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New Perspectives in Prenatal Diagnosis of Sickle Cell Anemia

Ebru Dündar Yenilmez and Abdullah Tuli

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Abstract

Hemoglobin disorders such as thalassemias and sickle cell anemias can be avoided by detecting carriers, ensuring genetic counseling and prenatal diagnosis. Nowadays Chorionic villus sampling (CVS amniocentesis, and cordocentesis are still the most widely used invasive sampling methods for prenatal diagnosis of the fetus. These traditional methods are associated with a risk of fetal loss. The revelation of cell-free fetal DNA (cffDNA) in maternal plasma and serum provides the opportunity of noninvasive prenatal diagnosis (NIPD). Different encouraging clinical applications have arose such as noninvasive identification of fetal sexing, fetal Rhesus D, and the determination of the paternal alleles in maternal plasma. The determination of the presence or absence of paternally inherited alleles in maternal plasma of sickle cell disease (SCD) and β-thalassemia would allow the diagnosis of autosomal dominant diseases or the exclusion of autosomal recessive diseases of the fetuses, respectively. prenatal diagnosis of genetic diseases. Analysis of cffDNA in maternal plasma for NIPD has the advantage of being safer versus the invasive methods. Different technologies were used since the discovery of cffDNA for NIPD—especially high-resolution melting (HRM) analysis is one of those methods. Genotyping can be done with HRM without using labeled probes and more complex regions can be analyzed with unlabeled hybridization probes. High-resolution melting is a rapid and useful method to detect paternal alleles for the NIPD of SCD and thalassemias when the fetus has a risk for double heterozygote.

Keywords: noninvasive prenatal diagnosis, sickle cell disease, cell-free fetal DNA, high-resolution melting, paternal mutation, maternal plasma

1. Introduction

Hemoglobinopathies caused by mutations in the α- or β-like globin gene clusters are the most common inherited disorders in humans, with around 7% of the world population being carriers of a globin gene mutation [1].

Hemoglobinopathies are caused by variants that affect the direct synthesis of the globin chains of hemoglobin, and may result in different synthesis (thalassemia syndromes, etc.) or structural changes (sickling of the red blood cells, hemolytic anemia). Thalassemia variants and various abnormal hemoglobins interact to produce a wide variety of disorders. Sickle cell disease was first described in 1910, and in the following years, similar cases were described, supporting the idea that this was a new disease and providing enough evidence for a preliminary clinical and pathological description [2]. Linus Pauling was the first to hypothesize in 1945 that the disease might originate from an abnormality in the hemoglobin molecule [3]. The sickle mutation was characterized several years later by Ingram et al. as a glutamine to valine substitution at the sixth residue of the β-globin polypeptide [4].

Sickle cell disease causes a very destructive condition and is an autosomal recessive-inherited hemoglobinopathy. The disease affects millions of people which results in serious complications due to vaso-occlusive phenomenon and hemolysis [5].

Prevention of the disease through carrier identification, genetic counseling, and prenatal diagnosis (PD) remains the only realistic approach to diminish the impact of the disease and allows better use of available resources for the existing patient populations [6–8]. In addition, for monogenic diseases the parental mutation(s) have to be characterized before analysis of the fetal sample [9].

Polymerase chain reaction (PCR) is commonly in use as a traditional molecular method for prenatal diagnosis of hemoglobinopathies. The PCR-based technologies differ in genotyping hemoglobin variants. Amplification refractory mutation system (ARMS), denaturing gradient gel electrophoresis (DGGE), restriction endonuclease PCR (RE-PCR), sequencing analysis (Sanger), microarrays, pyrosequencing, real-time PCR, and high-resolution melting analysis (HRM) can be counted among these PCR-based detecting methods [10].

2. Prenatal diagnosis in sickle cell disease

The prenatal diagnosis (PD) for the disease gives the opportunity for expectant couples to have an accurate, rapid result about the genotype of their fetus. This process offers an option for the parents to terminate the pregnancy at an early period in case of positive result and to prepare them psychologically and medically for the arrival of the new child when abortion is not an option. This practice is usually carried out using either chorionic villus sampling (CVS) or amniocentesis. Both procedures are invasive with CVS being done between the 10th and 12th week of pregnancy while amniocentesis is usually carried out later (between the 14th and 20th week) [5, 11].

Four main categories have been identified for severe disease states, for which genetic counseling, and possibly prenatal diagnosis, is indicated. The category for some SCD is shown in **Table 1** [10, 12].

Genotype interaction	Disorder expected	Appropriate to offer PND
Homozygous		
Hb S	Sickle cell disease	Yes
Compound heterozygous		
Hb S/β°- or severe β⁺-thalassemia	Sickle cell disease	Yes
Hb S/mild β⁺-thalassemia	Mild sickle cell disease	Occasionally*
Hb S/Hb C	Sickle cell disease (variable severity)	Yes
Hb S/Hb D-Punjab	Sickle cell disease	Yes
Hb S/Hbs I-Toulouse, Shelby, Hope, North Shore	Hemolytic anemia	No
Hb S/Hb E	Mild to severe sickle cell disease	Occasionally*

Note: The decision to have prenatal diagnosis belongs to the couple, once they have had comprehensive counseling.
*Couples with genotypes that may lead to offspring with unpredictable phenotypes occasionally select to have prenatal diagnosis or PGD.

Table 1. Sickle cell disorders—interactions and indications for prenatal diagnosis and preimplantation genetic diagnosis (PND) [10].

These conventional methods for sampling fetal genetic material are invasive and associated with a risk for fetal miscarriage [13] (**Table 2**).

Sample	Fetal risk	Analysis	Current status
Fetal blood	1–2%	Hb separation	Disused
Amniotic cells	>0.5%	DNA	Clinically available
Trophoblastic cells	>0.5%	DNA	Clinically available
Fetal Cell/DNA in maternal circulation	0%	DNA	Investigational

Table 2. The sampling methods used in prenatal diagnosis for hemoglobinopathies at past and present [6].

2.1. Invasive methods

The main testing procedures include amniocentesis and CVS, together with ultrasonography, ultrasound, serum markers, and genetic screening. Amniocentesis and CVS continue to be the gold standards for prenatal diagnosis of genetic disorders. Though these procedures are minimally invasive and cause some risk to the mother and fetus, they are routinely and safely conducted. Each of these procedures must be applied during a specific time period to achieve accurate results, and the test sensitivity of these tests is limited. Chorionic villus sampling is

not applicable before 9 weeks of gestation. On the other hand, for amniocentesis the correct time interval is proposed to 15 and 20 weeks of gestation. Both these procedures were associated with a risk of fetal miscarriage of <1.0%. Fetal sexing cannot be determined in the early first trimester using ultrasonography. Thus, the noninvasive applications that clarify fetal sexing, fetal Rhesus D, single gene disorders, and chromosome abnormalities at the early stage of first trimester were a fascinating development [14].

2.2. Noninvasive methods

The well-known presence of fetal cells and free fetal nucleic acids (including DNA and RNA) in the maternal circulation has accelerated the research area toward developing new methods for noninvasive prenatal diagnosis (NIPD), applicable to the exclusion of both single gene and chromosome disorders [15]. This is in contrast to cell-free fetal DNA (cffDNA) where only paternally inherited alleles that differ from those carried by the mother can be distinguished with most current methods [12, 15].

2.2.1. Circulating fetal cells in maternal plasma

Fetal cells represent the ideal source of fetal genetic material for NIPD, since they offer the potential of achieving a "full" genetic analysis. Among fetal cell categories found in the circulation are trophoblasts, fetal leukocytes, and fetal nucleated erythroblasts (nucleated red blood cells (NRBCs)). Fetal nucleated cells that are present in the maternal circulation have been explored as a source of fetal genetic materials for NIPD [16, 17]. Typically, fetal cells exist at a concentration of several cells per milliliter of maternal blood. The rarity of circulating fetal cells has prevented their robust detection, thus hampering the general use of this approach. Due to their limitations, an alternative form of fetal genetic materials for diagnostic test development would be needed [18].

2.2.2. Cell-free fetal DNA in maternal plasma

The discovery of cell-free fetal DNA (cffDNA) in the maternal blood circulation has offered new possibilities for NIPD [19]. Many fascinating clinical experiments such as noninvasive detection of fetal sexing and fetal Rhesus D status have been developed which is the bases for the detection of paternal alleles in maternal plasma [20, 21]. Detecting the presence or absence of paternally inherited alleles in maternal plasma in inherited diseases such as β-thalassemia and SCD would allow the diagnosis or the exclusion of those diseases in the fetus, respectively [22–24].

3. Genotyping applications with high-resolution melting

High-resolution melting (HRM) is a novel, closed-tube, post-PCR technique allowing genomic researchers to easily analyze genetic variations in PCR amplicons. This method was introduced in 2002 as a simplest approach for genotyping and mutation scanning. After PCR amplification,

melting curves are generated by monitoring the fluorescence of a saturating dye that does not inhibit PCR [25].

This technique enables researchers to rapidly and efficiently discover genetic variations (e.g., single nucleotide polymorphisms (SNPs), mutations, methylations). In HRM experiments, the target sequence is amplified by PCR in the presence of a saturating fluorescent dye (e.g., LightCycler® 480 ResoLight Dye). Dyes that stain double-stranded DNA are commonly used to identify products by their melting temperature (T_m). Alternatively, hybridization probes allow genotyping by melting of product/probe duplexes [26].

High-resolution DNA melting analysis with saturation dyes for either mutation detection of PCR products or genotyping with unlabeled probes, PCR product scanning, and probe genotyping in the same reaction has been reported [27]. Modern HRM is facilitated by novel saturation dyes and high-resolution instruments. Asymmetric cyanine dyes such as SYBR Green I and LCGreen are dyes of choice in fluorescence melting analysis [26].
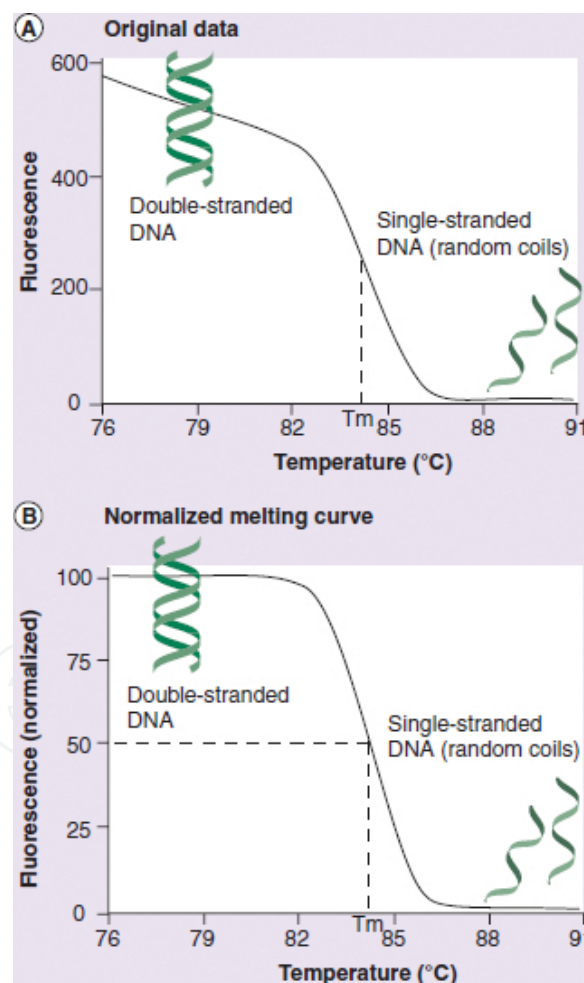

Figure 1. (A) The linear decrease of fluorescence at low temperature and a rapid decrease at melting temperature (T_m). (B) The normalized data (between 0 and 100%) shown after the background subtraction and the curve is seen horizontal outside of the transition period [26].

High-resolution melting analysis requires only the usual unlabeled primers and a generic double-stranded DNA dye added before PCR for amplicon genotyping, and is a promising method for mutation screening.

The HRM analysis of the related sequence (amplicon) has a unique DNA melting temperature in the presence of saturating DNA-binding dyes. The melting behavior depends on the base content (primarily the GC bases) and the length of the sequence when the temperature of the solution is increased. The graph of the fluorescence signal against the temperature plotted as the intensity decreases and the double-stranded DNA becomes single stranded as the dye is released (**Figure 1**). To estimate the T_m at which 50% of the DNA is in the double-stranded state, the derivative of the curve can be considered. The difference or the derivative plot and the melting curve may be used for analysis of the sequence (**Figure 2**) [28].
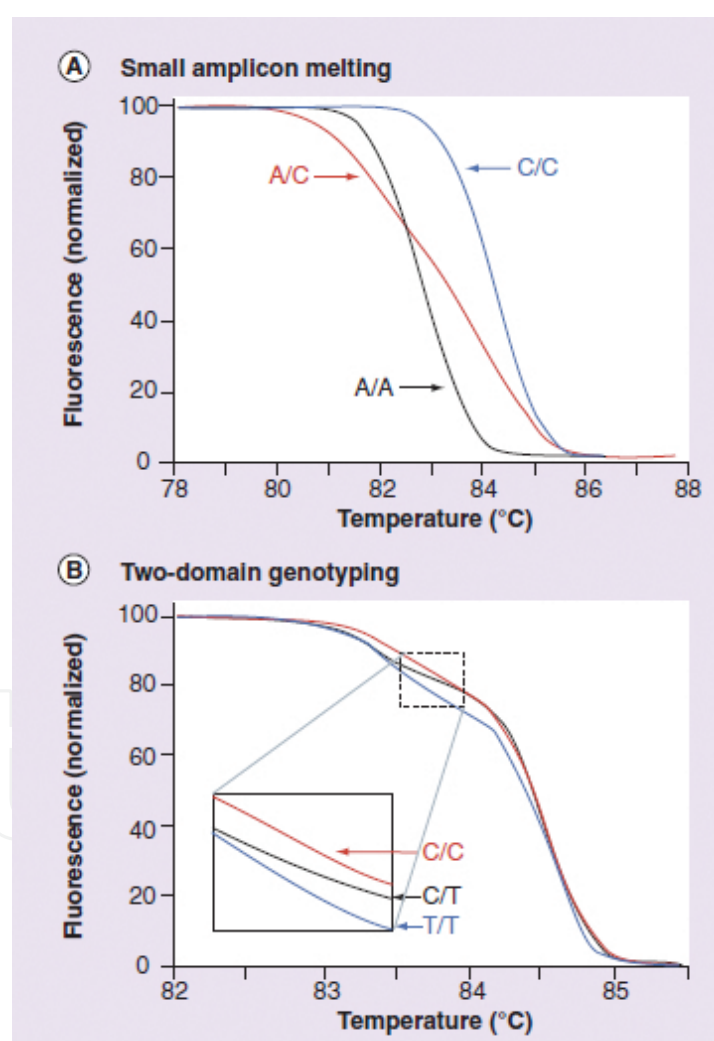
Figure 2. (A) Melting of a small amplicon (B) A large amplicon melting that melts in two-domains.

High-resolution method has been successfully applied in many studies for NIPD [29, 30]. Specific primers that are used in the assay can detect the mutations when compared to

hybridization or restriction enzyme-based methods [31, 32]. Recently, it has also been used in the detection of α- and β-thalassemia variants [32, 33].

4. Materials

4.1. Equipment

The method described in this chapter was performed with a LightCycler LC 480 instrument and version 1.5 (Roche Diagnostics, Basel, Switzerland). The other important equipment includes the following:

- LightCycler 480 Multiwell Plate 96 (Roche Diagnostics 4729692001), in which the real-time PCR reactions are run in LightCycler instrument.

- LightCycler 480 Sealing Foil—50 foils (Roche Diagnostics 04729757001)—is used to cover the multiwell plates.

- MagNa pure compact instrument (Roche Diagnostics, Basel, Switzerland), for the extraction of couples genomic DNA, plasma cffDNA, and chorion villus DNA of fetus.

- A bench centrifuge, to separate the maternal plasma and to centrifuge the multiwell plates before real-time PCR.

4.2. Reagents

The reagents used are as follows:

- MagNA Pure LC DNA Extraction Kit—Large Volume (Roche).

- Hemoglobin S/C ToolSet™ for LightCycler™ (Roche Diagnostics) (β-globin, sickle cells, Hb S, Hb C) (see **Table 2**).

- LightCycler master hybridization probes (Roche).

- LightCycler HRM master mix ×2 (Roche).

- Primers for HRM analysis of beta-globin gene mutations (see **Table 4**).

4.3. Storage of the PCR reagents

Storage of the PCR reagents is as follows:

- The PCR primers to be used on the LightCycler are diluted from 100-μM stock solutions. The diluted primers are divided into aliquots and stored at −20°C. The stock solutions are diluted to 10 μM for preparing primer working solutions. These working solutions can be stored at 4°C for up to 3 months.

- LightCycler master hybridization probes and HRM master mix are stored in aliquots of small volume (e.g., 20 μL) at −20°C (when thawed, an aliquot does not refreeze; it can be used up to 1 month at 4°C.

5. Methods

5.1. Cell-free fetal DNA extraction

Maternal blood (10 mL) and peripheral blood (5 mL) samples from parents of each fetus were collected in ethylenediaminetetraacetic acid (EDTA) tubes. Two steps were used during centrifugation (1600 g for 10 min and 16,000 g for 10 min) for separating the plasma from maternal blood within 1 h. The plasma samples were stored at −20°C for the next step [33]. The plasma samples were taken before chorionic villus sampling. Magna Pure Large Volume Isolation Kit (Roche Diagnostics, Basel, Switzerland) according to the total nucleic acid plasma extraction protocol of the MagNa Pure Compact instrument is used for DNA extraction. Extracted DNA was eluted in elution buffer (50 μL) and stored at −80°C. Whole blood (500 μL) of each parent's DNA was extracted by the same method.
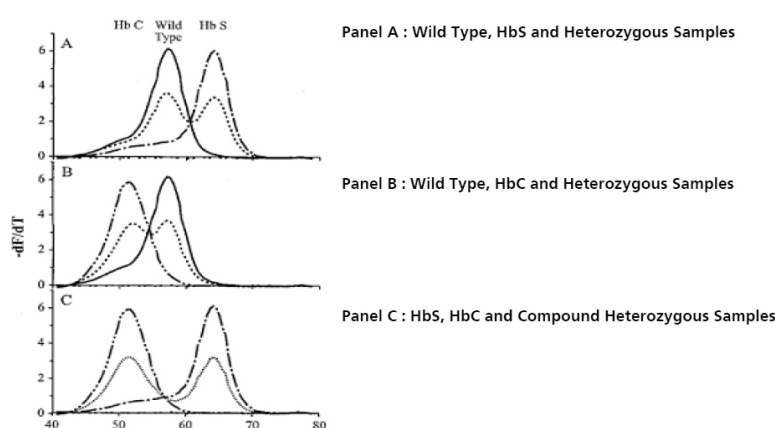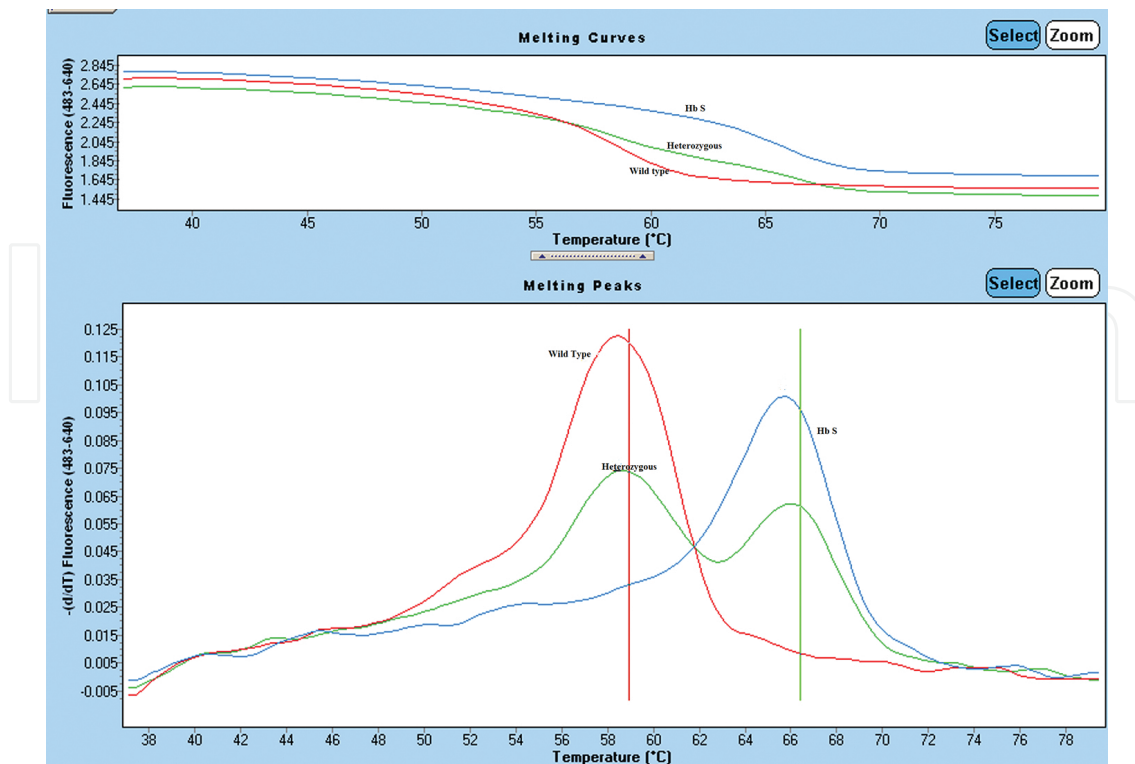

Figure 3. Melting curve analysis of Hb S/Hb C locus in β-globin gene.

5.2. Real-time PCR and melting curve analysis

5.2.1. Genotyping Hb S mutation using Hb S/C Toolset

Genotyping Hb S mutation using Hb S/C Toolset includes the following:

1. The Hb S/C genotyping in cffDNA was detected by real-time PCR and HRM analyses in the same run in a LightCycler 480 (Roche Applied Science) instrument.

2. The hemoglobin S/C kit (Ratiogen) for the LightCycler™ (Neftenbach, Switzerland) was used to examine the human β-globin gene for the presence of Hb S/C variant using LightCycler PCR with melting curve analysis (**Figures 3** and **4**).

3. The primer pair and fluorescent detection/anchor probes were optimized to specifically amplify a 214-bp segment of exon 1 of the human β-globin gene. In a final volume of 20 μL, the reaction mixture included 9.6μL of Hb S/C Solvent, 2.8μL of HbS/C Oligo Tool, 25mM 1.6 μL MgCl2, and 2 μL Master Hybridization probe (10×) (**Table 3**).

Figure 4. Genotyping of Hb S. Melting curve analysis of Hb S genotypes of the Hb S locus in the beta-globin gene. (T_m values: wild type 56°C; Hb S: 63°C; Hb C: 50°C (not shown). Note: The values for the respective melting temperatures may vary for ±2.5°C).

Reagent	Volume (µL)
OligoTool Hb S/C	2.8
Solvent Hb S/C	9.6
$MgCl_2$, 25 mM	1.6 (final 3mM)
Master hybridization probes 10×	2
Total reaction mix	16
DNA or control Hb S heterozygous	4
Total	20

Table 3. Reaction mix preparation.

5.2.1.1. PCR protocol

- The PCR protocol consisted of an initial denaturation step at 95°C for 1 min, followed by 35 cycles of 1 s at 95°C, 30 s at 63°C, and 1 s at 72°C.

- After the amplification step, the LightCycler is programmed for the melting step; 95°C for 60 s, 35°C for 20 s, and melting at 80°C with continuous fluorescence reading at 25 acquisitions per 1°C.

- Control samples (negative and positive) were suggested to use for each run.

5.2.2. High-resolution melting design and gene scanning for Hb S/beta thalassemia

- Four overlapping DNA fragments were synthesized to cover the regions of interest in the β-globin gene. The oligonucleotide primers are shown in **Table 4**.

- Perform PCR amplifications in a total volume of 20 μL, use high-resolution melting master ×2 (Roche Diagnostics) and 5 μL of DNA from the plasma samples.

- For the β-globin gene mutation assay, we use 300-nM primers and 2.5 mM $MgCl_2$. Control samples with known β-globin gene mutations and the wild type should be included in each assay.

Location	Amplicon length (bp)	Primer sequences	
		Forward primer (5′–3′)	Reverse primer (5′–3′)
P1 (promoter-exon 1)	351	F1-CAATTTGTACTGATGGTATGG	R1-CTTCATCCACGTTCACCTTGC
P2 (5′ UTR-Exon 2)	425	F2-CACTAGCAACCTCAAACAGAC	R2-CACTCAGTGTGGCAAAGGTG
P3 (Exon 2-IVS 2)	318	F3-TTTGAGTCCTTTGGGGATCTG	R3-CCACACTGATGCAATCATTCG
P4 (IVS 2-3′ UTR)	354	F4-GTTAAGGCAATAGCAATATTTCT	R4-TGGACAGCAAGAAAGCGAGC

Table 4. Primers for HRM analysis of beta-globin gene mutations.

5.2.2.1. PCR protocol

1. The PCR program requires SYBR Green I (533 nm) and it consists of an initial denaturation-activation step at 95°C for 10 min followed by 45 cycles of 3 s at 95°C, 5 s at 58°C, and 20 s at 72°C.

2. The melting step at 95°C for 60 s, 35°C for 20 s, and the melting at 80°C with continuous fluorescence reading at 25 acquisitions per 1°C.

3. Gene Scanning software was used to perform the melting curve analysis in three steps: normalization, shifting of the temperature axis of the normalized melting curves, and analysis of the difference plot of the difference between the melting curve shapes derived by subtracting the wild type and mutant DNA curves.

4. The difference plots cluster the samples into groups. High-resolution melting results were confirmed by sequencing in samples that had not been identified using conventional PCR.

5.2.3. High-resolution melting analysis

- The HRM technique was standardized by analyzing genomic DNA from Hb S and β-thalassemia heterozygous parents with known mutations. A melting curve program is used to detect the Hb S mutation in the samples. The melting temperature (T_m) was 63°C for the S allele and 56°C for the A allele.

- The related primer sets P1-P4 (**Table 4**) were used to analyze the β-thalassemia mutations by HRM analysis. Heterozygous and homozygous samples were included in each PCR run as a positive control to check the mutation.

- The contamination was monitored using a DNA-free blank. In all steps in the assay, for each mutation, the wild-type samples were separated from the heterozygous or homozygous samples by the melting curves, as expected (**Figure 4**).

- The majority of mutations seen in our Mediterranean population can be detected using P1, P2, and P3 set primers. Paternal alleles have been detected in cffDNA using primer set P1 in exon 1 and promoter region (ex. -30 (T>A), -101 (C>T), CAP+1 (A>C)_ of beta-globin gene. The mutations of 5′ UTR-exon 2 region (ex. HbS (A>T), IVSI-1 (G>A), IVSI-5 (G>A), IVSI-6 (T>C), IVSI-110 (G>A), Cd8 (-AA), Cd9/10 (+T), Cd15 (G>A), Cd39 (C>T)) were detected with primer set P2. The most detected mutations in exon 2-IVS 2 region were IVSII-1 (G>A), IVSII-745 (C>G), and IVSII-848 (C>A) using primer set P3 [33].

6. Conclusion

The invention of cffDNA in maternal plasma provided new opportunities for NIPD during pregnancy [19]. Many fascinating clinical studies such as fetal sexing and fetal Rhesus D genotyping have been developed according to the detection of the paternal alleles, which differ from the mother in maternal plasma [34]. The parents with different carrier status have a chance of 50% for having a sick (thalassemia or SCD) fetus, if the paternal allele is detected in maternal plasma. If the mutation of the father is not detected in maternal plasma, there is no need to perform invasive prenatal processes. High-resolution melting analysis can be applicable to find paternal mutations in cffDNA that differs from the mother. The double-heterozygote-affected fetuses can be diagnosed using HRM analysis. The results should be confirmed by invasive methods. The maternal background could affect the results in cffDNA when the gestational age is under <7 weeks. The low levels of cffDNA may be the reason at this point.

Determination of the paternal alleles in cffDNA avoids the risk for a double heterozygous fetus. High-resolution melting method is easy to practice when compared to other complicated methods. This method is useful for NIPD of hemoglobinopathies and does not require any modification of PCR protocols. The couples that carry the same mutation, genotype determination of the cffDNA in maternal plasma is difficult but not impossible. Specific SNPs might be used instead of mutations for the best accuracy of the HRM method. In conclusion to compare with invasive methods, HRM has the lowest risk for PCR contamination because of being a closed-tube method. Small amounts of fetal DNA in maternal plasma can be detected and analyzed for mutations of single-gene disorders such as hemoglobinopathies in the early stage of pregnancy. The HRM method is applicable for other genetic disorders to detect the known mutations in cffDNA from maternal plasma.

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