

PCR-DHPLC for Non-Invasive Prenatal Evaluation of the IVS-II-1 (G>A) Mutation in Fetal DNA of β -Thalassemia Major: A Pilot Diagnostic Performance Study

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Abstract- β -thalassemia is one of the most common autosomal recessive single-gene disorders, particularly in high-prevalence regions. This study aimed to explore the feasibility and diagnostic performance of polymerase chain reaction-denaturing high-performance liquid chromatography (PCR-DHPLC) for non-invasive prenatal detection of the IVS-II-1 (G>A) mutation in fetal DNA. Fifty pregnant women at risk of carrying a fetus with β -thalassemia major were enrolled. Cell-free DNA was extracted from maternal plasma and analyzed using high-resolution melting (HRM) real-time PCR, followed by PCR amplification. Amplicons were assessed by electrophoresis, purified using a PCR purification kit, and subsequently analyzed by DHPLC under predefined buffer and temperature conditions to detect the IVS-II-1 (G>A) mutation. DHPLC analysis identified 13 true positive, 11 true negative, 19 false negative, and 7 false positive cases compared with the invasive reference method. The calculated sensitivity and specificity were 40.6% and 61.1%, respectively, indicating limited diagnostic performance in the current setting. PCR-DHPLC does not provide sufficient sensitivity and specificity for reliable non-invasive prenatal detection of the IVS-II-1 (G>A) mutation in β -thalassemia major. Further methodological optimization and extended mutation panels in larger cohorts are necessary to assess its potential for clinical validation.

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Introduction

Thalassemia encompasses a group of inherited disorders resulting from deficiencies in α and β globin chain synthesis, leading to impaired hemoglobin production (1). Caused by various mutations including deletions, insertions, and point mutations in the corresponding globin genes, the clinical spectrum of the disease is wide, ranging from asymptomatic cases to severe anemia, splenomegaly, and fatal outcomes (1-6). Beta-thalassemia is the most prevalent autosomal recessive single-gene disorder globally, resulting from mutations in the β -globin gene and showing marked molecular heterogeneity with more than 200 identified causative variants to date (7-10). Iran represents a high-prevalence region for β -thalassemia, with approximately 25,000 affected patients and 3 million carriers, imposing substantial health, economic, and social burdens (11-13). The distribution of β -globin mutations varies worldwide; however, in the Iranian population, five mutations IVS-I-5(G>C), IVS-II-1(G>A), FSC36/37(-T), IVS-I-110(G>A), and FSC8-9(+G) account for nearly 70% of all cases (14-17). Among these, the IVS-II-1(G>A) mutation is particularly noteworthy due to its high prevalence across most regions of the country, making it a highly informative target for molecular diagnosis and prenatal risk assessment programs (7,14-17). Traditionally, this has relied on invasive procedures such as chorionic villus sampling and amniocentesis. Although accurate, these methods carry procedure-related risks including pregnancy loss, bleeding, infection, and significant maternal anxiety (18-20). The discovery of cell-free fetal DNA (cffDNA) in maternal plasma has opened avenues for non-invasive prenatal diagnosis (NIPD). While advanced techniques like digital PCR and next-generation sequencing (NGS) offer high accuracy, their cost, technical complexity, and infrastructure demands limit widespread implementation in resource-limited settings (21,22). This technological gap underscores the need for accessible NIPD alternatives suitable for high-burden regions. Denaturing high-performance liquid chromatography (DHPLC), a mutation-scanning technique that separates DNA heteroduplexes under partially denaturing conditions, presents a compelling option. Its advantages including relative affordability, semi-automated operation, and high-throughput capacity make it a practical platform for analyzing cffDNA in maternal plasma, particularly where advanced sequencing is not feasible. Given the high prevalence of the IVS-II-1 (G>A) mutation in Iran and

the need for implementable NIPD methods, this pilot study aimed to assess the diagnostic performance and feasibility of PCR-DHPLC analysis of maternal plasma for the non-invasive prenatal detection of this mutation in β -thalassemia carrier couples.

Materials and Methods

Study population and design

This study aimed to evaluate the diagnostic utility of PCR-DHPLC for prenatal and non-invasive detection of the IVS-II-1 (G>A) mutation in β -thalassemia. The present work was designed as a diagnostic accuracy pilot study building on our previous HRM-based non-invasive prenatal diagnosis study conducted in the same center and on the same clinical cohort. In that earlier study, 50 at-risk β -thalassemia minor couples (50 pregnancies) were enrolled for HRM analysis of fetal IVS-II-1 (G>A) in maternal plasma, and fetal genotypes were determined by invasive prenatal diagnosis using reverse dot blot (RDB) as the gold standard. In the current DHPLC study, we used the same cohort and the same reference results; therefore, a total of 50 at-risk pregnancies with available archived maternal plasma were included (23).

Inclusion criteria were: (1) maternal β -thalassemia minor with various β -globin mutations, (2) paternal carrier status for the IVS-II-1 (G>A) mutation, (3) singleton pregnancy, and (4) gestational age between 10 and 12 weeks at the time of sampling. Venous blood (10 ml) was collected from each pregnant woman into EDTA tubes, processed to obtain plasma, and stored at -80°C until analysis. Fetal β -globin genotypes for all at-risk pregnancies were determined by chorionic villus sampling and RDB analysis, which served as the reference (gold standard) method for classification of DHPLC results.

HRM-DHPLC workflow

HRM real-time PCR was used as the initial amplification and screening step to generate specific PCR products encompassing the IVS-II-1 (G>A) site and to verify assay performance with three standard genomic DNA controls (normal, heterozygous IVS-II-1, homozygous IVS-II-1), following the protocol previously validated by our group (23). The resulting 186 bp amplicons were then analyzed by DHPLC for mutation detection in maternal plasma samples.

DNA extraction

After thawing plasma at room temperature, 200 μ l of

maternal plasma was transferred to a microtube. Cell-free DNA was extracted using the QIAamp DNA Mini and Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instructions, identical to the procedure in our previous HRM study. DNA concentration and purity were assessed by NanoDrop spectrophotometry, and the presence of amplifiable DNA was confirmed by agarose gel electrophoresis of a control PCR product.

HRM real-time PCR

Primers targeting the β -globin gene region containing the IVS-II-1 (G>A) mutation were designed based on the NCBI reference sequence NG-000007.3 (HBB). The forward primer was 5'-TCACCTGGACAACCTCAAG-3' and the reverse primer was 5'-CCCATTCTAAACTGTACCC-3', identical to our previously published HRM assay. HRM real-time PCR was performed using a Rotor-gene 6000™ real time rotary analyzer (Corbett Research, USA) system and a Type-it HRM PCR (Qiagen) 2× HRM master mix. Each 25 μ L reaction contained 12.5 μ L of 2× HRM master mix (1× final concentration), 1.75 μ L of each primer (10 μ M; 0.7 μ M final concentration), 8 μ L of nuclease-free water, and 5 μ L of extracted plasma DNA. Three standard genomic DNA controls (normal, heterozygous IVS-II-1, homozygous IVS-II-1) were included in each run. After amplification, the expected 186 bp band was confirmed by agarose gel electrophoresis prior to DHPLC analysis. In the present study, HRM served to ensure specific amplification and quality control rather than to provide an independent diagnostic readout, in contrast to our prior HRM-focused publication (23) in which HRM itself was the primary method under evaluation.

DHPLC assay

For DHPLC analysis, the 186 bp amplicons from all 50 maternal plasma samples and the three standard controls (normal, heterozygous, and homozygous IVS-II-1) were first verified by electrophoresis and then purified using the AccuPrep PCR Purification Kit (Cat. No. K-3034, Bioneer, Korea) according to the manufacturer's protocol. DHPLC analysis was performed on a Transgenomic WAVE® System 4500 (Germany, 2012) equipped with a DNASep® HT cartridge (Transgenomic, Inc.). The mobile phase consisted of a binary buffer system: Buffer A (0.1 M triethylammonium acetate, TEAA) and Buffer B (0.1 M TEAA with 25% acetonitrile). The flow rate was maintained constant at 0.9 mL/min throughout the analysis. Each PCR product (5-10 μ L) was automatically loaded onto the column with a loading duration of 0.5 minutes. The fragment was eluted

under partially denaturing conditions optimized for mutation detection using a linear acetonitrile gradient. The gradient was initiated with a 5% drop ("Drop for Loading") followed by a linear slope of 2% B per minute over a gradient duration of 4.5 minutes, resulting in an increase of Buffer B from 49.9% to 53.5% during the run. The column oven temperature for the DNASep® column was set at 57.2° C, the optimal melting temperature determined for this amplicon using the instrument's Navigator™ melt algorithm based on the HBB reference sequence, target mutation, and primer sequences, and was empirically verified. Under these optimized conditions, the denaturing elution time of approximately 4.5 minutes corresponded to the elution of heteroduplexes, while the non-denaturing elution time of approximately 6.0 minutes corresponded to homoduplexes. A normal cleaning protocol ("Clean Type: NORMAL") was used between injections to maintain column performance and reproducibility.

Analytical specificity was established by running normal, heterozygous, and homozygous IVS-II-1 control samples under the optimized conditions described above and using their chromatographic profiles as reference patterns. Analytical sensitivity (limit of detection, LOD) was evaluated by preparing serial dilutions of heterozygous IVS-II-1 DNA in normal DNA at ratios of 1:10, 1:100, 1:1000, and 1:10000, with each dilution analyzed in duplicate. The lowest dilution at which heteroduplex peaks remained clearly distinguishable was defined as the LOD.

Diagnostic definitions

The DHPLC results obtained from maternal plasma were compared with fetal β -globin genotypes established by the reference reverse dot blot (RDB) method. Fetal genotyping was performed by RDB analysis of invasive samples, specifically chorionic villus sampling (CVS) or amniocentesis. Briefly, this process entailed PCR amplification of the β -globin gene and subsequent hybridization to membrane-bound, allele-specific oligonucleotide probes (14). Based on this comparison, cases were classified into the following four categories:

- True positive (TP): samples in which both DHPLC and RDB identified the fetus as carrying the IVS-II-1 (G>A) mutation (heterozygous or homozygous).
- True negative (TN): samples in which both DHPLC and RDB indicated absence of the fetal IVS-II-1 (G>A) mutation.
- False positive (FP): samples with a positive DHPLC result but a negative RDB result.
- False negative (FN): samples with a negative

DHPLC result but a positive RDB result.

Diagnostic accuracy indices were calculated using standard formulas as follows:

- Sensitivity=TP/(TP+FN)
- Specificity=TN/(TN+FP)
- Positive predictive value (PPV)=TP/(TP+FP)
- Negative predictive value (NPV)=TN/(TN+FN)
- Positive likelihood ratio (LR+)=Sensitivity/(1-

Specificity)

- Negative likelihood ratio (LR-)=(1-Sensitivity)/Specificity

Statistical analysis

Statistical analyses were performed using SPSS software version 22. Categorical variables were presented

as frequencies and percentages. Sensitivity, specificity, PPV, NPV, LR+, and LR- were calculated with their corresponding 95% confidence intervals (95% CIs) based on the exact (Clopper–Pearson) method for binomial proportions. Where appropriate, diagnostic accuracy measures reported in the tables were checked for internal consistency with the underlying counts of TP, TN, FP, and FN.

Ethical consideration

This study was approved by the Ethics Committee of Mazandaran University of Medical Sciences (IR.MASUMS.REC.1393.926). Informed consent was obtained from all participants. The study procedures were fully explained to all enrolled cases, and any questions were answered.

Table 1. Primers sequences

NCBI Reference Sequence: NG_000007.3 (Homo sapiens beta globin region (HBB))	
Reverse primer	Sequences
Forward primer	5'CCCATTCTAAACTGTACCC3'
	5'TCACCTGGACAACCTCAAG3'

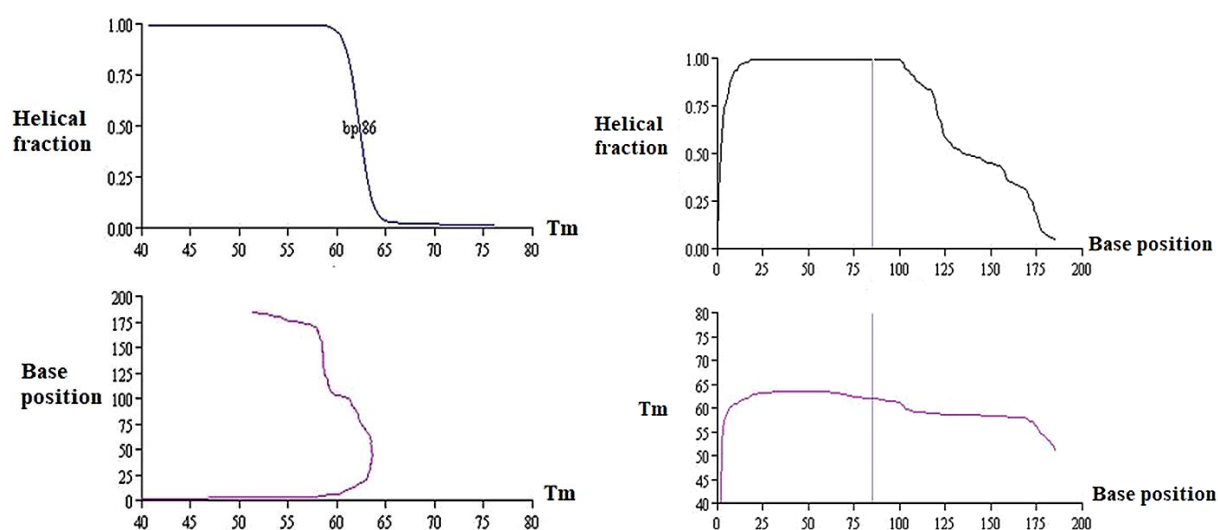


Figure 1. Melting curve analysis and optimal temperature determination for the HBB-IVS-II-1 amplicon. The graph shows the predicted melting profiles of the 186 bp HBB-IVS-II-1 amplicon generated by the Navigator™ software. Each curve represents the melting behavior of the DNA fragment at different column temperatures (55.7 °C to 59.7 °C). The vertical line at base position 86 marks the location of the IVS-II-1 (G>A) mutation site within the amplicon. The partially denaturing temperature of 57.2 °C (indicated by arrow) was selected for subsequent DHPLC analyses as it provides maximum resolution between heteroduplex and homoduplex species

Results

HRM-PCR amplification

All 50 maternal plasma samples yielded a single HRM-PCR product of the expected size. Agarose gel

electrophoresis confirmed a 186 bp band (approximately 200 bp) in all cases (Figure 2).

Analytical specificity of the DHPLC method

The analytical specificity of the DHPLC assay was evaluated using three genomic DNA standards: normal, heterozygous IVS-II-1 (G>A), and homozygous IVS-II-1 (G>A). Under the optimized partially denaturing conditions, each genotype produced a distinct and reproducible chromatographic profile that was used as a reference pattern for interpretation of maternal plasma chromatograms (Figure 3). The β -globin reference sequence, IVS-II-1 (G>A) mutation site, and primer sequences were entered into the DHPLC software to define the optimal separation conditions.

Analytical sensitivity (limit of detection)

To assess analytical sensitivity, serial dilutions of heterozygous IVS-II-1 (G>A) DNA in normal DNA (1:10, 1:100, 1:1000, 1:10000) were analyzed by DHPLC. Heteroduplex peaks were clearly detectable up to the 1:10 dilution (corresponding to an approximate 10% mutant allele fraction, 2 ng/ μ l), whereas at the 1:1000 dilution only one of the two expected peaks was observed, and at 1:10000 a single predominant peak was present (Figure 4). These findings indicate a limited analytical sensitivity of the assay for detecting low-

proportion mutant alleles in a background of normal DNA under the current conditions.

Clinical diagnostic performance in maternal plasma

DHPLC results on maternal plasma were compared with fetal genotypes obtained by reverse dot blot (gold standard). Overall, 13 true positive, 11 true negative, 19 false negative, and 7 false positive cases were observed (Table 2). Thus, a substantial number of affected/carrier fetuses were missed by DHPLC, reflecting a high false-negative rate.

The diagnostic accuracy indices for DHPLC in this non-invasive prenatal setting were calculated (Table 3). The assay demonstrated a sensitivity of 40.6% (13/32; 95% CI: 23.7%-59.4%) and a specificity of 61.1% (11/18; 95% CI: 35.7%-82.7%). The positive predictive value (PPV) was 65.0% (13/20; 95% CI: 40.8%-84.6%), and the negative predictive value (NPV) was 36.7% (11/30; 95% CI: 19.9%-56.1%). The positive likelihood ratio (LR+) was 1.04 (95% CI: 0.59-1.82), and the negative likelihood ratio (LR-) was 0.97 (95% CI: 0.62-1.52), indicating minimal discriminative ability in this pilot setting.

Table 3. Diagnostic performance of DHPLC for non-invasive detection of IVS-II-1 (G>A)

Parameter	Estimate (%)	95% CI (%)
Sensitivity	40.6	23.7-59.4
Specificity	61.1	35.7-82.7
PPV	65.0	40.8-84.6
NPV	36.7	19.9-56.1
LR+	1.04	0.59-1.82*
LR-	0.97	0.62-1.52*

Table 2. Cross-tabulation of DHPLC results versus reverse dot blot (gold standard)

	RDB: IVS-II-1 positive	RDB: IVS-II-1 negative
DHPLC: IVS-II-1 positive	13(TP)	7(FP)
DHPLC: IVS-II-1 negative	19(FN)	11(TN)

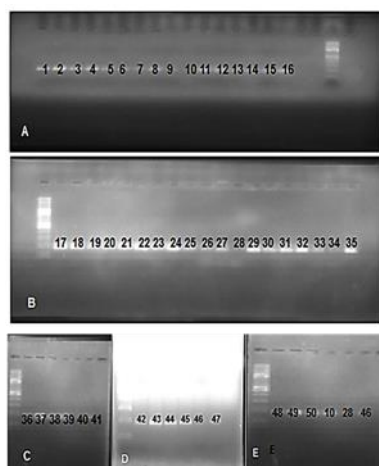
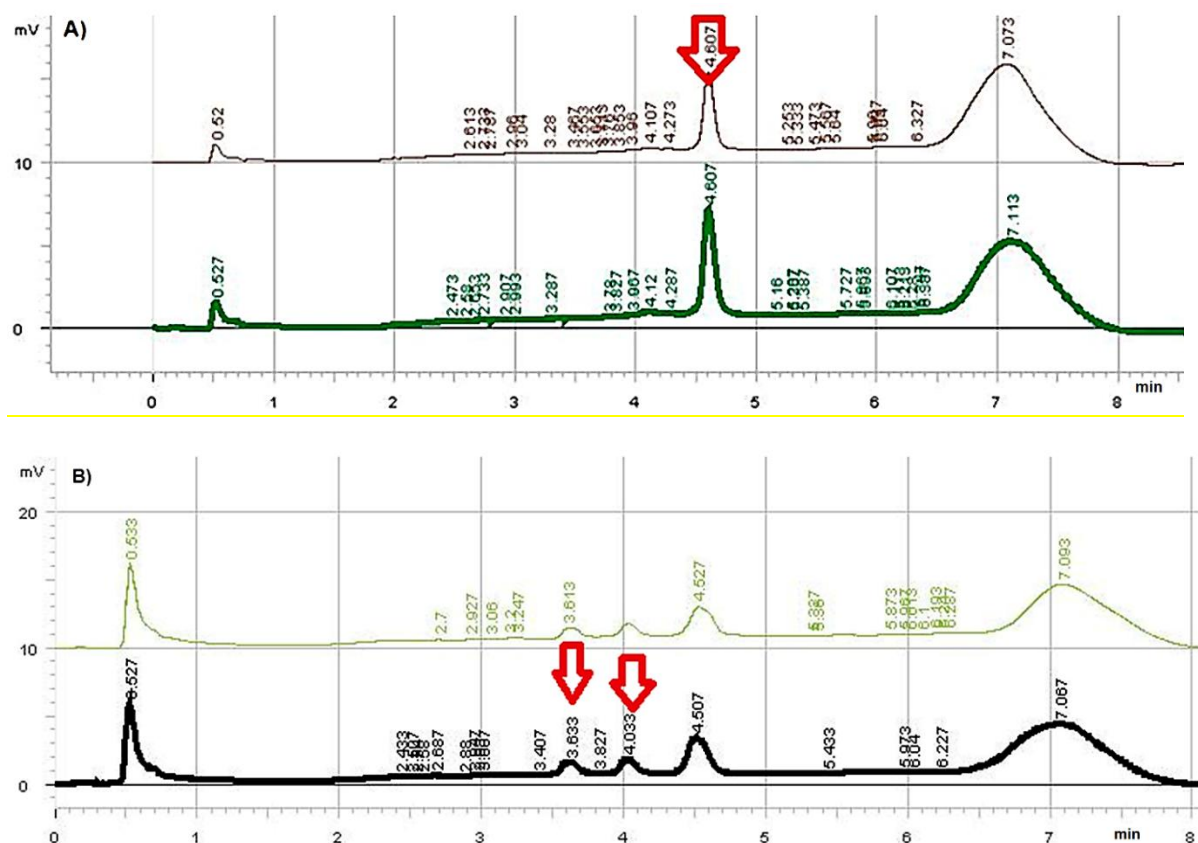


Figure 2. Agarose gel electrophoresis of HRM-PCR products from maternal plasma samples. PCR amplification of the HBB gene fragment (IVS-II region) from 50 maternal plasma samples and controls was performed, and products were analyzed on an agarose gel. Lanes 1-50 represent individual maternal plasma samples arranged in five sections (A: lanes 1-16; B: lanes 17-35; C: lanes 36-41; D: lanes 42-47; E: lanes 48-50), all showing a single band of approximately 186 bp, confirming successful amplification of the target region



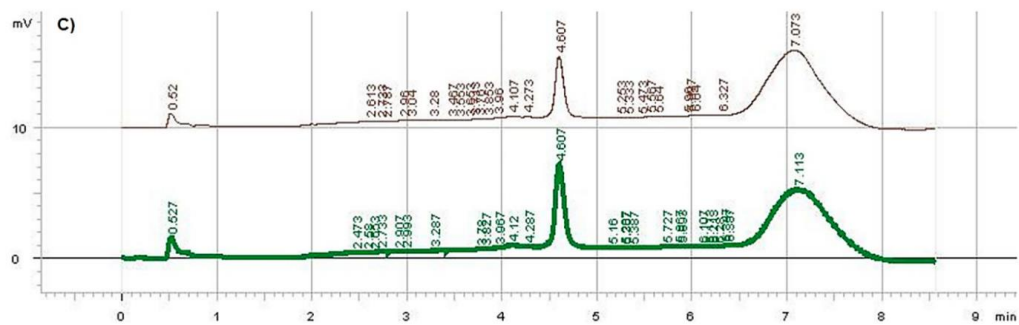
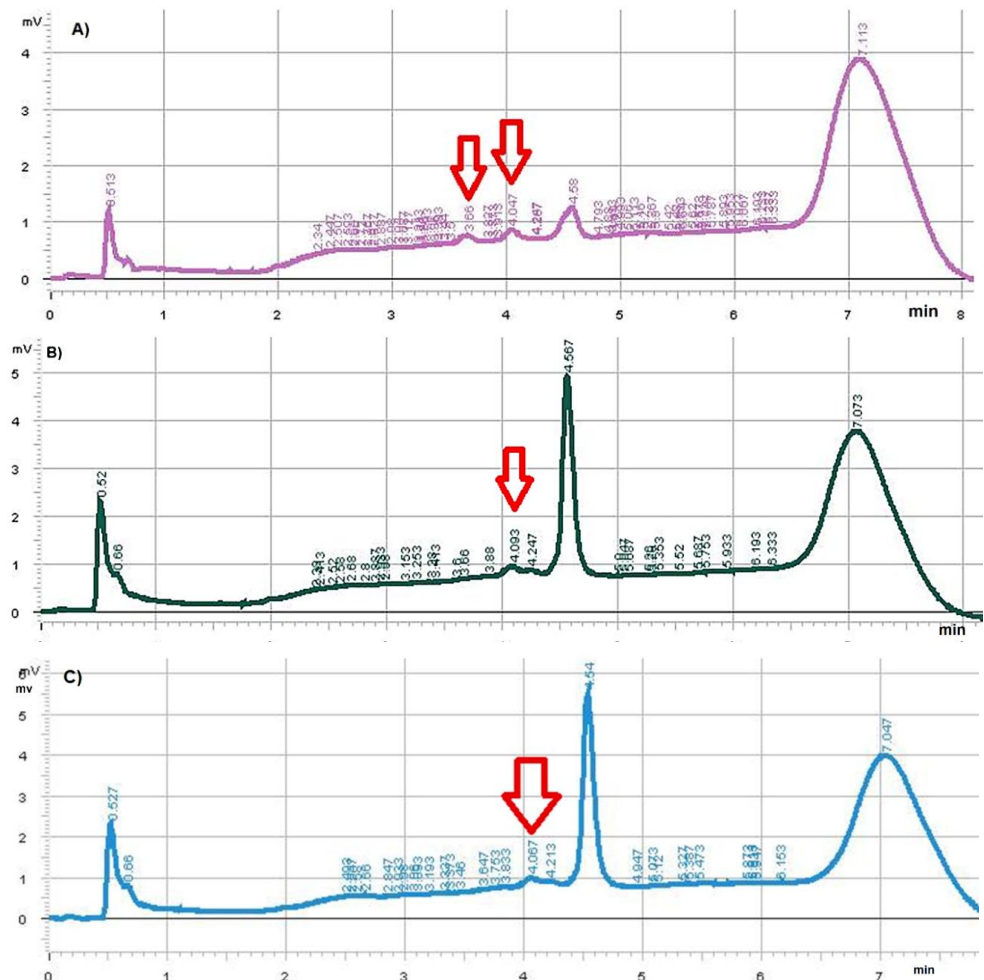


Figure 3. DHPLC chromatograms of genomic DNA standards: (A) normal, (B) heterozygous IVS-II-1 (G>A), and (C) homozygous IVS-II-1 (G>A), demonstrating distinct elution profiles used as reference patterns. DHPLC analysis was performed on the Transgenomic WAVE® System 4500 at a partially denaturing temperature of 57.2 °C with a linear acetonitrile gradient from 49.9% to 53.5% Buffer B over 4.5 minutes at a flow rate of 0.9 mL/min. (A) Normal homozygous control showing a single homoduplex peak at approximately 4.5 minutes retention time. (B) Heterozygous IVS-II-1 control displaying a mixed profile with two distinct peaks: an earlier eluting heteroduplex peak (~4.0 minutes) and a later eluting homoduplex peak (~4.5 minutes), confirming the presence of the mutation. (C) Homozygous IVS-II-1 mutant control showing a single homoduplex peak with a slightly shifted retention time compared to the normal control, consistent with homozygous sequence variation



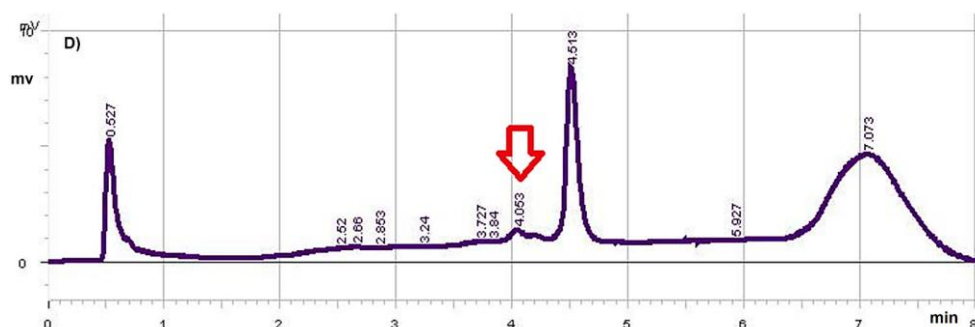


Figure 4. DHPLC chromatograms of serial dilutions of heterozygous IVS-II-1 (G>A) DNA in normal DNA: (A) 1:10, (B) 1:100, (C) 1:1000, and (D) 1:10000. Heteroduplex peaks are clearly visible at 1:10 but are no longer reliably detectable at higher dilutions

Discussion

In this pilot diagnostic accuracy study, we evaluated PCR–DHPLC as a possible non-invasive approach for prenatal detection of the IVS-II-1 (G>A) mutation in maternal plasma of β -thalassemia carrier couples. DHPLC is a non-gel-based, non-fluorescence-based chromatographic method that can separate heteroduplex and homoduplex DNA fragments under partially denaturing conditions and has been widely used as a sensitive and specific mutation-scanning technique (24). It has shown good performance for detecting β -thalassemia and hemoglobin E mutations in genomic DNA in both prenatal and postnatal settings, often with high concordance to standard molecular methods (25,26). However, our findings indicate that its diagnostic performance in the context of cell-free fetal DNA (cffDNA) in maternal plasma is limited. This study provides preliminary, hypothesis-generating data and was not powered to yield definitive estimates of diagnostic accuracy. In the present cohort of 50 at-risk pregnancies, DHPLC analysis of maternal plasma targeting the paternal IVS-II-1 (G>A) mutation yielded 13 true positives, 11 true negatives, 19 false negatives, and 7 false positives. The resulting sensitivity of 40.6%, specificity of 61.1%, and negative predictive value of 36.7% demonstrate that a substantial proportion of affected or carrier fetuses were missed, and a non-negligible number of unaffected fetuses were incorrectly classified as positive. This high false-negative rate markedly undermines the clinical utility of PCR-DHPLC as a stand-alone tool for non-invasive prenatal diagnosis (NIPD) of β -thalassemia major in its current form. The discrepancy between our results and previous reports of excellent performance of DHPLC and related methods can be largely explained by differences in the biological material and analytical context. Studies such as Prajantasen et al. used DHPLC after allele-specific PCR for prenatal and

postnatal screening of β -thalassemia and Hb E genes in genomic DNA and reported 100% agreement with amplification refractory mutation system (ARMS)–based prenatal diagnosis (25). Similarly, other groups have successfully applied PCR-based methods (including HRM) for detecting paternal β -thalassemia mutations in cffDNA under carefully controlled conditions (26,27). In these settings, either high-quality genomic DNA is analyzed or highly optimized assays are used for a limited set of mutations. In contrast, cffDNA represents only a small fraction of total circulating DNA in maternal plasma, generally around 10% and often lower in early gestation (28). This low fetal fraction is a key challenge for NIPD of monogenic disorders (28,29). Our analytical sensitivity experiments showed that heteroduplex peaks were clearly detectable only down to approximately a 1:10 dilution of heterozygous DNA in normal DNA, suggesting that the limit of detection of our DHPLC setup is close to the typical range of fetal fractions encountered in clinical practice. Under such conditions, small fluctuations in fetal fraction, DNA quality, or pre-analytical handling can easily lead to missed mutant alleles and, consequently, false-negative clinical results. This study has several notable limitations. First, fetal fraction was not quantitatively measured using targeted polymorphic markers or digital PCR, which represents a critical determinant of NIPD reliability (28,29). Second, this evaluation was restricted to a single mutation site in a modest cohort ($n=50$). While this pilot design provided essential feasibility parameters, the small sample size widens the confidence intervals and limits the statistical precision of the accuracy metrics. The absence of fetal fraction quantification, the exclusive focus on a single common mutation (IVS-II-1(G>A)), and the modest sample size are important limitations that restrict the interpretability and generalizability of our findings. These constraints are consistent with the exploratory nature of the study and support its interpretation as a feasibility

assessment rather than a definitive evaluation of clinical performance. At the same time, substantial progress has been made in NIPD using more sensitive technologies. Massively parallel sequencing of cffDNA in maternal plasma can accurately detect common chromosomal aneuploidies and has been implemented in clinical practice for trisomy screening (30). Molecular prenatal diagnosis has also benefited from advances in targeted sequencing and digital PCR, which can improve detection of low-frequency alleles in mixed maternal–fetal samples (29). Although these methods require sophisticated infrastructure and may not be readily available in all high-prevalence, resource-limited regions, they highlight the performance benchmarks that any new NIPD approach must meet. Taken together, our findings suggest that, under the conditions tested, PCR-DHPLC alone does not achieve the level of sensitivity and reliability needed for clinical NIPD of β -thalassemia major. However, they also confirm that cffDNA-based detection of paternal β -globin mutations is technically feasible and that DHPLC can generate genotype-specific chromatographic patterns. Building on successful experiences with other cffDNA-based assays for paternal mutations, fetal sex, and RhD status, PCR-DHPLC might still be explored as a component of multi-step screening strategies, provided that key pre-analytical and analytical limitations are addressed. Future work should therefore focus on: (i) optimizing pre-analytical processing to maximize fetal DNA yield and minimize maternal background, (ii) routinely quantifying fetal fraction and defining minimum thresholds for reliable DHPLC analysis, (iii) systematically refining DHPLC temperature and gradient conditions to enhance analytical sensitivity, and (iv) expanding the mutation panel to include other prevalent β -globin gene variants characterized in Iranian and regional populations. Larger, statistically robust studies will be required to determine whether PCR-DHPLC can play a limited, adjunctive role within broader NIPD algorithms, rather than serving as a stand-alone diagnostic test.

The advent of cffDNA analysis in maternal plasma has transformed the landscape of prenatal diagnosis by enabling non-invasive testing for selected genetic conditions, particularly chromosomal aneuploidies and certain paternally inherited mutations (28-31). In this exploratory pilot study, we assessed the diagnostic value of PCR-DHPLC for detecting the paternal IVS-II-1(G>A) β -globin mutation in maternal plasma of β -thalassemia carrier couples. Although the assay was technically feasible and distinct DHPLC profiles could be obtained for control genotypes, the clinical diagnostic

performance in maternal plasma was limited, with low sensitivity, modest specificity, and a high false-negative rate. Under these circumstances, PCR-DHPLC cannot be recommended as a stand-alone non-invasive prenatal diagnostic method for β -thalassemia major. Our results should therefore be interpreted as preliminary feasibility data rather than evidence for clinical implementation. More extensive investigations are needed, incorporating larger sample sizes, fetal fraction quantification, optimized pre-analytical and DHPLC conditions, and inclusion of additional common β -globin mutations. Such studies will be essential to clarify whether PCR-DHPLC might contribute in a supporting capacity to future NIPD strategies in high-prevalence, resource-limited settings, while routine invasive prenatal diagnosis remains necessary for definitive fetal genotyping.

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