



***HBB* FSC 36-37 (-T) Gene Mutation Detection in Carriers of Thalassemia Minor Using High Resolution Melting Analysis**

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Abstract

Beta-thalassemia is one of the most common autosomal recessive disorders in the world population resulting from over 200 different mutations of *HBB* gene. Beta-thalassemias are caused by point mutations or, more rarely, deletions in the *HBB* gene leading to reduced (beta+) or absent (beta0) synthesis of the beta chains of hemoglobin (Hb). High-resolution melting of polymerase chain reaction (PCR) products can detect heterozygous and most homozygous mutations without electrophoretic or chromatographic separations.

In the current study, blood samples collected from 20 individuals carrying minor thalassemia were genotyped using HRM technique. The genotype of each sample had been previously determined via the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), amplification-refractory mutation system (ARMS) or sequencing method. This study aimed to determine the specificity and sensitivity of HRM method in the diagnosis of carriers of FSC 36-37 (-T) mutation from carriers who do not have this mutation. DNA extraction from peripheral blood was performed and HRM method was used to genotype samples. The results were analyzed according to the normalized and difference plot. High-resolution melting analysis could correctly identify all carriers of FSC 36-37 (-T) from who did not have this mutation. In summary, HRM is a technique associated with high sensitivity and specificity for the identification of FSC 36-37 (-T) mutation.

Key words: Beta thalassemia minor; Difference plot; Genotyping; Hemoglobin; HRM; Normalized plot

Introduction

Hemoglobinopathies are a group of autosomal recessive disorders that are classified into two main groups of synthesis alterations (α - and β -thalassemia) and structural alterations of hemoglobins (such as Hbs) (Weatherall, 2001). Beta-thalassemias are a heterogeneous group of inherited anemias that includes three main forms (Fig.1): Thalassemia major, thalassemia intermedia and thalassemia minor (Birgens and Ljung, 2007). Thalassemia major patients suffer from anemia, and pathological complications such as bone deformation,

hepatomegaly, splenomegaly and growth retardation (Urbinati *et al.*, 2006). Intermediate beta thalassemia is approximately intermediate between thalassemia major and minor, in most cases not requiring blood transfusions (Lahiry *et al.*, 2008). Carriers are generally asymptomatic, but may suffer anemia during childhood, pregnancy and physiological stresses (Bunn and Forget, 1986). The main importance of diagnosing thalassemia minor form is the need for genetic counseling and to prevent new born of thalassemia major children if their parents are the carriers of the trait (Hashemizadeh and Noori, 2013).

HBB (hemoglobin subunit beta) is localized on the short-arm of chromosome 11 (*11p15.5*) with an arrangement of 5' ϵ - γ - γ - δ - β 3' (Das and Talukder., 2001). The genomic sequence of *HBB* includes three exons, two intervening sequences (IVS1 and IVS2) and the 5' and 3' untranslated regions (UTRs) (Grosveld *et al.*, 1998). Beta thalassemia is highly prevalent and is a major public health problem in the malarial, tropical and sub-tropical regions of Mediterranean countries, the Middle East, Transcaucasus, South Asia, including SriLanka and the Maldives, Southeast Asia, Melanesia, South China, Taiwan (Weatherall and Clegg.,

2001; Lahiry *et al.*, 2008) and one of the widespread hereditary disorders in Iran (Akhavan-Niaki *et al.*, 2011).

High-resolution amplicon melting analysis is a closed-tube method to detect single base sequence variations such as SNPs (single nucleotide polymorphisms) or to discover unknown genetic mutations (Wittwer *et al.*, 2003). This study aimed to determine the specificity and sensitivity of HRM method in the diagnosis of carriers of FSC 36-37 (-T) mutation from carriers who do not have this mutation.

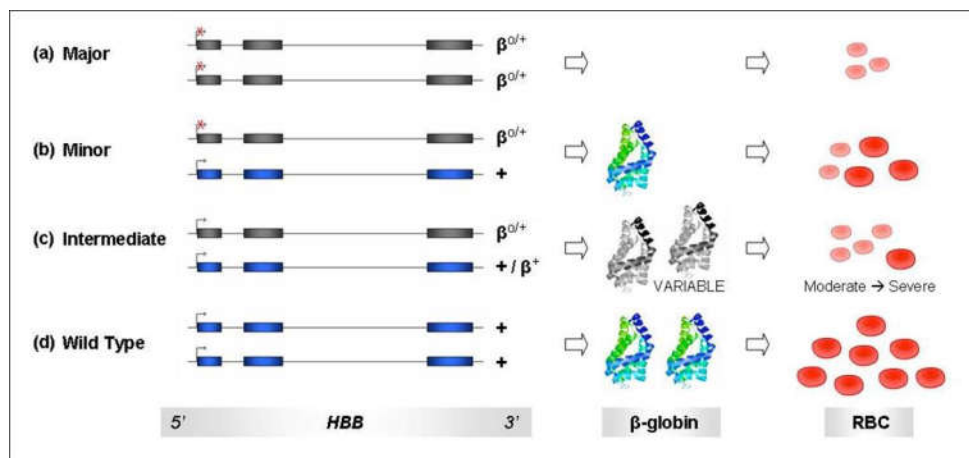


Fig 1. Schematic representation of inherited beta-globin variants and related beta-chain and red blood cell (RBC) phenotype. The *HBB* variants are displayed in grey exons while the wild type alleles are displayed in blue exons (Lahiry *et al.*, 2008).

Materials and Methods

In this study, blood samples collected from 20 individuals diagnosed as thalassemia minor of which 15 samples had FSC 36-37 (-T) and 5 samples had the other mutations such as IVS1-5. The latter samples were considered as controls in our assay.

Genomic DNA was extracted from white blood cells (WBCs) using the Diatome kit (Isogen Laboratory, Russia) according to the vendor's recommended protocol. Oligonucleotide primers were designed using gene runner 4.0.9. Forward and reverse primers simultaneously bound to *HBB* and only forward primer bound to *HBD* according to the data obtained from the BLAST server (Basic Local Alignment Search Tool) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Primers were designed to amplify a small fragment (134bps) surrounding the mutation

and SNPs were checked to avoid the presence of other important sequence variations in the primer region.

The PCR reaction was performed in a 25 μ l volume containing 2 μ l genomic DNA, 2.5 μ l 10X PCR buffer, 0.75 μ l $MgCl_2$ (50 mM), 0.5 μ l (40 mM) dNTP mix, 0.5 μ l of forward, (CTCTGGGTTTCTGATAGGC) and reverse (TAACAGCATCAGGAGTGGAC) primers (10 μ M), 0.25 μ l *Taq* DNA polymerase primers (5U/ μ l) (KBC, Iran). The PCR conditions were as follows: incubation in a DNA thermal cycler Bioer (Hangzhou, China) for an initial denaturation of 5 min at 95°C, succeeded by 35 cycles of denaturation at 95°C for 30s, annealing at 55°C for 30s and extension at 72°C for 30s followed by a final extension of 5 min at 72°C. PCR amplicons were then detected on a 1% agarose gel, stained by DNA green viewer (Afratoos, Iran).

After optimizing PCR reaction, The HRM assays were performed in 10µL volumes in a Rotor gene 6000 (Corbett, Australia). The amplification mixture included 0.5 µl (50ng) of genomic DNA as template, 5 µl Type-it HRM PCR master mix (Qiagen Co, Germany), 0.3 µl of each primer (10µM) and RNase-Free water up to 10 µL. The PCR program started with an initial denaturation at 95 °C for 5 min, followed by 40 cycles denaturation at 95°C for 10s, annealing temperatures at 55 for 30s and extension at 72 °C for 10 s. After denaturation at 94 °C for 1 min and cooling down to 40 °C for 1 min., a melting curve was generated on the Rotor gene 6000, consisting of a temperature ramp from 78 °C to 95 °C at a rate of 0.01 °C /s for 1 cycle. The temperature at which a peak occurs on the plot corresponds to the melting temperature (T_m) of the DNA duplex.

Results

In this descriptive-analytical research, the extracted DNA from WBC was separated on a 1% agarose gel as shown in Fig. 2. Due to the good quality of the extracted DNA, the conventional PCR was performed to optimize the amplification conditions. As shown in Fig. 3, the expected 134 bps band was observed on the 1% agarose gel.

To rule out the potential effect of the SNPs in the amplification, the frequency of some of the SNPs in the amplicon checked out by the SNP check3 (www.snpcheck.net/) as shown in Table1.

By finding the optimized condition for amplification, HRM was performed and the results were analyzed according to the normalized and difference plot (Fig 4 and Fig 5). Melting curve analysis was performed using the Rotor gene 6000, software version 2.02. The melting curve aberrations from wild type samples indicated the presence of FSC 36-37 (-T) mutation. The sensitivity and

specificity of HRM was 100 as it identified all the carriers.

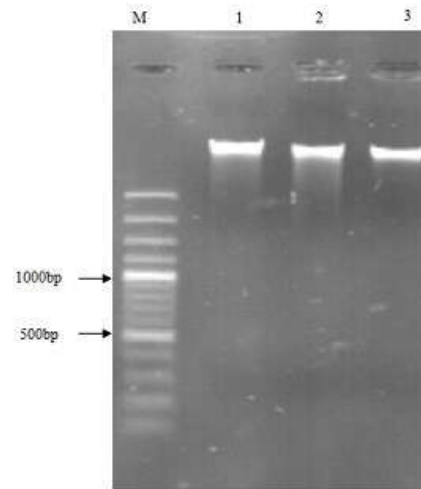


Fig 2. Extracted total genomic DNA on agarose gel electrophoresis: lane1 FSC 36-37 (-T) carrier; lanes 2 and 3 controls.

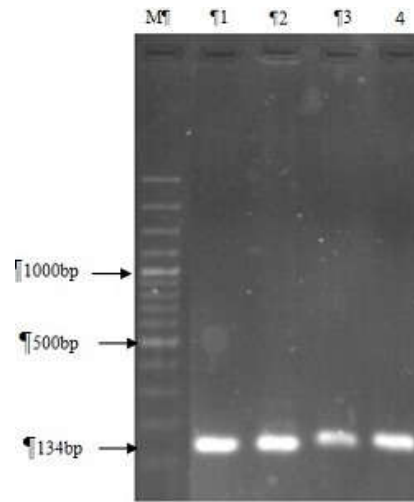


Fig 3. Electrophoresis of the 134bp PCR products on agarose gel. Lanes 1 and 2 FSC 36-37 (-T); Lanes 3 and 4 control.

Table1. Results of SNP check 3 tool

Chromosome	RS	Sequence	Global MAF
11:5226822	rs111851677	CTCTGCCTA[C/T]TGGTCT	0.0064/32
11:5226763	rs281864900	GAGGTT[-/CTTT]GAGTCC	0.0010/5
11:5226835	rs199587927	GAGAGA[A/C/G]TCAGTGC	0.0002/1
11:5226774	rs11549407	TTGGACC[A/C/G/T]AGA	0.0002/1
11:5226799	rs1135071	CTTAG[C/G/T]CTGCTG	0.0002/1

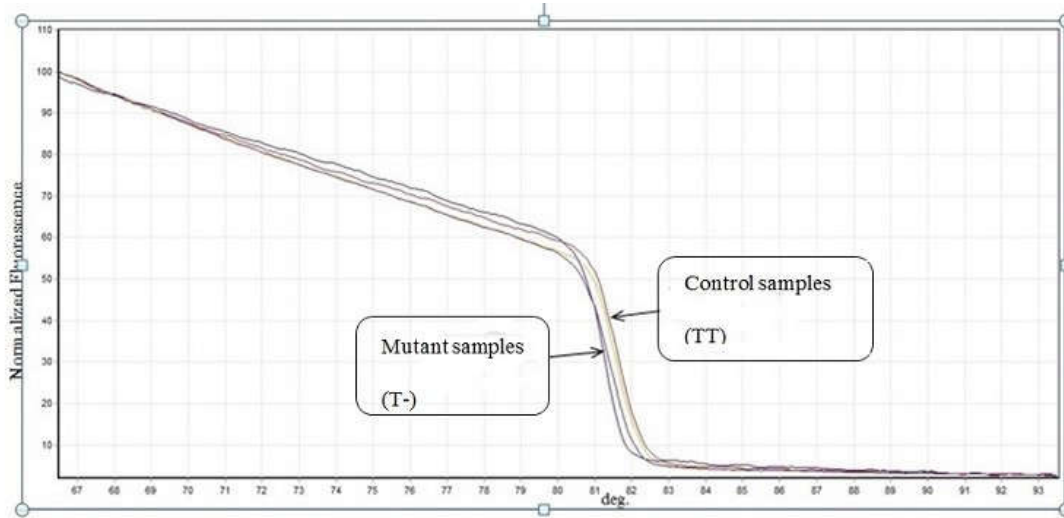


Fig 4. Normalized melt curves of control and mutant samples of FSC 36/37(-T) that differentiated primarily by a shift in the curve on the temperature axis (TM shift).

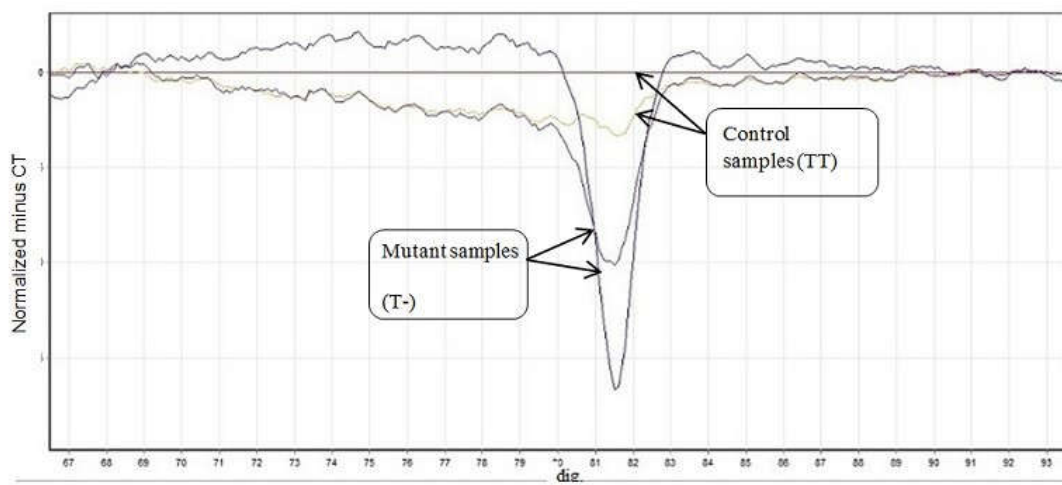


Fig 5. Samples viewed as a difference plot against one of the control.

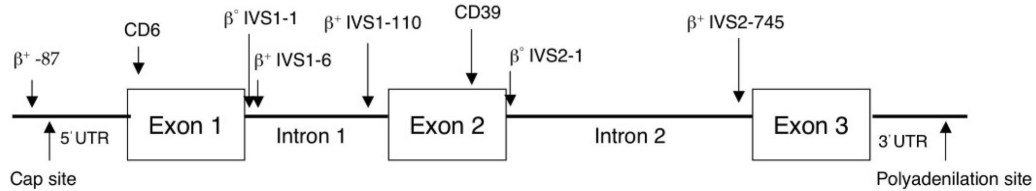


Fig 6. Schematic representation of the β -globin gene. The arrows show the positions of the β -thalassemia mutations in the Mediterranean area (Rosatelli *et al.*, 1992).

Discussion and Conclusion

As a common autosomal recessive disorder, β -thalassemia is resulting from over 200 point mutations which may occur in exon or intron sequences, as well as in the promoter or the 5'

and 3' flanking UTR (Untranslated region) sequences (Grosso *et al.*, 2012) (Fig. 6).

All over the earth, 150 million people carry beta thalassemia genes. The gene frequency of beta thalassemia is prevalent in Sardinia (11-34%), Sicily (10%), Greece (5-15%), and Iran (4-10%) (Habibzadeh *et al.*, 1998).

Beta Thalassemia has a high prevalence (around 10%) in north and South of Iran. The prevalence of the disease in most parts of the country has been estimated to be 4–8% (Habibzadeh *et al.*, 1998). There are 20,000 homozygotes and 3,750,000 carriers of β -thalassaemia in Iran (Rahimi., 2013). According to the previous studies, FSC 36-37 (-T) is the most frequent mutation for beta thalassemia in Shadegan City (40.24%) (Doosti *et al.*, 2015) and in Lorestan province (33.8%) (Najmabadi *et al.*, 2001).

Commonly used techniques for molecular diagnosis of β -thalassaemia subjects include PCR-based strategies consisting of high performance liquid chromatography (HPLC) (Reichert *et al.*, 2008), capillary electrophoresis (CE) (Chang *et al.*, 2004), restriction fragment length polymorphisms (RFLP) (Ward *et al.*, 1991), allele-specific oligonucleotide (ASO) hybridization (Baig *et al.*, 2006), reverse dot-blot (RDB) (Najmabadi *et al.*, 2002), allele-specific PCR, denaturing gradient gel electrophoresis (DGGE) (Dozy and Kan, 1994), amplification refractory mutation system (ARMS) (Baig, 2007) and direct sequencing (Chern and Chen, 2000).

In the current study, HRM Type it PCR master mix containing novel double-stranded DNA-binding fluorescent dye, EvaGreen, was used as a suitable dye to monitor the fluorescence signal while the amplicons were being slowly heated from 78 °C to 95 °C. Amplicons heterozygous for a FSC 36-37 (-T) yielded altered melting curves compared with control samples. As stated earlier, we successfully detected all the carrier of the FSC 36-37 (-T) mutation using HRM.

Two different categories of outputs were observed from the analysis of the HRM melting curve: The carriers of FSC 36-37 (-T) mutation and the carriers who did not have this mutation (TT); therefore, they were considered as control samples. Furthermore, we did not have thalassemia major patients, then we could not detect the (-T-T) genotypes in our samples. Moreover, according to the results of the SNP check 3, none of the observed SNPs in the amplicon, had high frequency.

HRM is a suitable technique for small amplicons genotyping with high sensitivity and specificity and have been used for SNP genotyping (Liew *et al.*, 2004), unlabeled probe genotyping (Zhou *et al.*, 2004), HLA

matching (Zhou *et al.*, 2004), and mutation scanning of various genes including *MCAD* (McKinney *et al.*, 2004), *c-kit* (Willmore *et al.*, 2004) and *PAH* (Amir *et al.*, 2017) genes. In a study conducted by Marashi *et al.*, 2012, the phenotype and genotype frequency of the most common β -globin mutations among the thalassemia major (TM) patients including FSC 36-37 (-T) mutation was successfully determined using HRM. Furthermore, HRM was used to detect beta thalassemia 3.5 kb deletion (Prathomtanapong *et al.*, 2009). Moreover, we have detected IVSII-1(G\A) mutation in *HBB* gene in the carriers of thalassemia (Akhondi *et al.*, 2016).

The main limitation of HRM method is that the accurate mutation cannot be easily identified and thus it needs to be used in combination with a sequencing method. Furthermore, it mildly affects with primer design constraints (Shih *et al.*, 2009). Its advantage over other genotyping and scanning methods is the fact that PCR amplification and melting curve analysis are performed within the same tube or plate and identifies sequences alterations without requiring post-PCR processing steps (gel electrophoresis and ethidium bromide staining). This makes it more convenient than other scanning methodology (Vrettou *et al.*, 2003).

In conclusion, high resolution melting is simple, fast, flexible and inexpensive over those methods previously described (Liew *et al.*, 2004; Prathomtanapong *et al.*, 2009; Marashi *et al.*, 2012).

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