**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.

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**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-γ0-γA-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 Sri Lanka 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 μl volume containing 2 μl genomic DNA, 2.5 μl 10X PCR buffer, 0.75 μl MgCl2 (50 mM), 0.5 μl (40 mM) dNTP mix, 0.5 μl of forward, (CTCTTGGGTTTCTGATAGGC) and reverse (TAACAGCATCAGGAGTGGAC) primers (10µM), 0.25 μl Taq DNA polymerase primers (5U/µl) (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 (Tm) 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.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>RS</th>
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<th>Global MAF</th>
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<tr>
<td>11:5226822</td>
<td>rs111851677</td>
<td>CTCTGCCTA[C/T]TGGTCT</td>
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</tr>
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</tr>
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<td>GAGAGA[A/C/G]TCAGTGC</td>
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<tr>
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<td>TTGGACC[A/C/G/T]AGA</td>
<td>0.0002/1</td>
</tr>
<tr>
<td>11:5226799</td>
<td>rs1135071</td>
<td>CTTAG[C/G/T]CTGCTG</td>
<td>0.0002/1</td>
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</tbody>
</table>
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 thalassaemia 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-thalassemia 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 thalassaemia 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 (Akondi 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).

Acknowledgements

This paper was derived from ZSA Master’s thesis and supported in part by a research grant from Shahrekord University, Shahrekord, Iran.

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