Single-cell polymerase chain reaction-based pre-implantation genetic diagnosis using fragment analysis for β-thalassemia in an Indian couple with β-globin gene mutations

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ABSTRACT

Despite advances in diagnostic techniques, approximately 10,000 babies with β-thalassemia major are born annually in India. Pre-implantation genetic diagnosis (PGD), an alternative to prenatal diagnosis, helps in negative selection of affected embryos prior to implantation. Hereby, we report the first successful β-thalassemia PGD pregnancy in an Indian carrier couple. β-Thalassemia mutation analysis by Amplification-Refractory Mutation Sequence (ARMS)-polymerase chain reaction (PCR) in the parents, followed by PGD for β-thalassemia mutation in embryos in two consequent *in vitro* fertilization (IVF) cycles, with transfer for three β-thalassemia minor embryos, resulted in singleton successful pregnancy, the results of which were confirmed on prenatal diagnosis. With advances in assisted reproductive techniques and molecular diagnosis, PGD for monogenic diseases is feasible in high-risk couples. The methodology in the current study included two rounds of PCR using fluorescently labeled primers, fragment analysis using the ABI 3100 nucleotide sequencer and the GeneMapper software, purification, and concentration of PCR product, which enabled distinct clear peaks making the analysis and interpretation non-ambiguous.

KEY WORDS: Amplification-refractory mutation sequence-polymerase chain reaction, β-thalassemia, pre-implantation genetic diagnosis, single-cell polymerase chain reaction

INTRODUCTION

With advances in medical science and better control of infectious diseases, congenital malformations and genetic disorders are becoming an important cause of morbidity and mortality in developing countries, including India. Additionally, high birth rate and traditional arranged and/or consanguineous marriages result in an increase in the mutated gene pool in communities. Compounded by inadequate diagnostic, management, and rehabilitation facilities, the burden of these disorders is higher in India than in Western countries.

β-Thalassemia is an autosomal recessive disorder that occurs due to β-globin gene mutations. Homozygotes and compound heterozygotes for β-globin gene mutation suffer from β-thalassemia major, which presents with severe anemia requiring repeated blood transfusions and expensive iron chelation therapy.[1] About 200 β-globin gene mutations, primarily point mutations or small deletions, have been identified.[1] Of these, 35 mutations have been identified in ethnic Indians. In India, the carrier frequency of β-thalassemia varies from 1%-17% (mean 3.3%), with an estimated 40 million β-thalassemia carriers in the population, and approximately 10,000 babies affected with β-thalassemia are born annually in India.[2,3] Affected pregnancies are diagnosed by DNA mutation analysis on amniotic fluid samples at 16 weeks or chorionic villous biopsy at 10 weeks.[4,5] The children of heterozygous carrier parents inherit a 25% risk of β-thalassemia major, 50% risk of β-thalassemia minor, and 25% children are born with a normal β-globin gene. Genetic counseling and prenatal diagnosis enable informed decisions by clinicians and parents with respect to the conceived pregnancy.

Pre-implantation genetic diagnosis (PGD) is an alternative option to prenatal diagnosis, with negative selection of affected embryos, prior to implantation. *In vitro* fertilization (IVF), followed by determination of the β-globin gene mutation status of the embryos, facilitates transfer of disease-free embryos or carrier status embryo transplantation. Thus, clinical application of single-cell genetic analysis and PGD prevents the risk of affected children in parents with a high risk of β-thalassemia major or minor children.[6-9]

In the current report, the first successful application of PGD for β-thalassemia in India for a couple at risk of transmitting an IVS1-5 (G-C) mutation to the offspring is presented.

CASE REPORT

A β-thalassemia carrier couple comprising a 28-year-old female and a 29-year-old male partner, married for 7 years, with one 5-year-old β-thalassemia major child, were referred to our Molecular Medicine laboratory for PGD. Following the birth of a β-thalassemia major child, the couple was counseled for prenatal diagnosis (PND) in subsequent pregnancies. PND on chorionic villus sampling (CVS) in two consecutive pregnancies had confirmed β-thalassemia major diagnosis, and following genetic counseling, the couple opted for medical termination of the pregnancies. Having gone through the mental, emotional, and psychological trauma of having one β-thalassemia major child, and two medical terminations due to diagnosis of β-thalassemia major status of the fetus in two subsequent pregnancies, before planning the next pregnancy, the couple was referred for PGD counseling. Post-counseling the couple opted for IVF and PGD.

The patient underwent two IVF/PGD cycles after informed written consent, involving controlled ovarian stimulation using a gonadotropin-releasing hormone (GnRH) downregulation protocol. Following ultrasound-guided transvaginal oocyte retrieval, mature oocytes were fertilized by intracytoplasmic sperm injection (ICSI) as the IVF center where the couple was undertaking the treatment had a policy of "ICSI for all". Cleavage stage embryo biopsy on day 3 embryos was performed using non-contact Saturn 3™ laser system (Research Instruments, Cornwall, UK), followed by aspiration of a single blastomere from each embryo. The biopsied embryo was washed and put for culture to blastocyst stage. The biopsied blastomere was washed in wash buffer and transferred to a 0.2 ml PCR tube containing 1 µl dissociation medium containing phosphate-buffered saline with 0.1 mg/ml phenol red (Sigma-Aldrich, Bangalore, India). The PCR tubes containing the blastomere were transported to the laboratory at 2 - 8°C, in 2.5 µl alkaline lysis buffer (5 mmol dithiothreitol and 20 mmol KOH). The cells were further subjected to a single freeze-thaw cycle, with freezing at -80°C for 30 min and thawing at 65°C for 10 min, to ensure maximum release of DNA from the single cell.

A two-step Amplification Refractory Mutation Sequence (ARMS)-polymerase chain reaction (PCR) protocol was used for single-cell PCR to identify β-thalassemia mutations as per standard protocol.[10] The target DNA was amplified using normal and mutant allele-specific 5′-primers and 3′-primers to amplify 285-bp and 384-bp fragments representing normal or IVS1 - 5 (G-C) mutations. The sequence of the primers[10] is as follows:

Common forward primer - FP1: ACC TCA CCC TGT GGA GCC AC

Common forward primer - FP2: GGG GCC AAG AGA TAT ATC TTA GAG GG

Normal reverse primer: CTC CTT AAA CCT GTC TTG TAA CCT TGT TAC

Mutant reverse primer: CTC CTT AAA CCT GTC TTG TAA CCT TGT TAG

The amplification of two independent fragments ensured false-negative interpretations due to allele dropout. The mutant primer was labeled with the 6-carboxy-hexachlorofluorescein (HEX) fluorescent dye resulting in a green peak, and the normal primer was labeled with the carboxy-fluorescein (FAM) fluorescent dye or carboxy-tetramethylrhodamine (TAMRA), resulting in blue or black peaks, respectively.

The lysis buffer was neutralized by neutralization buffer (90 mmol Tris-HCl, 30 mmol KCl, 20 mmol HCl). A 21.5-µl volume of the reaction buffer was added to 3.5 µl of the sample DNA. The first-round PCR used reaction buffer containing 10 mmol Tris-HCl, 2 mmol $MgCl_2$, 0.01% gelatin, 1.25 U AmpliTaq Gold DNA Polymerase (Applied Biosystems, CA, USA), 200 µmol dNTP, and 0.2 µmol of each of the primers. In the second-round PCR re-amplification, a 3-µl aliquot of the first-round PCR product was added to the reaction buffer consisting of 10 mmol Tris-HCl, 50 mmol KCl, 2 mmol $MgCl_2$, 0.01% gelatin, 2 U AmpliTaq Gold DNA Polymerase (Applied Biosystems, CA, USA), 200 µmol dNTPs, and 0.4 µmol of each primer as detailed earlier. Table 1 details the PCR program used in the first and second round.

PCR products were purified using a HighPure PCR product purification kit (Roche Applied Science, India). The purified products were eluted in a small volume of low-salt buffer. The purified PCR products were concentrated using sodium acetate and ethanol, re-suspended in UltraPure diethylpyrocarbonate (DEPC) water, and subjected to fragment analysis on an ABI 3100 Genetic Analyzer (Tokyo, Japan) using GeneMapper Software

Table 1: Cycling conditions employed for single-cell polymerase chain reactionfor β-thalassemia

PCR protocol	First-round PCR	Second-round PCR
Initial denaturation	94°C, 5 min	-
Denaturation	94°C, 45 s	94°C, 60 s
Annealing	60°C, 45 s	60°C, 60 s
Extension	72°C, 60 s	72°C, 90 s
Number of cycles	10	25
Final extension	72°C, 5 min	72°C, 3 min
Hold	4°C, ∞	4°C, ∞

PCR = Polymerase chain reaction

version v3.5. Thus, 2 µl of the purified PCR products was added to a mixture containing 15 µl of Hi Di Formamide and 0.5 µl of Rox standard, denatured at 94°C for 5 min, followed by immediate chilling. The product was loaded on an ABI MicroAmp Optical 96-well reaction plate and electrophoresed on an ABI 3100 Genetic Analyzer using the POP6 (Performance Optimized Polymer 6) separation matrix at 50°C, in a 50 cm capillary array.

PND was performed on amniotic fluid sample collected under ultrasonography guidance. The PCR was performed in two sets, one for the mutant allele and one for the normal allele with the β-globin gene as the internal control using the standard ARMS-PCR protocol. The PCR products were electrophoresed on 2% agarose gel with molecular weight markers to size the specific fragment.

The patient was a poor responder to controlled ovarian stimulation and hence, both IVF-PGD cycles resulted in the retrieval of only four oocytes each. In the first IVF cycle, of the four oocytes retrieved two oocytes were immature, and hence, were excluded from further procedure. One of the two mature oocytes fertilized following ICSI and was subjected to embryo biopsy on day 3 post fertilization. Molecular testing on the blastomere by fragment analysis revealed the presence of two peaks for the mutant allele at 285 bp and 384 bp for the IVS1 - 5 (G-C) mutation and absence of peaks for the normal allele, thus identifying the embryo to be β-thalassemia major. Hence, the embryo was not transferred.

The second PGD cycle, 5 months later, resulted in four mature oocytes. Post ICSI, four embryos were available for biopsy. On day 3 post fertilization, the four embryos were subjected to embryo biopsy and one blastomere was removed from each of the embryos. Molecular genetic testing on the blastomere revealed the presence of peaks both for the mutant and the normal allele in the four embryos, indicating β-thalassemia carrier status of the four embryos [Figure 1]. Earlier pregnancies in the patient had resulted in a β-thalassemia major child, and two prenatally diagnosed β-thalassemia major fetuses in consecutive pregnancies. The couple was keen on transfer

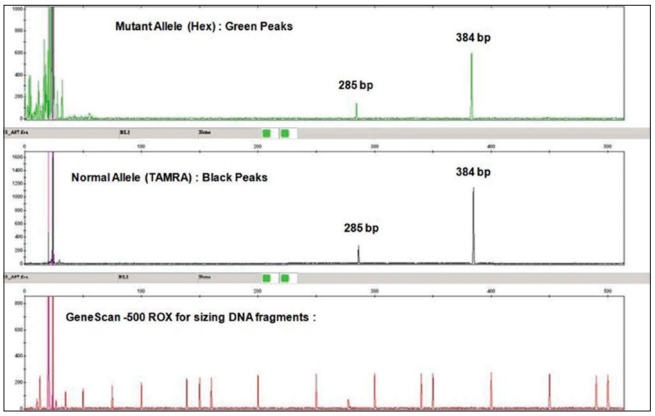

Figure 1: Fragment analysis for β-Thalassemia IVS 1-5(G-C) mutation in blastomere sample. Peaks for IVS1-5 (G-C) mutation and the normal allele were observed indicating β-thalassemia minor: (i) Presence of two Hex-labeled green peaks for the mutant allele at 285-bp and 384-bp size. (ii) Presence of two TAMRA-labeled black peaks for the normal allele at 285-bp and 384-bp size. GeneScan-500 Rox marker

of the β-thalassemia minor embryos. A singleton pregnancy resulted following transfer of three embryos. The PGD results were verified on PND.

DISCUSSION

This report documents the first successful pregnancy following PGD for β-thalassemia in India. Despite rapid progress in assisted reproduction technologies, India has failed to keep pace with developed counties in the area of PGD. In the present study, PGD enabled the β-thalassemia carrier couple to an informed decision for conception and full-term pregnancy of a carrier child following the birth of one child with β-thalassemia major and two terminated pregnancies as PND of β-thalassemia major was confirmed in the fetal sample.

PGD is not a technique for all. PGD is intended to enable at-risk couples to embark on pregnancies with unaffected fetuses. Although prenatal diagnostic techniques can diagnose genetic disorders when the patient is pregnant, however, in the case of untoward test results, the couple needs to undergo medical termination of pregnancy where the cost to the patient and the family is immeasurable. Additionally, in certain religious communities, medical termination of pregnancy is not acceptable, resulting in a β-thalassemia major child. The consequent child is subject to tremendous physical, medical, and social problems, and the family is subjected to mental anguish, emotional trauma, and economic problems.

Diagnosis in the case of PGD is carried out on a single cell, which may be associated with misdiagnosis due to allele dropout or contamination; it is strongly recommended to confirm the diagnostic results with PND. Misdiagnosis rates have been usually quoted as <1% for PCR-based tests.[11] It

is essential to avoid contamination from either the cumulus cells or extraneous sperm cells, which can be precluded by performing ICSI, which helps in avoiding both paternal contamination from sperm DNA as well as removal of cumulus cells prior to biopsy, which helps to avoid maternal contamination.[11]

For monogenic disorders, multiplex fluorescent PCR is perceived as the gold standard. In the current case, the multiplex PCR technique developed included few changes incorporated in the standard protocol to increase efficiency. These included two rounds of PCR, i.e., re-amplification of the first-round PCR product aliquots. In addition, mutant and normal primers were labeled with different color fluorescent dyes, followed with fragment analysis using the ABI 3100 nucleotide sequencer and the GeneMapper software. Further, an additional common 3′-primer representing a normal or mutation sequence was added to the reaction for confirmation of negative test results and absence of false-negative interpretations due to allele dropout. Also, the PCR products were purified to remove the excess of unbound fluorescent primer, thus reducing the possibility of non-specific peaks. The purified PCR products were concentrated, which enabled the formation of distinct clear peaks on fragment analysis, making the analysis and interpretation non-ambiguous.

With advances in assisted reproductive techniques as well as molecular biology techniques, PGD is a practical alternative for couples at risk of transmitting genetic aberrations to the offspring.

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