normal (αα/αα), carrier (αα/- -^{SEA} or αα/- -^{FIL}) and affected (- -^{SEA}/- -^{SEA}) genotyping results from embryo blastomeres are shown. Allele fragment sizes are indicated in base pairs.

and percentage of embryos with the three different genotypes. Amplification success and ADO are affected by many biological and technical factors that are unrelated to the accuracy of the PCR assay itself. Unlike preclinical data generated using lymphoblasts or lymphocytes picked under identical controlled conditions, clinical PGD data are generated from blastomeres of variable biological quality, derived from embryos cultured and biopsied using different techniques in different IVF laboratories. The different amplification efficiencies and ADO rates observed from the different patients' lymphocytes also reflect the different unlinked markers used and allele size differences of the three markers in the different cases, as amplification efficiencies and ADO rates would be expected to differ between the different triplex PCR assays and also vary according to the size differences between alleles at each of the three marker loci.

Of the three pregnancies arising from this study, the first pregnancy resulted in a healthy baby girl whose genotype has been confirmed as αα/- -^{SEA}. The second pregnancy resulted in a healthy baby boy, for whom parental consent for genotype confirmation could not be obtained but whose current clinically normal status is entirely consistent with a homozygous normal or carrier genotype. This is because homozygosity or compound heterozygosity for the double α-globin gene deletion is incompatible with post-natal life even if the fetus were to survive gestation to term. For the third pregnancy, the patient has also opted against prenatal genotype confirmation of the fetus. However, the pregnancy is now into its third trimester and there is no clinical evidence of fetal distress to suggest a Hb Bart's conception. Therefore, it is concluded that the earlier protocol has been successfully adapted and a simple and reliable single-cell assay applicable to PGD of Hb Bart's hydrops fetalis syndrome, regardless of type of deletion, has been developed.

Acknowledgements

This work was supported by grants from the Health Services Development Program, Ministry of Health, Singapore (MOH HSDP 05/X01), the Biomedical Research Council, Agency for Science, Technology and Research, Singapore (A*STAR BMRC 03/1/21/18/222), to CHAY and SSC.

References

Cai, R., Li, L., Liang, X., et al., 2002. Prevalence survey and molecular characterization of alpha and beta thalassemia in Liuzhou city of Guangxi. Zhonghua Liu Xing Bing Xue Za Zhi, 281—285.

Chan, V., Ng, E.H., Yam, I., et al., 2006. Experience in preimplantation genetic diagnosis for exclusion of homozygous alpha degrees thalassemia. Prenat. Diagn., 1029—1036.

Chui, D.H., Waye, J.S., 1998. Hydrops fetalis caused by alpha-thalassemia: an emerging health care problem. Blood, 2213—2222.

Deng, J., Peng, W.L., Li, J., et al., 2006. Successful preimplantation genetic diagnosis for alpha- and beta-thalassemia in China. Prenat. Diagn., 1021—1028.

Dumoulin, J.C., Bras, M., Coonen, E., et al., 1998. Effect of Ca^{2+}/Mg^{2+}-free medium on the biopsy procedure for preimplan-

tation genetic diagnosis and further development of human embryos. Hum. Reprod., 2880—2883.
Goh, D.L., Zhou, Y., Chong, S.S., et al., 2007. Novel CFTR gene mutation in a patient with CBAVD. J. Cyst. Fibros., 423—425.
Guy, G., Coady, D.J., Jansen, V., et al., 1985. Alpha-thalassemia hydrops fetalis: clinical and ultrasonographic considerations. Am. J. Obstet. Gynecol., 500—504.
Higgs, D.R., Ayyub, H., Chong, S.S., 1999. The—THAI and—FIL determinants of alpha thalassemia in Taiwan [letter; comment]. Am. J. Hematol., 80—81.
Ho, S.S., Chong, S.S., Koay, E.S., et al., 2007. Microsatellite markers within—SEA breakpoints for prenatal diagnosis of HbBarts hydrops fetalis. Clin. Chem., 173—179.
Ko, T.M., Hsieh, F.J., Hsu, P.M., et al., 1991. Molecular characterization of severe alpha-thalassemias causing hydrops fetalis in Taiwan. Am. J. Med. Genet., 317—320.
Kuliev, A., Rechitsky, S., Verlinsky, O., et al., 1998. Preimplantation diagnosis of thalassemias. J. Assist. Reprod. Genet., 219—225.
Kuliev, A., Rechitsky, S., Verlinsky, O., et al., 2005. Preimplantation diagnosis and HLA typing for haemoglobin disorders. Reprod. Biomed. Online, 362—370.
Liang, S.T., Wong, V.C., So, W.W., et al., 1985. Homozygous alpha-thalassaemia: clinical presentation, diagnosis and management. A review of 46 cases. BJOG, 680—684.
Ng, I.S., Law, H.Y., 2003. Challenges in screening and prevention of thalassemia in Singapore. Asian-Oceanian J. Pediatr. Child Health, 29—38.
Weatherall, D.J., 2001. Phenotype-genotype relationships in monogenic disease: lessons from the thalassaemias. Nat. Rev. Genet., 245—255.
Weatherall, D.J., Clegg, J.B., 2001. The Thalassaemia Syndromes. Blackwell Science, Oxford.
Xu, Y.W., Zeng, Y.H., Deng, J., et al., 2009. Preimplantation genetic diagnosis for alpha-thalassaemia in China. J. Assist. Reprod. Genet., 399—403.
Yap, C., Tan, A.S., Wang, W., et al., 2009a. First successful preimplantation genetic diagnosis in Singapore — avoidance of beta-thalassaemia major. Ann. Acad. Med. Singapore, 720—723.
Yap, C., Wang, W., Tan, A.S., et al., 2009b. Successful preimplantation genetic diagnosis of Hb Bart's hydrops fetalis in Singapore after fresh and frozen embryo replacement cycles. Ann. Acad. Med. Singapore, 910—913.

Declaration: The authors report no financial or commercial conflicts of interest.

Received 11 January 2010; refereed 3 June 2010; accepted 8 June 2010.