Original Article

Assessment of high resolution melt analysis feasibility for evaluation of beta-globin gene mutations as a reproducible, cost-efficient and fast alternative to the present conventional method

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Abstract

Background: Beta-thalassemia is the most prevalent monogenic disease throughout the world. It was the first genetic disorder nominated for nation-wide prevention programs involving population screening for heterozygotes and prenatal diagnosis (PND) in Iran. Due to the high prevalence of beta-thalassemia, the shift from conventional mutation detection methods to more recently developed techniques based on novel innovative technologies are essential. We aimed to develop a real-time polymerase chain reaction (PCR) based protocol using high resolution melting (HRM) analysis for diagnosis of common beta-thalassemia mutations.

Materials and Methods: Forty DNA samples extracted from peripheral blood of suspected beta-thalassemia carriers participated in this study were subjected to amplification refractory mutation system (ARMS). We then used 20 of these samples for HRM optimization. When 100% sensitivity and specificity was obtained with HRM procedure, we applied the technique for mutation detection on another remaining 20 samples as thalassemia cases with unknown mutations (detected mutations with ARMS-PCR kept confidential). Finally, the HRM procedure applied on 2 chorionic villous sample (CVS) biopsied from 12 weeks gestational age pregnant women for routine PND analysis.

Results: In the first step of study, Fr 8/9 (+G), IVSI-1 (G > A), IVSI-5 (G > C), IVSI-110 (G > A), and CD44 (−C) mutations were diagnosed in samples under study using ARMS-PCR technique. Finally, the HRM procedure applied on 20 unknown samples and 2 CVS The results of HRM were in complete concordance with ARMS and confirmed by sequencing.

Conclusions: The advantages of HRM analysis over conventional methods is high throughput, rapid, accurate, cost-effective, and reproducible.

Key Words: Amplification refractory mutation system, beta-thalassemia mutations, high resolution melting, sequencing

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INTRODUCTION

Beta-thalassemia is one of the prevalent inherited hemoglobinopathies resulting from reduced or complete absence of beta-globin chain due to any one of the several reported types of mutations in the beta-globin gene located on human chromosome 11. More than 200 mutations have been recognized, but a limited number of them are common in any particular ethnic group.[1] The disease is a significant health complication in 71% of 229 countries worldwide including Iran. Due to the clinical severity and high prevalence of beta-thalassemia major and relatively high consanguinity marriage in Iran, the disease was the first genetic disorder nominated for nation-wide prevention programs involving premarriage screening for heterozygotes and offering optional prenatal diagnosis (PND) for carrier couples.[2] In all countries with high prevalence of carriers, there is the high birth rate of thalassemia minor individuals that should be referred to genetic diagnostic laboratories for mutation detection. Currently, traditional polymerase chain reaction (PCR)-based strategies such as restriction fragment length polymorphisms, amplification refractory mutation system (ARMS), and direct sequencing are widely carried out for molecular diagnosis of beta-thalassemia mutations. Sequencing provides both genotyping and scanning at the same time, but requires extensive automation, expensive instrumentation, and is time taking on the basis of fee for service setup. Other methods are involved extensive post-PCR manipulations such as enzymatic digestion, separation on a gel, or other comparable matrices which are time taking and labor intensive. Advances in molecular diagnosis have been developed in which both PCR amplification and genotyping can be performed within the same reaction tube, and no other post-PCR processing is required. Using these methods, limited sample handling steps provide less chance for contamination. Moreover, elimination of post-PCR analysis step provide a useful high throughput option amenable to automation with quick gene scan tools.[3-6] High resolution melting (HRM) is a simple and reliable method for genotyping and mutation scanning. If all of the technical considerations observed carefully, HRM strategy is highly reliable and sensitive method for mutation screening.[7,8] Berenstein *et al*. performed a comparative study on various PCR-based methods, including ARMS, for DNMT3A and IDH1/2 mutation detection and concluded that HRM analysis was the most accurate, time-saving, and cost-efficient method.[9] In another extensive comparative study for detection of K-ras mutations, direct sequencing, pyrosequencing, HRM analysis, and some more complicated methods were compared. HRM proved superior compared to direct sequencing and pyrosequencing on sensitivity basis and percentage of the detected mutations in samples under study.[10] In some comparative studies on HRM and some other advanced methods, HRM represented more sensitive approach than direct DNA sequencing to detect somatic mutations in tumoral tissues with sensitivity similar to that observed for the snapshot.[11]

In the present study, we utilized HRM analysis for detection of the most prevalent HBB gene mutations in Iranian beta-thalassemia carriers as well as chorionic villous samples (CVS). The results from unknown samples evaluation using optimized HRM protocol were in complete concordance with ARMS and direct sequencing methods.

MATERIALS AND METHODS

DNA samples

Ethical approval of the study was obtained from the Ethical Committee of Isfahan University of Medical Sciences (code: 392226). Blood samples were collected from 40 individuals who were referred for beta-thalassemia mutation detection. Each sample was given a code and recorded confidentially. ARMS-PCR strategy was used to discover the mutation of each individual as a conventional method for mutation detection. Later, we used 20 of these samples as known mutation samples for optimizing our real-time PCR-HRM analysis strategy. We used the rest of 20 samples that their mutations kept confidential as unknown samples to test the efficiency of our developed HRM protocol. To further confirm the discovered mutations in unknown samples, they were subjected to DNA sequencing. Finally, two available CVS samples were also included in our procedure to evaluate the efficiency of the optimized HRM protocol in PND trial. For calculation of sensitivity and specificity values following formulas were used; sensitivity = TP/TP + FN; specificity = TN/TN + FP, where T is true, P is positive, F is false, and N is negative.

High resolution melting analysis

Polymerase chain reaction

Optimization of PCR reaction was carried out carefully through conventional PCR prior to conducting real-time PCR. Three pairs of primers were designed by online Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) [Table 1] for detection of mutations in exon 1, Fr 8/9 (P1 + P2), exon 2, CD44 (P5 + P6), and intron 1, IVSI-1, IVSI-5, IVSI-110 (P3 + P4). Real-time PCR reaction was performed in duplicate in 10 μl final volume using 5 μl 2 × HRM PCR master mix (Type-it® HRM™ PCR kit-QIAGEN). To each reaction tube 50 ng DNA and 10 pM

Table 1: Used primers for HRM analysis of HBB gene mutations

Location	Sequence (5'–3')	Length of PCR amplicon (bp)	Annealing temperature (°C)
Exon1 (FR8/9)	P1 5'-CATAAAAGTCAGGGCAGAGCC-3' (forward)	160	60
	P2 5'-ACCACCAACTTCATCCACGTTCACC-3' (reverse)		
Intron 1 (IVSI-1, IVSI-5, IVSI-110)	P3 5'-GCAAGGTGAACGTGGATGAAG-3' (forward)	184	60
	P4 5'-AGCAGCCTAAGGGTGGGAAA-3' (reverse)		
Exon 2 (CD44)	P5 5'-GTCTATTTTCCCACCCTTA-3' (forward)	120	60
	P6 5'-ATGAGCCTTCACCTTAGGGTT-3' (reverse)		

HRM: High resolution melting, PCR: Polymerase chain reaction

of appropriate primer mix were added. The HRM assay was performed using the Rotor-Gene 6000 instrument provided with the Rotor-Gene 6000 Series Software Version 1.7 (Corbett). The PCR program consisted of an initial denaturation–activation step at 95°C for 5 min, followed by a 40-cycle program (denaturation at 95°C for 10 s, annealing/extension at 60°C 30 s). PCR amplification cycles were automatically followed by HRM channel within the same reaction tube in which increasing temperature from 65°C to 95°C at the ramp rate of 0.2°C/2s. For HRM analysis appropriate positive controls with known mutations, wild type DNA, and no-template negative controls were included.

Gene scanning and analysis

The HRM analysis performed by the related software was including three steps: Normalized graph, equaling to 100% the initial premelt fluorescence and to 0% the postmelt fluorescence remnant, shifting of temperature axis of the normalized graph to the point where the entire double-stranded DNA is completely denatured and, difference graphs. Mutation scanning by HRM depends on the melting of heteroduplex (mutant DNA) that distort the shape of the melting curve. This distortion can be seen by comparing the normalized melting curves of a homozygous standard to a heterozygous sample.[12,13] As the difference between curves is very small, it is often apparent by plotting the difference between samples. Each curve is usually subtracted from the homozygous wild-type reference curve. ARM-PCR was performed as reported previously.[14,15] ARMS is an amplification strategy in which primer is designed in such a way that it can discriminate between templates that differ by a single nucleotide residue at its 3' terminal. Thus, an ARMS primer can be designed to amplify a specific member of a multi-allelic system while remaining refractory to amplification of another allele that may differ by as little as a single base from the former.

RESULTS

Based on ARMS-PCR results, mutation of samples in initial phase of study was determined as Fr8/9 (+G), IVSI-1 (G > A), IVSI-5 (G > C), IVSI-110 (G > A), and CD44 (–C). For identifying HBB gene mutations with HRM in exon 1, Fr 8/9, exon 2, CD44, and intron 1, IVSI-1, IVSI-5, IVSI-110 the final combination of primer sets P1 + P2, P5 + P6, and P3 + P4 were used. Using this, HRM profile for each beta-thalassemia mutation was obtained and mutations were identified successfully using these primer sets [Figure 1]. The results were in 100% concordance with ARMS and direct DNA sequencing results. Finally, after confirming PCR reaction and HRM analysis with specific primer set for each mutation, for evaluation of HRM analysis, the optimized protocol was used for detection of 20 beta-thalassemia cases acting as unknown mutations as well as two CVS cases. The results were in 100% concordance with ARMS and sequencing results [Figures 2 and 3, Table 2].

DISCUSSION

Although more than 200 causative mutations have been reported for beta-thalassemia syndromes, the spectrum of mutations and their frequencies in almost all populations is consisting of a limited number of common mutations and of course some less frequent and rare mutations.[1] In all multiethnic societies like Iran beta-thalassemia mutation spectrum are complex combinations of various types making the diagnostic procedure much more difficult and time taking with presently practicing procedures. Mutation detection in carriers is an essential task when offering PND to couples at risk for having a child with thalassemia major and frequently needs to be detected as quickly as possible, especially when at-risk couples referral for PND made after establishment of a pregnancy. Considering the importance of rapid, reproducible with high sensitivity DNA analysis, real-time PCR and subsequent melting curve analysis using HRM has become an important tool in research as well as clinical diagnostics.

To produce accurate and reproducible HRM results, the size of amplicon, careful design of primers to act well in PCR reaction and highly optimized PCR protocol is playing a central role.[16-18] We

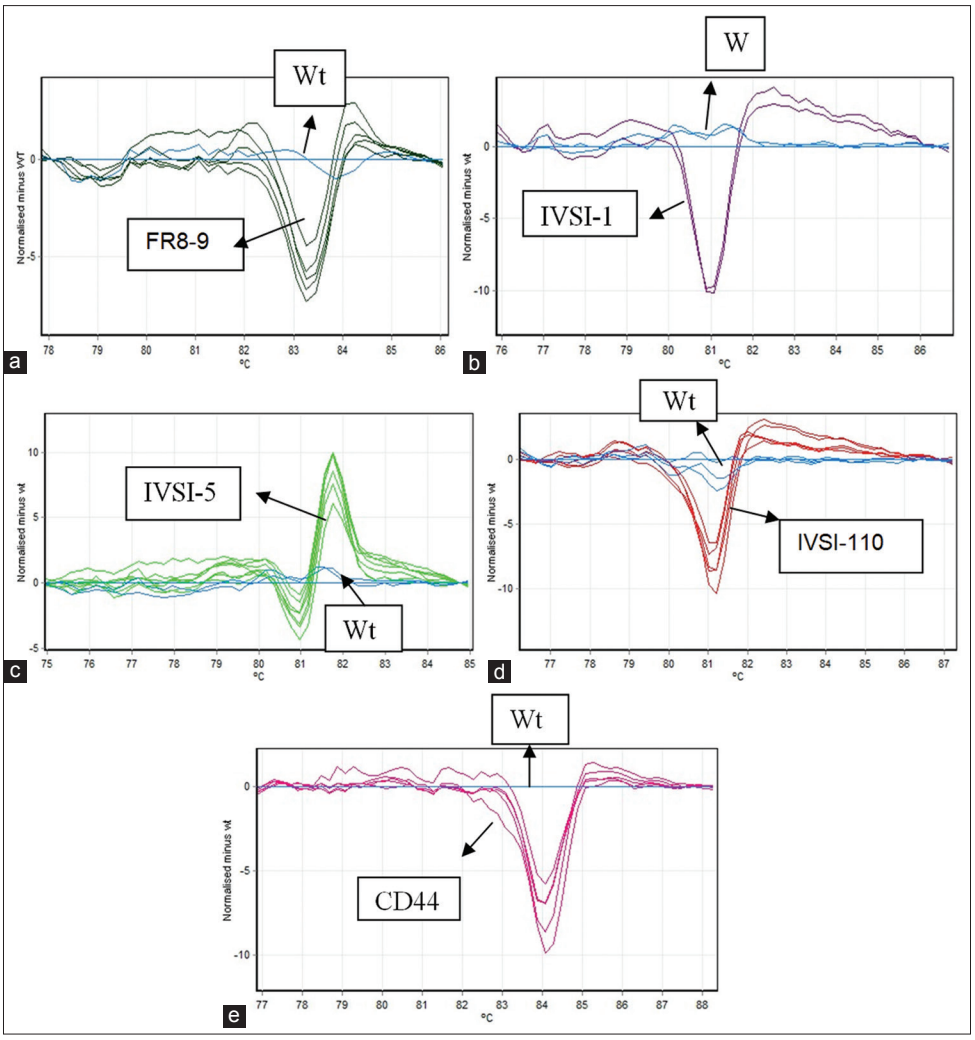

Figure 1: Representative resolution melting profiles for HBB gene mutations using difference curves against one control sample. The wild type (Wt) DNA was used as reference. Fr 8/9 mutation using primer sets P1, P2 (a), IVSI-1, IVSI-5, IVSI-110 mutations using primer sets P3, P4 (b-d), and CD44 using primer sets P5, P6 (e)

Table 2: Results of validation of HRM assays on 22 samples with various beta-thalassemia status

Beta-thalassemia status (*n*)	Evaluation with HRM (*n*)	Evaluation with ARMS (*n*)
Wild-type (4) (including 1 CVS)	Wild-type (4)	Wild-type (4)
IVSI-5 (4)	IVSI-5 (4)	IVSI-5 (4)
CD44 (5)	CD44 (5)	CD44 (5)
IVSI-110 (3) (including 1 CVS)	IVSI-110 (3)	IVSI-110 (3)
Fr8/9 (6)	Fr8/9 (6)	Fr8/9 (6)

HRM: High resolution melting, ARMS: Amplification refractory mutation system, CVS: Chorionic villous sample

used alternative primer sequences, various PCR condition and careful adjustment of PCR thermal cycling condition. Using primers that specifically were designed to amplify a segment of beta-globin gene harboring some prevalent mutations, like; IVS I-1, IVS I-5, IVS I-110, Fr 8/9, CD44, a single real-time PCR reaction in combination with the subsequent HRM analysis would be able to detect one of the mutations that may be present in this segment of the HBB. However, the same task if would be done using ARMS method required at least two primer sets (mutant and normal) for each mutation in conjunction with internal controls and lengthy postPCR analytical processes. This study is in concordance with the study conducted by Reed and Wittwer that for PCR products <400 bp, sensitivity and specificity of scanning for heterozygous single base changes were 100%.[8] Larger amplicon size provides lower resolution in HRM. The reason is that a single base variation has a greater effect on the melting behavior of small DNA fragment than the larger one. In this study, also we designed several different primer sets and finally obtained the present cohort of primers with excellent performance. We realized that with point mutations that their melting curves remained very similar, melting curve resolution was not sufficient for detection of such a narrow range of thermal differences (data not shown). For getting higher resolution, better-performing primer with

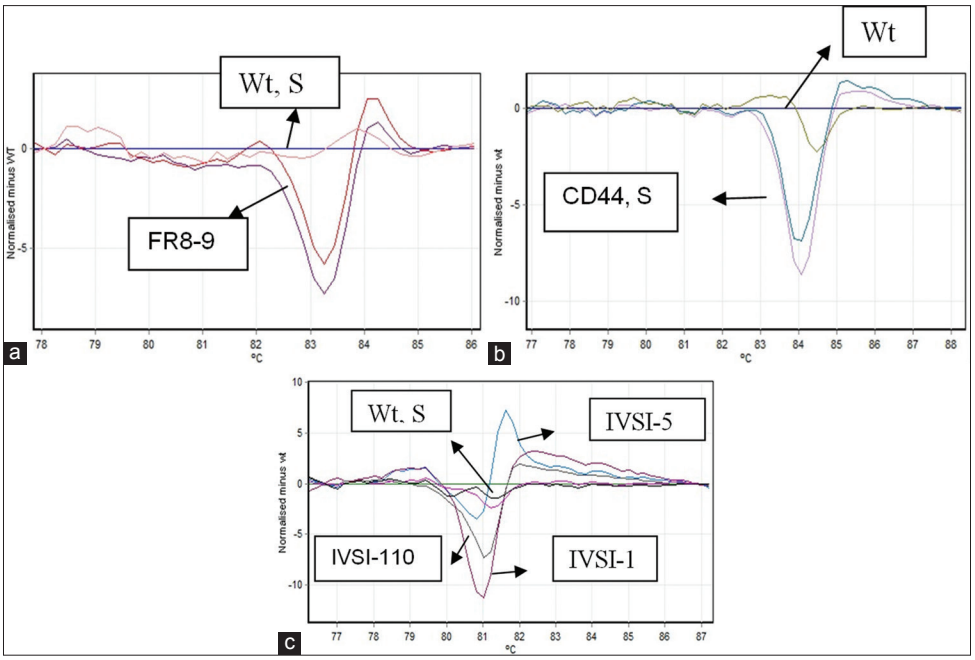

Figure 2: Unknown sample evaluated for beta-thalassemia mutations. Difference curves have been produced by primer sets P1 + P2 (a), P5 + P6 (b), P3 + P4 (c). As it is evident, the samples can easily being categorized as CD44.Wt: Wild type, S: Unknown sample

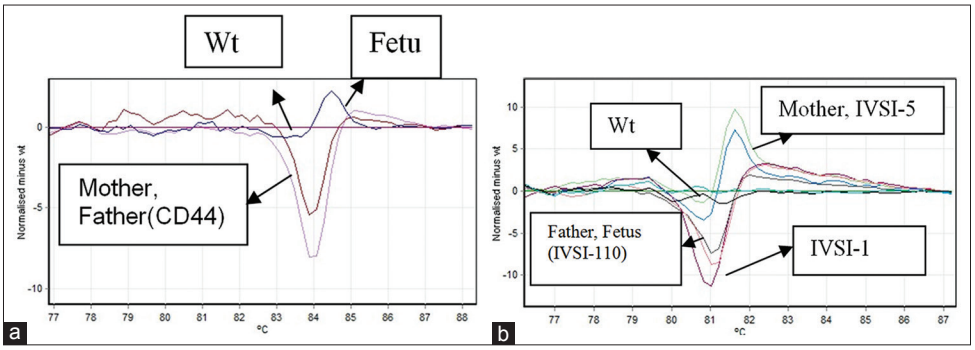

Figure 3: Assessment of the developed resolution melting protocol on DNA extracted from two chorionic villous sample samples. (a) The parents were both carrier of CD44 mutation. According to the resolution melting profiles the fetus diagnosed as unaffected homozygous with primer sets P5 + P6. (b) The father was a carrier of IVSI-110 mutation, and the mother was an IVSI-5 trait. According to the resolution melting profiles the fetus diagnosed as a carrier of IVSI-110 mutation with primer set P3 + P4. CVS: Chorionic villous sampling

shorter amplicon size plays a crucial role. Therefore, in spite of the fact that many point mutations do not produce a significant melting temperature shift but this problem can be resolved by precise primer design, optimized PCR protocol and using accurate analyzing methodology of melting curves. Using this strategy specific and reproducible HRM profile for each beta-thalassemia mutations was obtained. We could differentially diagnose all the samples and assigned correct genotypes as heterozygote and wild-type as well as differentiation of heterozygotes from each other. The method applied to both mutation screening and PND of beta-thalassemia [Figures 2 and 3] which revealed complete concordance compared to routine allele-specific PCR assays and sequencing. In some studies, HRM analysis was compared with more complicated methods like a snapshot. Magnin *et al*. performed a comparative study including snapshot, sequencing, and HRM analysis. Based on their conclusion, HRM represented more sensitive approach than direct DNA sequencing to detect somatic mutations in tumoral tissues with sensitivity similar to that observed for the snapshot. Moreover, HRM seems to be a suitable, fast, closed-tube methodology for testing low quantity DNA.[11]

It is quite predictable that traditional mutation detection strategies in use in clinical laboratories are rapidly going to be replaced by HRM as a high throughput, fast, reproducible, accurate and amenable to automation method. Disease-associated variants or gene mutations, for instance CFTR gene mutations,[19]

RET proto-oncogene mutations,[20] epidermal growth factor receptor mutations,[21] K-ras mutations,[22] TP53 mutations,[23] BRCA1 and BRCA2 gene mutations[24] are now successfully detectable by HRM method. Other applications of HRM method is extended to infectious disease targets. *Staphylococcus aureus* genotypes,[25] influenza A subtypes,[26] *Bacillus anthracis* strains,[27] and Mycoplasma synoviae strains[28] and many others are examples of switching from traditional detection strategies to HRM analysis system.

CONCLUSIONS

Beta-thalassemia being a highly prevalent monogenic disease in many countries throughout the world. We assume that the shift from current traditional mutation detection methods to the newly novel developed techniques based on recent advancements is a positive event that should take place. This study can help in switching from presently practicing beta-thalassemia mutation detection to the real-time PCR with HRM analysis as robust, high throughput, rapid, and accurate method.

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Conflicts of interest

There are no conflicts of interest.

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