Int J Clin Exp Pathol 2017;10(7):7586-7595
www.ijcep.com /ISSN:1936-2625/IJCEP0053693

Original Article

Genetic diagnosis of β-thalassemia preimplantation using short tandem repeats in human cryopreserved blastocysts

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Received March 24, 2017; Accepted June 13, 2017; Epub July 1, 2017; Published July 15, 2017

Abstract: This study aimed to evaluate the application of short tandem repeats (STRs) for the preimplantation genetic diagnosis (PGD) of β-thalassemia. This was a prospective study performed at the Liuzhou Maternity and Child Healthcare Hospital. From May to December 2016, eight couples formed of two β-thalassemia carriers underwent in vitro fertilization (IVF) procedures and PGD. All couples and four family members/couple underwent blood testing. Whole genome amplification of trophectoderm cells was performed. PCR products were used for linkage analysis of 15 STR loci. From the eight couples, 147 embryos were obtained and 86 blastocysts were formed. The DNA from 82 blastocysts was successfully amplified (amplification efficiency of 95.4%). Eighty blastocysts obtained a definite diagnosis. Among them, 24 blastocysts were diagnosed as normal, 38 blastocysts were diagnosed as heterozygous for β-thalassemia, and 18 blastocysts were homozygous or compound heterozygous. Two patients received a thawed embryo and both had a clinical pregnancy. These results indicated that in the setting of PGD for β-thalassemia, after multiple displacement amplifications, reverse dot hybridization combined with STRs could be an effective, accurate, and practical clinical strategy to improve the detection of β-thalassemia in at-risk couples undergoing embryo transfer. These results have to be validated in a larger cohort.

Keywords: β-thalassemia, preimplantation genetic diagnosis, multiple displacement amplification, reverse dot hybridization, short tandem repeats

Introduction

β-thalassemia is an inherited hemoglobinopathy that can seriously threat human health and result in mortality and disability. It is one of the most common monogenic autosomal recessive genetic diseases [1]. It is reported by the World Health Organization that the carrier rate of β-thalassemia in the Mediterranean area, Africa, Middle-East, Indian, and southeast Asia ranges from 1% to 20% [2]. β-thalassemia is very common in China, with a prevalence of 4.51% in the southeast [3], 2.54% in Guangdong [4], and 6.7 8% in Guangxi [5]. Nevertheless, the genetic basis of β-thalassemia in China is still poorly understood [6, 7].

Hemoglobin transports oxygen in the human body. Each hemoglobin molecule is made of two α-globin chains and two β-globin chains. The β-globin (HBB) gene cluster is located on chromosome 11 (11p15.3). The molecular basis of β-thalassemia is that HBB gene mutations lead to decreased or absent β-globin chain synthesis, resulting in hemolysis and ineffective hematopoiesis.

Usually, the affected children have no symptoms at birth, but they will develop anemia and other symptoms before two years of age. Long-term regular blood transfusions and iron chelation therapy are required. The morbidity and psychological impacts for the children and parents are important. If left untreated, the children will die.

Because β-thalassemia lacks effective treatment, pregnancy termination can be consid-

Table 1. STR loci flanking the HBB gene

Name	Repeat motif	Alleles	Primer 5'->3'	Amplicon size (bp)
HBB4506	(AC)n	16	HEX-GTTTGACATATCTGTGAGGAAG	348-380
			CAGCAAGTAAATAGGGCACTG	
D11S988	(TG)n	17	FAM-GGACAAGAGAAAGTTGAACATACTG	82-126
			CCACCATTTAAGATGCCAATAAGC	
HBB4677	(AC)n	18	HEX-TGTAAAAGGGCCTCTAATCAG	154-196
			TCACTGATATACAAATGGCAAAGTG	
D11S2362	(AAT)n	13	HEX-CTTCCCTRATCTGGAATGAACTC	69-105
			GGGTTTCCCAGTCCTTTTAC	
HBB5089	(AC)n	12	FAM-CAATTTCCTTTTCTTCTCCCTATAC	220-244
			GTGAGTCTAGCATTTGTCTTGC	
D11S1243	(TG)n	19	HEX-CTGCCCTAATTCTGTCTACC	202-238
			GTTGTYCACRATGAAGATACAC	
HBB5138	(AC)n	13	HEX-AGAAATGTCCTTTAGAGAAATACCTTC	386-410
			GTGGAGAGGAATCTYTTACTG	
HBB5178	(TG)n	15	FAM-CGTAATTGCTTTCAGTACCATTTATG	137-171
			GATGTATTCGTCAACAGATAAATGG	
HBB5205	(AGAT)n	13	FAM-CCAGGGTAGGTGACATATAC	380-428
			GTAACTCAAAAAATGGGACCCAAAC	
D11S1760	(CA)n	21	FAM-ACCCTGAGTGTCTTCAAAACTC	174-220
			CAAMARTGCTGCATCATGACT	
HBB5576	(AAGG)n	24	FAM-TCCTTCAGGTAAGAAGGAGC	306-348
			CTTGAAGAGGCTAGGTGC	
HBB5655	(AC)n(AT)n	23	FAM-TCATTGTTTTGGTAGGTACTGAAAG	251-299
			AGTTGTAGTAAGTTTGTCAGGCTA	
HBB5820	(AC)n(AG)n	11	HEX-CTGAGAATTATTTATACAGCAACACTTG	293-313
			CCAGTTATTGGTTGCTTTAGATTACC	
HBB5859	(ATCT)n	12	FAM-CTGTCTATTTCATCTGTCAGCTTC	354-396
			AAAGTGTTGGCGTGAGC	
D11S1338	(AC)n	11	HEX-AAGGACACACAGATTCACTTAAAG	119-139
			GCTACTTATTTGGAGTGTGAATTTC	

ered. Taking into account the religious beliefs of different populations and the physical and psychological trauma caused by induced labor, preimplantation genetic diagnosis (PGD) is undoubtedly a good choice. PGD for β-thalassemia has been reported previously [8-14]. The screening for β-thalassemia carriers mainly rely on whole genome amplification (WGA) or nested polymerase chain reaction (PCR) combined with the reverse dot blot (RDB) technology for detection. Difficulties are mainly that the DNA content of the PGD sample is very limited, only one or a few cells. This may result in failed amplification and impossible detection. In addition, single-cell PCR may suffer from allele drop out (ADO), which can lead to a false negative and result in a pregnancy of children with β-thalassemia major.

Therefore, the present study aimed to improve the PGD screening for β-thalassemia. In order to increase the sample amount and improve the amplification efficiency, we selected trophectoderm cells as the material for genetic testing, and amplified the DNA using the multiple displacement amplification (MDA) method. In order to reduce the false negative rate caused by ADO, we selected 15 short tandem repeats (STRs) loci closely linked with the HBB gene for linkage analysis [15], and used the reverse dot blot technique to detect these STRs directly. The final screening was made through this PGD strategy in order to select the most suitable embryos for transplantation. This strategy could improve the detection of β-thalassemia in at-risk couples undergoing embryo transfer since the risk of ADO becomes close to zero with the use of multiple STRs. The use of single-cell DNA amplification and of multiple STRs are improvements over the other strategies that have been suggested for PGD of β-thalassemia [8-14].

Materials and methods

Patients

This was a prospective study performed at the Department of Reproduction, Liuzhou Maternity and Child Healthcare Hospital. From May to

Table 2. Characteristics of the couples

Couple	Age, female/ male (years)	Gravida	Para	Female genotype	Male genotype	Pregnancy history
1	30/31	3	0	CD41-42	CD-41-42	In 2012, spontaneous abortion occurred in early pregnancy. In 2013 and 2015, two induced abortion were conducted for prenatal diagnosis of fetal major β-thalassemia.
2	30/33	0	0	CD41-42	CD17	---
3	29/32	0	0	CD41-42	CD41-42	---
4	27/31	0	0	CD41-42	CD41-42	---
5	30/37	1	0	CD41-42	CD17	In 2014, induced abortion was conducted for prenatal diagnosis of fetal major β-thalassemia.
6	26/30	2	0	CD41-42	CD41-42; α-thalassemia (–SEA/αα)	In 2014 and 2016, two induced abortion were conducted for prenatal diagnosis of fetal major β-thalassemia at 11 weeks of gestation based on villus sampling.
7	27/30	1	0	-28	CD41-42	One spontaneous abortion in early pregnancy.
8	26/29	2	0	IVS-II-654; α-thalassemia (–SEA/αα)	-28	In 2013, one induced abortion in early pregnancy. In 2015, induced abortion was conducted for prenatal diagnosis of fetal major β-thalassemia in mid pregnancy.

All subjects were β-thalassemia carriers.

December 2016, eight couples made of two β-thalassemia carriers (heterozygotes for one defective allele among CD41-42/N, CD17/N, -28/N, and IVS-II-654/N) underwent in vitro fertilization (IVF) procedures and PGD. All couples and four family members per couple underwent blood testing for hemoglobin electrophoresis, β-thalassemia gene mutation detection, and STR locus linkage analysis. The inclusion criteria were: 1) couples made of two β-thalassemia carriers who underwent IVF; and 2) requested PGD. The exclusion criteria were: 1) reproductive or acute urinary system infection or unfavorable pregnancy; or 2) any contraindication to IVF.

The couples received genetic counseling from a clinical geneticist and signed the informed consent form. This study was approved by the Liuzhou Maternity and Child Healthcare Hospital ethics committee.

Pedigree linkage analysis

Each couple and four family members per couple underwent blood testing. Genomic DNA was extracted from the peripheral blood by phenol-chloroform (Shanghai Biological Engineering Co., Shanghai, China) extraction, diluted to 10 pg/μl with ultrapure water, and stored at -80°C. According to the literature [15], 15 STR loci (**Table 1**) that are closely linked to the HBB gene were selected. The primers were synthesized by Shanghai Shengong Biology Co. (Shanghai, China). The PCR protocol was: 1) pre-denaturation at 95°C for 3 min; 2) 30 cycles of 95°C for 45 s, 53°C for 45 s, and 72°C for 30 s; and 3) extension at 72°C for 30 min. Fragments were resolved by capillary electrophoresis using 0.5 μl of LIZ (Shanghai Biological Engineering Co., Shanghai, China), 9.0 μl of HI-DI formamide (Shanghai Biological Engineering Co., Shanghai, China), and 1.0 μl of PCR product on an ABI 3500Dx (Applied Biosystems, Foster City, CA, USA) and analyzed using Genemaper 4.1 (Applied Biosystems, Foster City, CA, USA).

In vitro fertilization and blastocyst biopsy

Gonadotropin-releasing hormone agonist (GnRHa) triptorelin (Diphereline; Ipsen Pharma Biotech, Boulogne-Billancourt, France) was used at days 5-7 after the natural cycle of ovulation or at day 21 of menstrual periods on oral contraceptives for pituitary down-regulation; GnRHa was used for ≥15 days. Standard down-regulation was considered when serum E2 was <50 pg/ml, FSH was <5 mIU/ml, LH was <5 mIU/ml, bilateral ovarian follicle diameter was <10 mm, and endometrial thickness was ≤5 mm. Then, recombinant FSH (Gonal-F; Merck Serono KGaA, Darmstadt, Germany) and menotropins (HUMOG, Bharat Serums And Vaccines Lt, Ambernath, India) were used to induce ovulation. In the presence of >3 follicle with diameters of <18 mm, recombinant human chorionic gonadotropin (Merck Serono KGaA, Darmstadt, Germany) was injected intramuscularly, and oocyte retrieval was performed 36 h

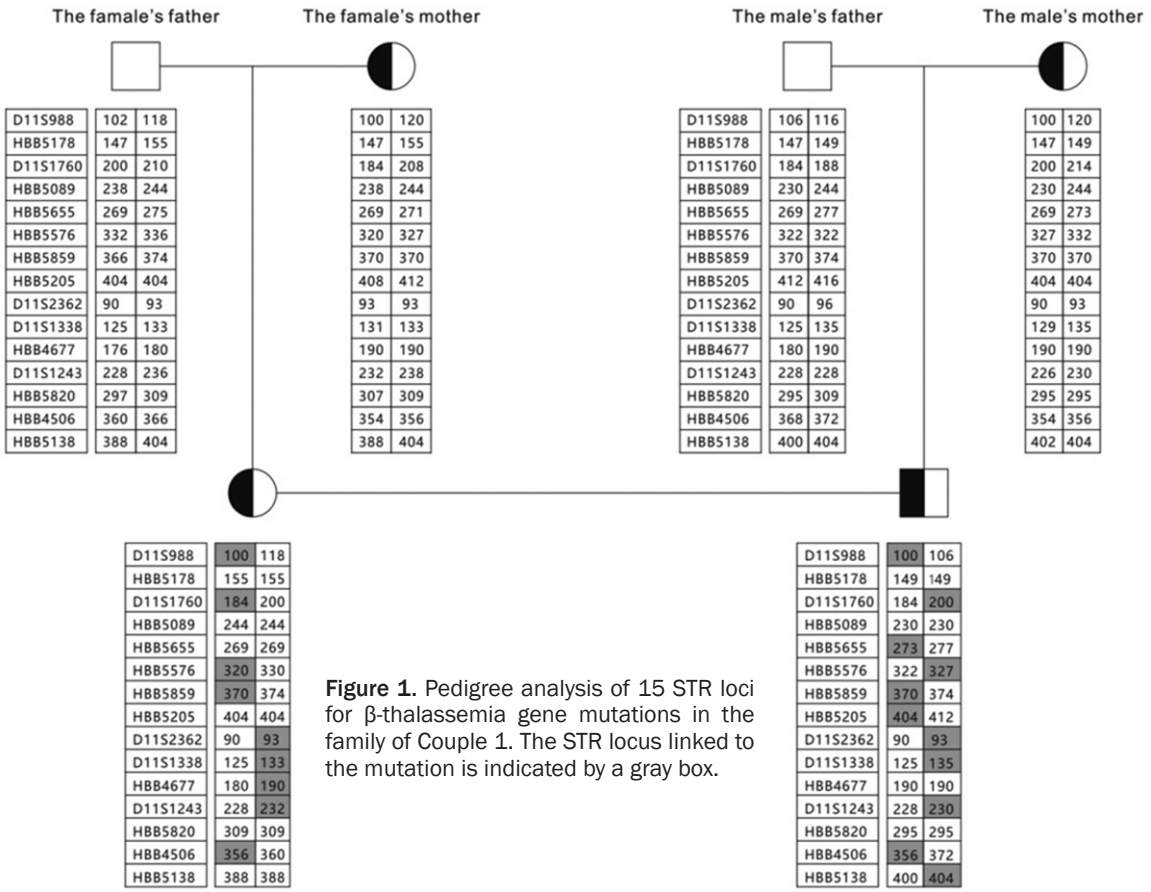

Figure 1. Pedigree analysis of 15 STR loci for β-thalassemia gene mutations in the family of Couple 1. The STR locus linked to the mutation is indicated by a gray box.

later. The obtained oocytes were subjected to intracytoplasmic sperm injection (ICSI). On the fifth day after oocyte retrieval, the blastocysts were biopsied mechanically. The extracted trophectoderm cells were washed three times in PBS, and then placed in a PCR reaction tube. The PBS solution (2 μl) from the last washing was used as negative control.

Preimplantation genetic diagnosis

Whole genome amplification of trophectoderm cells was performed using the REPLI-g single cell kit (Qiagen, Venlo, The Netherlands). The product was diluted 100-fold at 4°C and the remaining product was stored at -80°C. PCR products were used to detect the β-thalassemia gene by reverse dot blotting, and 2 μl of PCR products were used for linkage analysis with 15 STR loci.

Statistical analysis

Only descriptive statistics were used. Categorical data are expressed as frequencies and percentages. Data management was performed using SPSS 19.0 (IBM, Armonk, NY, USA).

Results

Pedigree analysis

Table 2 presents the characteristics of the couples. Eight couples and their family members used STR loci for linkage analysis. **Figure 1** presents the results from Couple 1.

Blastocysts

Table 3 presents the data about the blastocysts from the eight couples; 147 embryos were obtained and 86 blastocysts were formed (blastocyst formation rate of 58.5%). Eighty-two samples were successfully amplified by MDA (amplification efficiency of 95.4%).

Genetic analysis of the embryos

Direct detection of pathogenic genes was carried out using reverse dot hybridization. The RDB results are shown in **Figure 2**. **Figure 3**

Table 3. Summary of the blastocysts

Couple	Oocytes	Embryos	Blastocysts	Blastocyst formation rate (%)	Amplified	Amplification efficiency (%)
1	28	23	16	69.57	14	87.5
2	20	17	9	52.94	8	88.9
3	21	15	5	33.33	4	80.0
4	40	27	21	77.78	21	100.0
5	14	13	10	76.92	10	100.0
6	21	15	11	73.33	11	100.0
7	30	24	8	33.33	8	100.0
8	29	13	6	46.15	6	100.0
Total	203	147	86	58.5	82	95.4

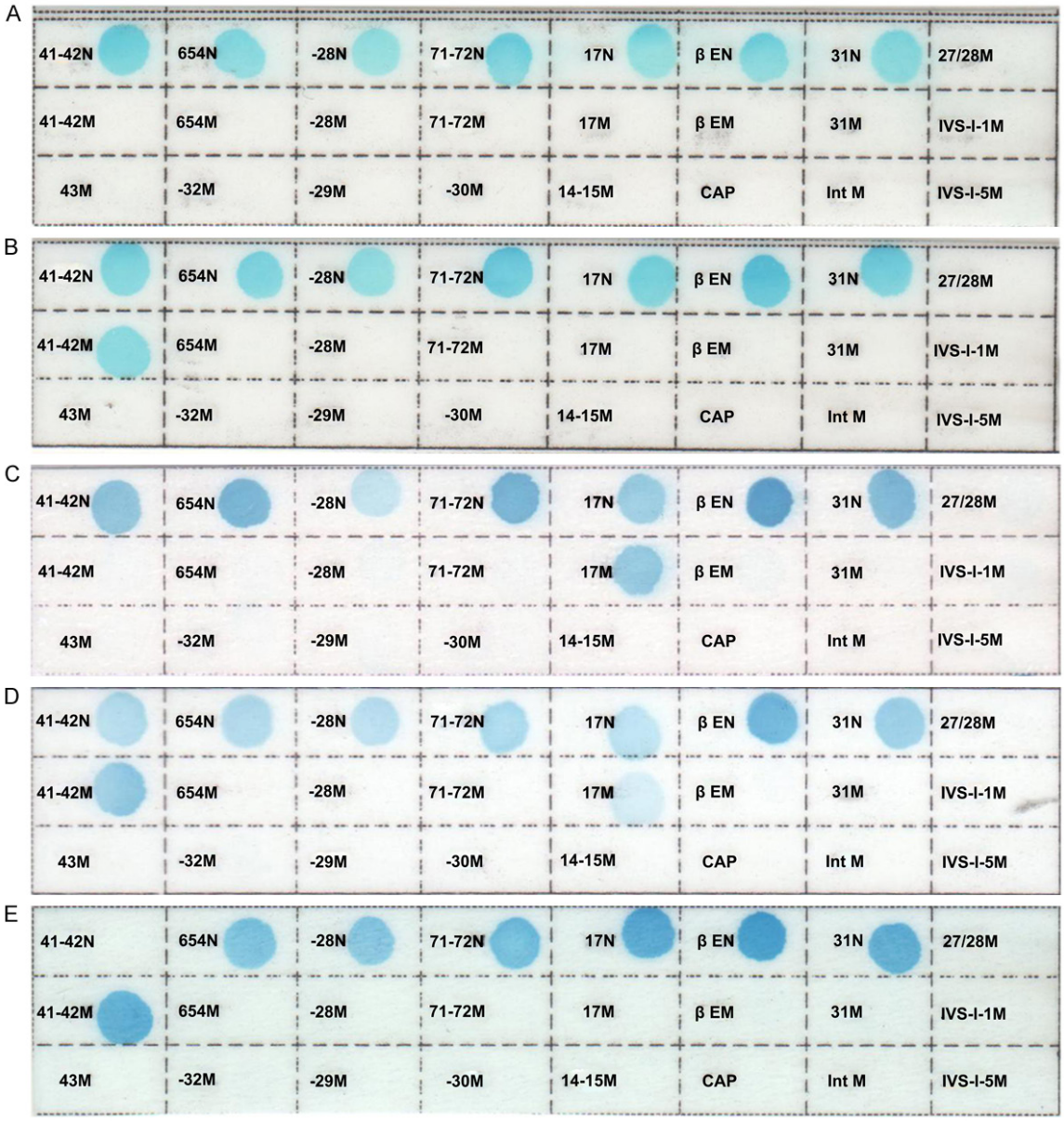

Figure 2. The results of RDB of trophectoderm cells in PGD. A. No β-thalassemia mutations were detected (normal). B. β-thalassemia CD41-42 heterozygote (carrier). C. β-thalassemia 17 heterozygote (carrier). D. Double heterozygotes for β-thalassemia CD41-42 and 17 (affected). E. β-thalassemia CD41-42 homozygote (affected).

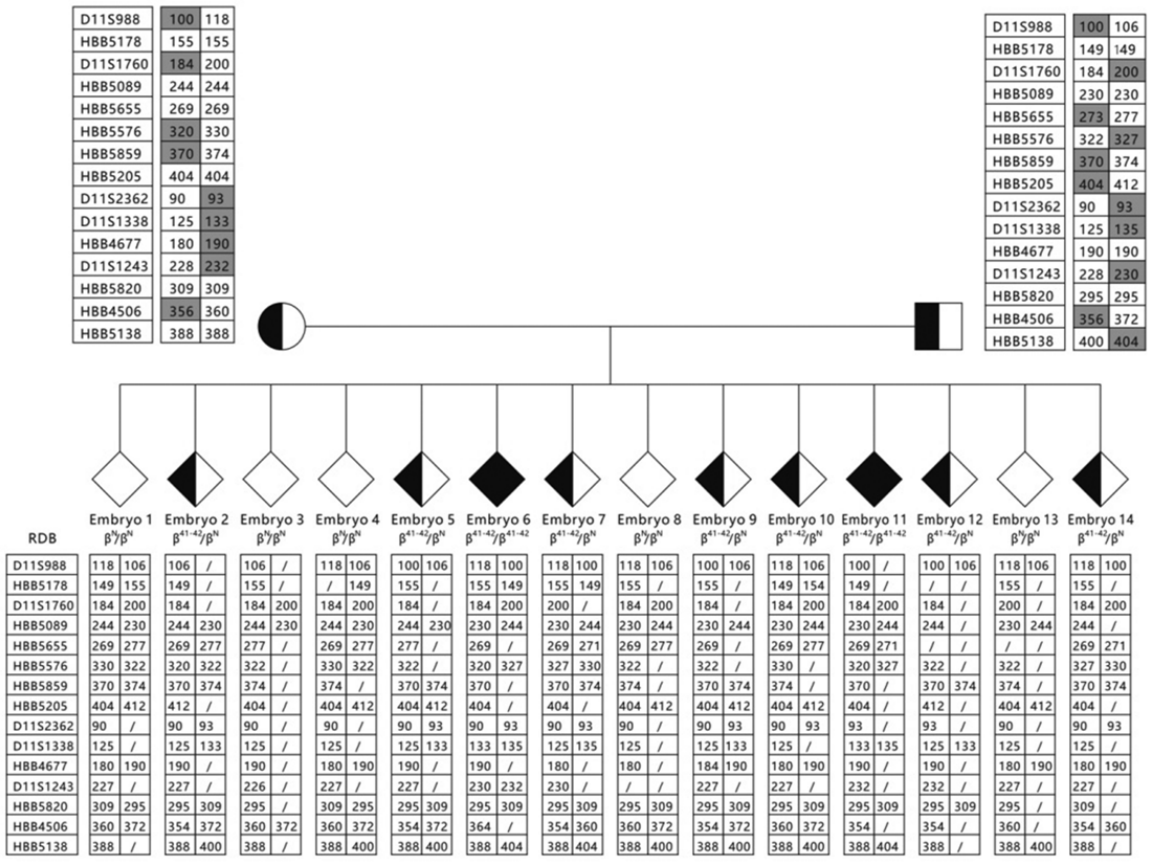

Figure 3. Pedigree linkage analysis of 15 STR loci of each blastocyst in Couple 1. For example, embryo 1: RDB test results were β^N/β^N. D11S988, HBB5576, and HBB4506 indicated that this blastocyst did not carry the pathogenic genes of both parents, so the final diagnosis of this blastocyst was β^N/β^N. Embryo 2: RDB test results showed β^{41-42}/β^N. HBB5576, D11S1338, and HBB4506 indicated that this blastocyst carried the maternal pathogenic gene. D11S988, HBB5655, HBB5576, HBB5205, HBB4506, and HBB5138 indicated that this blastocyst did not carry the paternal pathogenic gene. So, the final diagnosis of this blastocyst was β^{41-42}/β^N. Embryo 6: RDB test result showed $\beta^{41-42}/\beta^{41-42}$. HBB5576, D11S1338, and D11S1243 of the 15 STR loci indicated that this blastocyst carried the pathogenic genes of both parents. So the final diagnosis of this blastocyst was $\beta^{41-42}/\beta^{41-42}$.

shows the pedigree linkage analysis results of Couple 1. For example, embryo 1: RDB test results showed β^N/β^N. D11S988, HBB5576, and HBB4506 indicated that this blastocyst did not carry the pathogenic genes of both parents, so the final diagnosis of this blastocyst was β^N/β^N. Embryo 2: RDB test results showed β^{41-42}/β^N. HBB5576, D11S1338, and HBB4506 indicated that this blastocyst carried the maternal pathogenic gene. D11S988, HBB5655, HBB5576, HBB5205, HBB4506, and HBB5138 indicated that this blastocyst did not carry the paternal pathogenic gene. So, the final diagnosis of this blastocyst was β^{41-42}/β^N. Embryo 6: RDB test result showed $\beta^{41-42}/\beta^{41-42}$. HBB5576, D11S1338, and D11S1243 of the 15 STR loci indicated that this blastocyst carried the pathogenic genes from both parents. So, the final diagnosis of this blastocyst was $\beta^{41-42}/\beta^{41-42}$.

PGD results of blastocysts

In the present study, 86 blastocysts were tested (**Table 4**). DNA from 82 blastocysts was successfully amplified (amplification efficiency of 95.4%). Two blastocysts from Couple 2 failed to obtain a definitive diagnosis, while the remaining 80 blastocysts obtained a definite diagnosis. One of the two unconfirmed blastocysts failed the RDB test, which was ruled to be $\beta^{17/N}$ according to the STR linkage analysis results. The other blastocyst had a RDB test result of $\beta^{N/N}$, and the linkage analysis failed. Of the 80 blastocysts diagnosed, 24 blastocysts were diagnosed as normal (30.0%), 38 blastocysts were diagnosed as heterozygous for β-thalassemia (47.5%), and 18 blastocysts were diagnosed as mutant homozygotes or compound heterozygotes (22.5%).

Table 4. Summary of PGD results of the blastocysts

Couple	Blastocysts	Amplified	Diagnosed	Normal	Carrier	Affected
1	16	14	14	5	7	2
2	9	8	6	3	1	2
3	5	4	4	0	3	1
4	21	21	21	4	9	8
5	10	10	10	0	9	1
6	11	11	11	6	5	0
7	8	8	8	3	3	2
8	6	6	6	3	1	2
Total	86	82	80	24	38	18

Embryo transfer

Only two of the eight couples underwent thawed embryo transfer. Female 1 received a thawed blastocyst on September 21, 2016. The diagnosis was $\beta^{N/N}$. The transfer resulted into an intrauterine pregnancy and a single live fetus. At 22 weeks of pregnancy, prenatal diagnosis from amniotic fluid sampling showed consistent diagnosis. Female 2 received a thawed embryo on September 14, 2016. The diagnosis was $\beta^{N/N}$. The transfer resulted into an intrauterine pregnancy and a single live fetus. Prenatal diagnosis from amniotic fluid sampling was not done. The remaining 6 couples did not yet receive thawed embryo transfer. In Couple 6, the analysis of the embryo showed that it was $\beta^{41\text{-}42/N}$. Linkage analysis of the 15 loci showed consistent result with the mother, and UPD was suspected. A comprehensive analysis showed that it was 41-42/N and with uniparental disomy, and thus could not be transplanted.

Discussion

Current PGD strategies for β-thalassemia suffer from a risk of false negative results. Therefore, the aim of the present study was to evaluate the application of STRs for the PGD of β-thalassemia. Results showed that in the setting of PGD for β-thalassemia, the combination of MDA, reverse dot hybridization, and STRs could be an effective, accurate, and practical clinical strategy that could improve the detection of β-thalassemia in at-risk couples undergoing embryo transfer. Nevertheless, these results have to be validated in a larger cohort.

A previous retrospective study of a single family with β-thalassemia showed the potential of PGD using a single-cell technique [16]. A previous study also validated the technique used in the present study, i.e. the use of 15 STRs from single cells with or without whole genome amplification [15]. The study by Xu et al. showed that 97% of the embryos could be successfully sequenced [16], similar to the rate in the present study.

There are three sources of specimens for PGD: polar bodies, blastomeres, and trophectoderm cells. In the present study, trophectoderm cells were used in the blastocyst stage. Using these cells have some advantages. First, higher sample amount; indeed, the technical difficulty of PGD is the limited availability of DNA that may lead to amplification failure or ADO. In contrast, the polar body and blastomeres have 1 or 2 cells, while there are 5-10 trophectoderm cells in the blastocyst stage. Higher cell number will increase the rate of amplification success [17, 18]. Secondly, blastocysts have a higher developmental potential than embryos. Indeed, many embryos with development potential but low implantation rate will not develop into blastocyst. Finally, a paired randomized clinical trial showed that blastomeres biopsy significantly reduced the implantation rate, while trophectoderm cell biopsy in the blastocyst stage would not [19].

Since genetic diagnosis after blastocyst biopsy takes time, we adopted the clinical strategy of freezing all blastocysts after biopsy and waiting for genetic testing; ultimately, only the appropriate embryo is selected according to the results of PGD. Thanks to the development of the vitrification technology, the clinical outcome of frozen embryo transfer is not inferior to that of fresh embryo transfer [20-22]. In addition, the use of thawed embryo significantly reduces the likelihood of ovarian hyperstimulation syndrome (OHSS) [23], which is a common complication in assisted reproduction and may even be life-threatening in severe cases. During the process of PGD, the aim is to obtain more embryos in order to be able to select a normal embryo for transplantation. Therefore, the Gn dosage is very important, increasing the risks of OHSS. Hence, the use of thawed embryo was selected to decrease the risk of OHSS.

STRs are 2-7-base pair (bp) tandem repeats found throughout the human genome. Since these STRs are highly polymorphic among individuals, analysis of STR markers closely associated with a causative gene has been used in single-gene disease PGD (SGD-PGD) [24-35]. When PCR is performed, the STRs on the same chromosome will be amplified along with the pathogenic gene. The simultaneous analysis of marker loci and pathogenic loci can maximally monitor and eliminate the effects of ADO on diagnosis. The probability of the occurrence of ADO at the polymorphic marker loci and causative gene on a chromosome is much lower than that of a single locus. For example, the ADO probability of one locus of the causal gene is 0.01, and the ADO probability of the linkage polymorphic locus is 0.01. The probability of ADO at two loci on the same chromosome is 0.0001. If two or more polymorphic loci are linked, the probability of ADO becomes close to zero. It has been reported that STR loci are used in the PGD of β-thalassemia [10, 12, 13], but the numbers of STR loci in these previous studies were relatively small. A previous study showed that using STRs closely associated with the HBB gene increased the number of viable embryos otherwise rejected because of ADO [36]. In the present study, 15 STR loci associated with the HBB gene were used for PCR, and the STR polymorphisms were detected by capillary electrophoresis. In this way, the risk of misdiagnosis of β-thalassemia during PGD can greatly be reduced. Moreover, these 15 STR loci are close to the HBB gene (<1 Mb), which also reduces the possibility of gene recombination [15]. In the present study, 82 samples were successfully amplified; among them, RDB and STR results were consistent in 80 cases (diagnosis rate of 97.6%). Only two samples could not be diagnosed, because of RDB or STR failure.

The present study is not without limitations. First, the sample size was very small. There were only eight couples and only two proceeded with embryo implantation. Secondly, there are no live births yet and only one woman underwent amniotic fluid testing. Additional studies are necessary to confirm these results.

In conclusion, in the setting of the PGD of β-thalassemia, the combination of MDA, reverse dot hybridization, and STRs could be an effective, accurate, and practical clinical strategy that could improve the detection of β-thalassemia in at-risk couples undergoing embryo transfer. These results have to be validated in a larger cohort.

Acknowledgments

This study was supported by The Guangxi Zhuang Autonomous Region Health and Family Planning Commission self financing research (No. Z2016556).

Disclosure of conflict of interest

None.

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