Detection of β-Globin Gene Mutations by Syto-9

Real-Time PCR and High Resolution Melting

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Abstract

Thalassemias, as the most important inherited blood disorders, are a wide group of inherited anemias that are caused by decreased levels or no production of either types of globin chains (α or β) by mutations in their genes. Mutations in β-globin gene are more common than those in α-globin one, worldwide. Due to the pattern of inheritance of this disorder, parental detection and newborn screening would be very important in disease management. This study aimed to detect the mutations of β-globin gene by Real-Time PCR among patients in Tonekabon area in North of Iran. Whole blood samples were collected from 10 heterozygous people (A₂> 3.5%) and one healthy control from health centers in Tonekabon in North of Iran. The mutations in 5 segments of β-globin gene were recognized by High Resolution Melting following DNA extraction and Real Time PCR. As the result, only one mutation at exon 1, FSC 8/9 (+G), produced a unique heteroduplex in heterozygous form, which allowed to detect carriers from those who were homozygous. Based on the results obtained in this study, Syto-9 HRM technique is not a precise method to detect the carriers of β-globin gene mutations and have to be used together with other detection methods.

Keywords: β-thalassemia, mutation, HRM, Real Time PCR, Syto-9
1 Introduction

A genetic disease is a condition caused by alteration in either amount or function of a protein encoded by a gene [1,2]. Study on genetic diseases at different levels such as gene, protein, cell, tissue and whole body, not only improves our understanding about DNA and its role in the body system, but also leads us to the appropriate methods of treatment [2].

Sickle cell anemia was the first disease defined in a molecular point of view [2]. Hemoglobin is the iron-containing metalloprotein in the red blood cells to transport oxygen in the body of vertebrates [3]. Normal hemoglobin consists of two pairs of globins [2,4]. The type of normal hemoglobin can be different depending on the combination of a pair of α-chains together with a pair of either γ or σ or β-chains [2,4,5]. Human adult hemoglobin consists of 95% α2β2 (HbA), 3% α2σ2 (HbA2), and 2% α2γ2 (HbF), approximately [1,2,4,6].

Beta-thalassemia is a disease caused by the abnormal production of β-globin, leading to the sedimentation of β-chain both in Red Blood Cell (RBC) and its erythroid precursors. Destruction of erythroid precursors promotes the production of inefficient RBC, extra-production of erythropoietin, and bone marrow cell proliferation. Bone marrow extension leads to the skeletal disorder, which is another major problem attached to the β-thalassemia [2,4,5,7]. Major β-thalassemia patients are diagnosed by age two through severe hemolytic anemia. Microcytic hypochromic anemia, bone marrow erythroid hyperplasia, hepatomegaly and splenomegaly are the other problems caused by the disease [1,2,3,7,8].

Beta-thalassemia gene mutations are detected by different PCR based methods [7,9]. In this study, High Resolution Melting Real Time PCR method was used to detect β-globin gene mutations.

2 Materials and Methods

Whole blood samples were collected from 10 heterozygotes (hemoglobin A2 above 3.5) and one homozygote healthy people through different health centers located in Tonekabon in North of Iran. About 2 ml of peripheral blood samples into EDTA anticoagulant were collected from all participants. DNAs were extracted by “High Pure PCR Template Preparation Kit” (Roche, Germany). Four sets of primers designed by Chinchan and his colleagues [6] together with one set of primers designed by current researchers were used to amplify coding and regulatory domains of β-globin gene, in where the mutations lead no production of β-chain. The primer sequences are given in table 1. The PCR for all 5 fragments was carried out in a volume of 25 µl including 200 pmol of each primer (Forward and Reverse), 100-200 ng genomic DNA, 2.5 units Hot Start Taq DNA Polymersase (Promega, USA), 1.5 mM MgCl2, 10 µM
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PCRs program was included 95 °C for 10 minutes as the initial denaturation, 40 cycles at 95 °C for 30 seconds, annealing temperature for each fragment based on table 1 for 30 seconds, and 72 °C for 30 seconds; followed by the HRM conditions: temperature raised from 75 °C to 92 °C with a 0.1 °C/s shift in temperature (Corbett Rotor-gene 6000).

All samples that had different Normalized and Difference curves in compare to the normal samples were sent to the Macrogen Company (South Korea) for direct sequencing by sense and antisense primers. The sequences were analyzed by BLAST tool to detect the polymorphisms.

Table 1. Primer sequences, amplicon sizes, and annealing temperatures of 5 fragments amplified by PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Region</th>
<th>Primer Sequence</th>
<th>Amplicon Size (bp)</th>
<th>Annealing Temp.</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| β-globin | Promoter | F: 5’-AAG AGC CAA GGA CAG CTA-3’
R: 5’-TTG CTA GTG AAG ACA GTT GTG-3’ | 179                | 55              | 6    |
| β-globin | Exon 1  | F: 5’-CAT TTG CTT CTG ACA CAA CTG-3’
R: 5’-GTT CTC CTT AAA CCT GTC TTG-3’ | 178                | 55              | 6    |
| β-globin | Exon 2  | F: 5’-TGG GTT TCT GAT AGG CAC-3’
R: 5’-ACA TCA AGC GTC CCA TAG-3’ | 304                | 52              | Current Study |
| β-globin | Intron 2 | F: 5’-TAC AAT GTA TCA TGC CTC TTTG-3’
R: 5’-CTA AGA ATA ATC CAG CCT TATC-3’ | 194                | 55              | 6    |
| β-globin | Exon 3  | F: 5’-CAT GTT CAT ACC TCT TAT CCT C-3’
R: 5’-CCC CAG TTT AGT AGT TGT ACT TAG-3’ | 230                | 55              | 6    |

3 Results

Both the HRM normalised and difference graphs obtained for all 5 fragments showed differences between heterozygotes and homozyhote control samples. However, only one mutation at exon 1, FSC 8/9 (+G), produced a unique heteroduplex in heterozygous form that allowed to detect carriers from those who were homozygous (Figure 1), while for all other mutations the carriers were not detectable from homozygous genotypes due to lack of heteroduplex formation in heterozygote form.
Figure 1. Normalised graphs, Difference graphs, and Melt data obtained for the samples that were heterozygote for the exon 1 of β-globin gene and their comparison with normal homozygote sample. Only one mutation [FSC 8/9 (+G)] has produced a unique heteroduplex.

4 Discussion

Although there are many methods to screen hemoglobin gene mutations, however, a more rapid yet precise method to detect mutations in clinical samples is still on demand. Kukret et al. (2002) used Allele Specific PCR to detect polymorphisms at β-globin group of genes among Indian populations. They worked on five common mutations among 56 individuals, of which 85% were successfully genotyped while 15% remained undetected [10].

Foglieni and colleagues in 2003 used Microchip method to detect 9 common mutations in β-globin gene among 250 patients. All the patients had previously been studied by some other methods too and the comparison between results obtained by this method and those of other methods showed that this method is 100% valid [11]. Multiplex ARMS technique was also used by Pornprasert and colleagues in 2013 to detect mutations in β-globin gene [12].

Different methods have been used to detect mutations at this gene in Iran, too. Pourfaizi and his colleagues studied on 11 common mutations among the Mediterranean population by ARMS-PCR. Reverse Dot Blotting together with ARMS-PCR were also used to detect common β-globin gene mutation among Iranians by Hashemi and colleagues in 2000 [9].
High Resolution Melting (HRM) is considered as a new, fast and cost effective method to screen nucleotide polymorphism. Theoretically, this method is able to discriminate all nucleotide polymorphisms either in homozygote or heterozygote forms. However, the method has shown less effective in practice due to the influence of different parameters such as the position of nucleotide polymorphism in the amplified fragment as well as the PCR efficiency. In this study, although differences between melting points and fluorescence emission patterns were observed between the samples and those of normal homozygote, however, except for one mutation, the alleles were undetectable due to the lack of heteroduplex formation for heterozygote samples. The results obtained by this study show that HRM could not be considered as the method of choice to detect β-globin gene mutations and should be replaced by other PCR based methods such as Allele Specific PCR or ARMS.

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References


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