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Case Report

Allele Amplification Failure in the HBB Gene due to Allelic Dropout in a Pre-Preimplantation Genetic Testing (pre-PGT) Case

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Abstract

Beta-Thalassemia is one of the common autosomal recessive genetic conditions in the GCC region due to the increased rates of consanguinity. It is caused by reduced or absent synthesis of the globin chains of hemoglobin (Hb) [1,2]. β-Thalassemia is a condition caused by different types of mutations in the beta-globin gene (HBB) which is mapped to chromosome 11.

The clinical scenario of this hematological disease ranges from silent carriers to clinically manifested conditions needing continuous blood transfusions [3-4].

Keywords: *Beta-Thalassemia; Hemoglobin (Hb); Beta-Globin Gene (HBB)*

Introduction

Beta-Thalassemia is one of the common autosomal recessive genetic conditions in the GCC region due to the increased rates of consanguinity.

Case Presentation

The current case (Figure 1) represents a 30-year old gentleman who was informed at childhood to be a heterozygous carrier of *β*-thalassemia. At the clinic, peripheral blood was drawn for deoxyribonucleic acid (DNA) sequencing. Although the patient indicated that he was a carrier, sequencing results surprisingly showed him to be affected with the codon 39 mutation (C>T) which does not correspond at the genotype phenotype level indicating the possibility of an allele dropout.

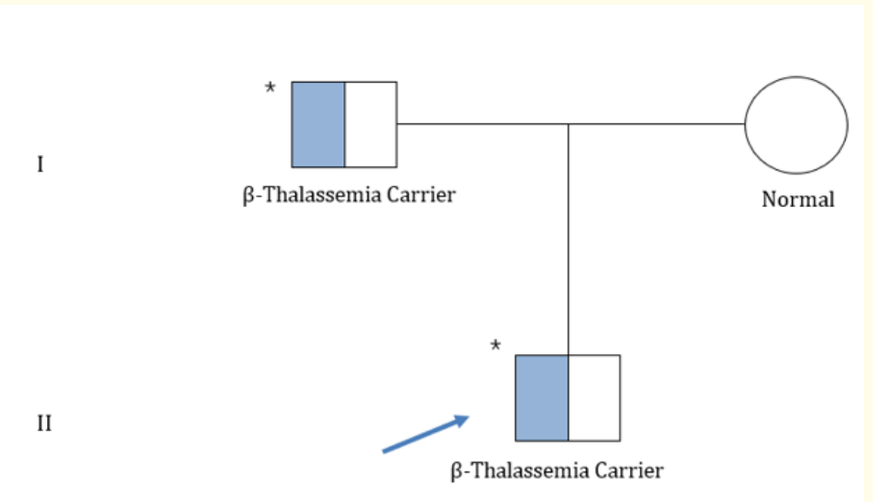

Figure 1: *Pedigree of the family studied as informed by the proband (blue arrow). *: Genetic Testing Performed.*

Further analysis was carried out using two different sets of designed primers to confirm the carrier status. This showed that the patient is indeed a carrier of *β*-thalassemia at codon 39 of the HBB gene.

Materials and Methods

Samples of venous blood were collected in two evacuated tubes containing K2 EDTA (Vacutainer®; BD, Franklin Lakes, NJ, USA), which were later on used for deoxyribonucleic acid (DNA) isolation. The genomic DNA was isolated from peripheral blood using the Qiagen QIAamp DNA Mini Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). The purified genomic DNA concentrations were measured in spectrophotometer (Invitrogen™ Qubit™ 3.0 Fluorometer). The samples were diluted to 20 ng/µl and stored at -20°C until analyses.

The samples were then amplified using the polymerase chain reaction (PCR) in a Veriti Thermal Cycle (Applied Biosystems.

PCR reactions were performed in 500 µl microtubes in which were added 2 µl of 10x PCR buffer (no $MgCl_2$), 1 µl of 25 nM $MgCl_2$, 1.5 µl of 2.5 mM deoxyribonucleotide triphosphate solution mix (dNTPs), 1 µl each of forward and reverse primers (0.5 pmol/µl), 8.5 µl of ultrapure water, 0.2 µl of Taq DNA Polymerase enzyme (FastStart™ Taq DNA Polymerase (5 U/µl)) and 5 µl of Sample DNA (20 ng/µl) to make a final reaction volume of 20.2 µl.

The tubes were placed in the thermal cycler under the following conditions: 10 minutes at 94°C for pre-denaturation; 35 cycles of 48 seconds at 95°C for denaturation, 48 seconds at 55°C for the annealing of primers, 48 seconds at 72°C for extension; 7 minutes at 72°C for one last extension cycle.

PCR mutations were genotyped, using in-house designed primer sets targeting the *β*-thalassemia codon 39 mutation region (Figure 2). Normal control with DNA, as well as blank solutions containing all reagent except DNA were analyzed concurrently with samples in order to avoid false-positives or false-negatives.

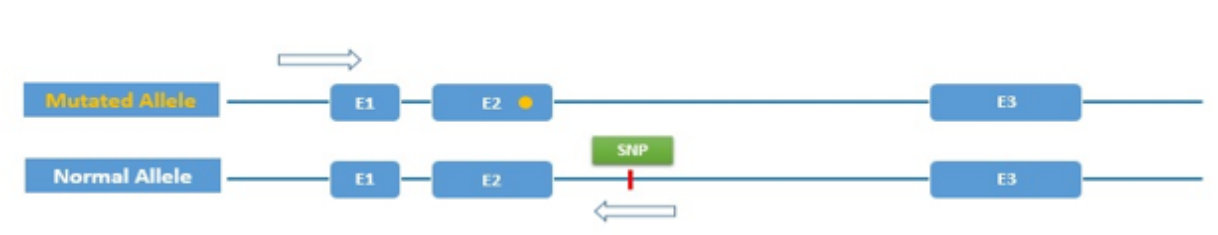

Figure 2: *Location of primers designed covering exon 2 of the HBB gene. SNP was detected in the region of the reverse primer.*

DNA amplification products were analysed in a 1% agarose gel, using 2 µl of PCR product with 5 µl buffer (SYBR green + loading dye) ran for 30 minutes at 100 volts and viewed on UV Trans illuminator (Biorad XR+ Gel documentation system).

The amplified PCR products were purified using the Qiagen PCR purification kit (QIAquick PCR Purification Kit) and diluted with 20 - 50 µl of deionized H_2O based on the concentration of the amplified PCR products viewed on UV Trans illuminator.

Sequencing reaction was then performed using the Big Dye Terminator V3.1 in 200 ul microtubes where 9.6 µl of the master mix (Big Dye) and 2.4 µl of 5 µM sequencing primer (Forward or Reverse) in each tube with 3 µl of diluted purified PCR products.

This mix in the tubes were placed in the thermal cycler under the following conditions: initial denaturation at 94°C for 10 minutes, 35 cycles, denaturation at 95°C for 10 seconds, annealed at 50°C for 5 seconds, extension at 60°C for 4 minutes and held at 4°C.

The sequencing products were the purified using the ethanol precipitation method with the DyeEx kit (Qiagen). Then denatured and dehydrated in the Vacufuge for 30 minutes. After drying in the vacufuge, 20 µl of Hi-Di Formamide were added to each tube then vortexed briefly, quick spun and transferred to the 96 well plate for denaturation. The denatured products were used for the capillary electrophoresis using the ABI 3130XL Genetic Analyzer (Applied Biosystems)

Final analysis (Figure 3) of the sequencing data was performed with the AB Genetic Analyzer Software (Sequencing Analysis Software v5.3.1).

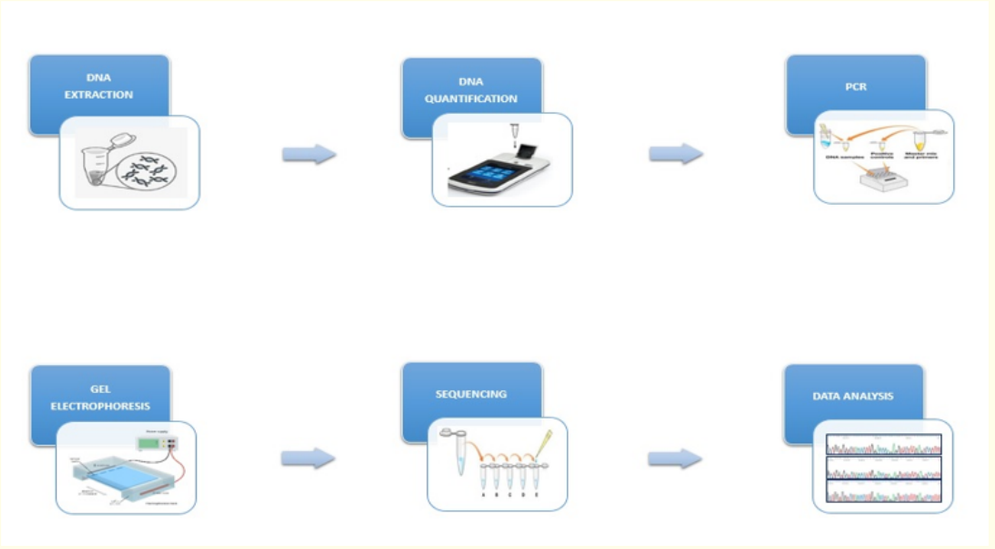

Figure 3: *Workflow from DNA extraction to data analysis.*

Results and Discussion

Although the patient indicated that he was a carrier and that he inherited the mutation from his father's side of the family, sequencing results (Figure 4) surprisingly showed him to be affected with the codon 39 (CD39) mutation (CAG>TAG). Which does not correspond at the genotype phenotype level.

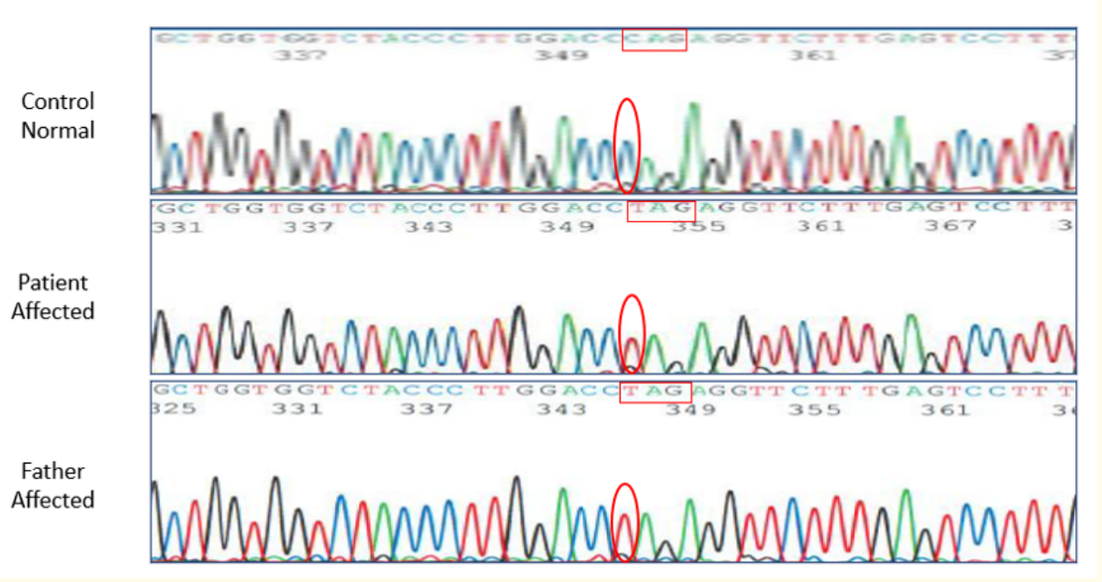

Figure 4: *Chromatogram showing the sequencing results.*
Top: Normal control.
Middle: Affected patient.
Bottom: Affected father.

Similar results were seen when his father's DNA was sequenced which raised the possibility of a case of an allele dropout due to the presence of a single-nucleotide polymorphism (SNP) in the primer annealing region.

The SNP location in the HBB gene was determined and new sets of primers (close to the mutation region yet away from the SNP) were designed. Sequencing results (Figure 5) clearly showed that both the patient and his father are indeed carriers of the codon 39 mutation and not affected (Figure 6).

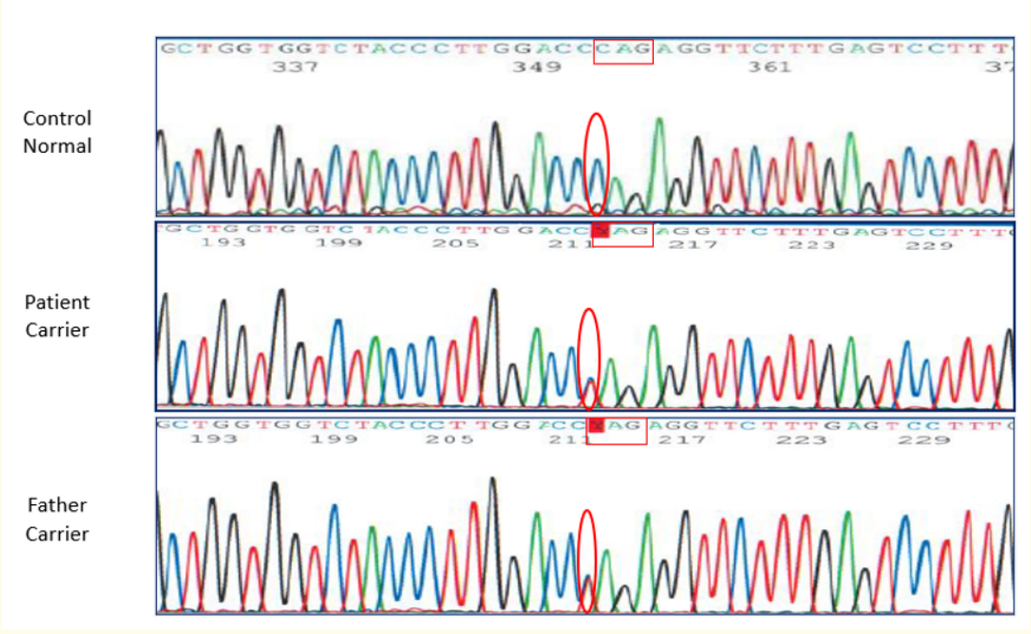

Figure 5: *Chromatogram showing the sequencing results suing the new set of primers.*
Top: Normal control.
Middle: Heterozygous patient.
Bottom: Heterozygous father.

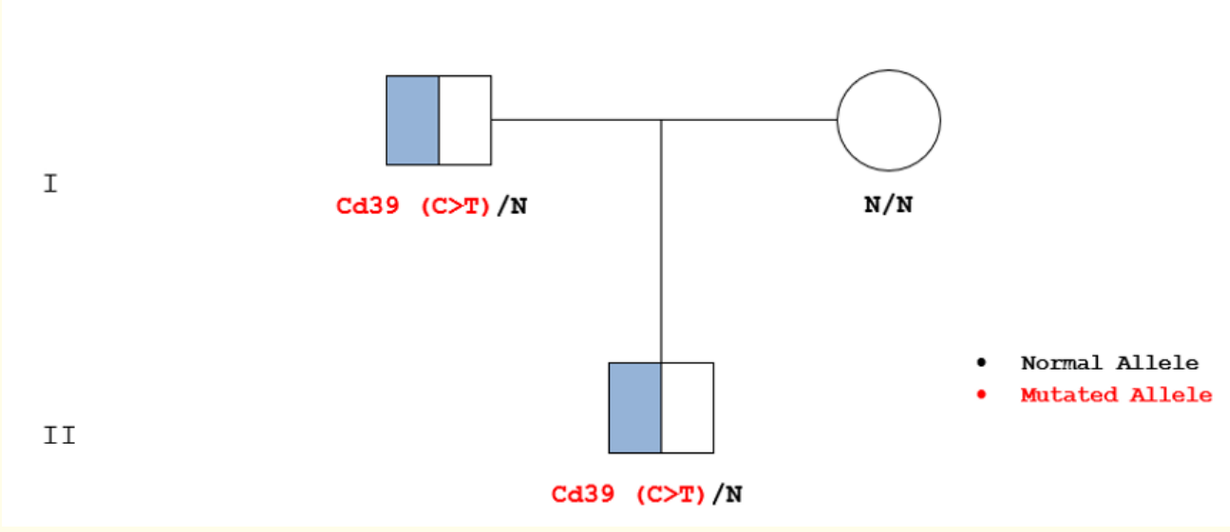

Figure 6: *Pedigree of the family after confirming the mutation in the HBB gene. Both patient and his father are carriers of the codon 39 mutation.*

Conclusion

The presence of a SNP in primer-annealing sequence interferes in the analysis of gene mutations leading to allele dropout. To overcome and minimize the incidence of an allele dropout event in the pre-PGT setting and during PGT-M, it is advisable to detect every mutation using at least two different sets of primers.

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