

Full Length Research Paper

Detection of eight common β -globin gene mutation in thalassemia major patients using real time polymerase chain reaction (PCR)-high resolution melting and EvaGreen™ dye

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The aim of the present study was to determine the phenotype and genotype frequency of the most common β -globin mutations among the thalassemia major (TM) patients, using high-resolution melting (HRM) curve analysis as a novel, reliable and rapid scanning method. In this descriptive-analytical study, real time polymerase chain reaction (PCR) was applied besides HRM assay in the same reaction tube for mutation detection by melting curve behavior analysis of EvaGreen™ dye. The phenotypic and genotypic frequency of β -globin mutations between 120 patients including IVS-II-1(G-A), IVS-I-110 (G-A), IVS-I-5(G-C), FSC 8/9, FSC 36/37, Codon 30, IVS-I-6 (T-C) and IVS-I-1(G-A) was successfully detected. TM patients showed significant genotype heterogeneity. The clinical outcomes of our TM patients were mostly explained by the genotypes linked to the β^0 type of β - thalassaemia. Genotypic analysis showed 70 patients with homozygous mutations TM (40 β^0/β^0 and 30 β^+/β^+) and 15 with compound heterozygous TM (14 β^+/β^0 and 1 $\beta^0/\text{Hb Variant}$). HRM method as a simpler and cost effective way, accurately characterized the molecular basis of the TM patients.

Key words: β -Globin, β -thalassaemia major, real time polymerase chain reaction, high-resolution melting analysis, melting curve, direct sequencing, single nucleotide polymorphism.

INTRODUCTION

β -Thalassaemia is a common autosomal recessive disorder among the hereditary diseases worldwide. The β -thalassemias refer to that group of inherited hemoglobin disorders which are characterized by a reduced synthesis (β^+ -thalassaemia) or absence (β^0 -thalassaemia) of β -globin chain production which causes

anemia (Weatherall and Clegg, 2001). It is mostly caused by point mutations, a small deletions or insertions within the β -globin gene which is located as a cluster on the short arm of chromosome 11 (Sack, 1999). More than 200 different mutations of β -globin genes have been identified (Trent, 1997; Ho and Thein, 2000). It is most prevalent around the Mediterranean Sea, that is, countries like Greece, Italy, Turkey, and North African, Middle East and South East Asian countries. It is also seen in Iran; the gene frequency of β -thalassaemia in Iran is high and alters significantly from area to area,

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but around the Caspian Sea and Persian Gulf, more than 10% have the highest rate (Orkin et al., 1982). Since the Iranian population is a mixture of different ethnic groups, it is necessary to determine the frequency and distribution of mutations in the different parts of the country.

Traditional polymerase chain reaction (PCR)-based strategies consisting of restriction fragment length polymorphisms (RFLP), temporal temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE), reverse dot blot hybridization, amplification refractory mutation system (ARMS) and direct sequencing have been carried out for molecular diagnosis of β -thalassemia subjects (Losekoot et al., 1990; Cai et al., 1994; Dozy and Kan, 1994; Tan et al., 1994; Shaji et al., 2003). Post-PCR processing steps require the gel electrophoresis and ethidium bromide staining. Recently, novel advances in molecular diagnosis have been developed in which both PCR amplification and genotyping can be performed within the same reaction tube (Vrettou et al., 2003).

Using high-resolution melting (HRM), a Rotor-Gene 6000 instrument can characterize samples based on sequence length, GC content and DNA sequence complementarily. The HRM process consists of performing the PCR in the presence of the DNA binding dye EvaGreen™, monitoring the progressive change in fluorescence caused by release of the dye from a DNA duplex as it is denatured by increasing the temperature, collecting a high resolution melting curve, and identifying the samples with melting curve aberrations which is indicative of the presence of a sequence variant. HRM is used to detect single base sequence variations such as SNPs or to discover unknown genetic mutations (White and Potts, 2006).

Thereby, we evaluated β -globin chromosomes of a total of 120 affected patients (120 β -thalassemia major) by using real time-PCR high resolution melting method. Our findings detected allele frequency, provided genetic insights of TM occurrence in Qazvin province of Iran and will be useful in genetic counseling, treatment and management.

MATERIALS AND METHODS

Clinical specimens

In this descriptive-analytical research, the whole blood of 120 transfusion dependent β -thalassemia major patients were collected in EDTA-containing tubes. Ethics clearance was obtained from the medical research ethical committee of the Faculty of Medicine and Health Sciences of Qazvin Medical University of Iran and University Putra Malaysia, also, the patients signed an informed consent. DNAs were extracted from the whole blood samples by using AccuPrep® Genomic DNA extraction kit (Bioneer, South Korea). Finally, the extraction containing purified DNA was used for PCR or the DNA extraction was kept at -70°C until analysis.

Table 1 shows the primer sets for the detection of HBB gene mutations which occurs in exon and intron. Six of them were

depicted previously (Sarookhani et al., 2009). We also designed two primer sets on the HBB DNA sequences (NCBI Reference Sequence: NG_000007.3) using primer 3 online software, according to Corbett research amplicon design protocol (CorProtocol™ 6000-1 July, 2006). The accuracy and efficiency of the designed primers were tested with the BLAST search tool. Specific PCR is critical when results depend on the PCR melting profile, so gradient thermal cycler and gel electrophoresis was applied to obtain the best annealing temperature for each primer.

High resolution melting

The eight known β -thalassemia mutations including IVS-II-1(G-A), IVS-I-110(G-A), IVS-I-5(G-C), FSC 8/9, FSC 36/37, Codon 30, IVS-I-6(T-C) and IVS-I-1(G-A) were applied by real time-PCR HRM method. Polymerase Chain Reaction was performed in a total of 25 μ l final volume containing 2 (μ l) of genomic DNA, 5 μ l of iDNA master mix (genomic DNA, 0.5 μ l 10 mM dNTP mix, 2.5 μ l 10X PCR buffer, 1.25 μ l 50 mM MgCl₂, 0.5 μ l Taq DNA polymerase), variable volume of forward primer and reverse primer, 1 μ l of EvaGreen™ dye and variable volume (μ l) of distilled water for each mutation (total volume 25 μ l).

The HRM assays were performed using the Rotor-Gene™ 6000 real time rotary analyzer (Corbett research, USA). PCR amplifications consisted of an initial denaturation at 95°C for 10 min, followed by 35 cycles denaturation at 94°C for 1 min, variable annealing temperatures (depending on each mutations) for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min to ensure complete extension. All the samples were analyzed simultaneously with DNAs of known HBB gene mutation genomes (positive control), wild-type control DNAs and non template control (NTC). Positive controls were applied in order to reliably analyze all of the samples for particular mutation. An 861 bp internal control band was also amplified in all the reactions indicating successful PCR.

PCR Amplification cycles were automatically followed by HRM channel within the same reaction tube consisting of a temperature ramp from 70 to 90°C at a rate of 0.1°C (Increment) per 2 s just for 1 cycle. The temperature at which a peak occurs on the plot corresponds to the melting temperature (T_m) of DNA duplex.

Mutation screening

For SNP analysis, homozygous allelic variants were characterized by a temperature (x-axis) shift in a HRM melt curve whereas heterozygotes were characterized by a change in melt curve shape. The analysis of the result was carried out using Rotor-Gene 6000 software. We have normalized the melting curves with shifting of the temperature axis; normalization allows all the curves to be compared with the same starting and ending fluorescent signal level to aid interpretation and analysis. The difference plot was demonstrated differences in melting curve shape by subtracting the wild-type curve from particular mutation curve.

Direct sequencing

Further sequence analysis was applied on β^0 and β^+ samples. The PCR products were purified with AccuPrep® Gel PCR purification kit (Bioneer Corporation, South Korea). To confirm the efficiency of real time-PCR HRM technique as well as the identification of each particular mutation, the purified samples were analyzed on an automated sequencer analyzer (ABI-3730XL Capillary system, USA). The sequence detection was performed in the Version 1.4 of Finch TV DNA sequence viewer (Geospiza, USA).

Table 1. Primer sequences used in the real time- PCR high resolution melting analysis assay.

Mutation	Primer sequence (5' to 3')	NCBI reference SNP	Amplicon size (bp)	Annealing temperature (°C)
IVS-1(G-C)5	F:5'- ACCTCACCTGTGGAGCCAC-3' RM:5' - CTCCTTAAACCTGTCTTGTAACCTTGTTAG-3' RN: 5' - CTCCTTAAACCTGTCTTGTAACCTTGTTAC-3'	rs33915217	285	61
IVS-1(G-A)1	F:5'- ACCTCACCTGTGGAGCCAC-3' RM:5' -TTAAACCTGTCTTGTAACCTTGATACCGAT-3' RN:5' -TTAAACCTGTCTTGTAACCTTGATACCCAC-3'	rs33971440	281	61
IVS-1(T-C)6	F:5'- ACCTCACCTGTGGAGCCAC-3' RM:5' -TCTCCTTAAACCTGTCTTGTAACCTTCATG-3' RN: 5' -TCTCCTTAAACCTGTCTTGTAACCTTCATA-3'	rs35724775	286	62
FSC(8/9)	F:5'- ACCTCACCTGTGGAGCCAC-3' RM:5' - CCTTGCCCCACAGGGCAGTAACGGCACACC-3' RN: 5' - CCTTGCCCCACAGGGCAGTAACGGCACACC-3'	rs35699606	225	64
Codon 30(G-C)	F:5'- ACCTCACCTGTGGAGCCAC-3' RM:5' -TAAACCTGTCTTGTAACCTTGATACCAACG-3' RN: 5' -TAAACCTGTCTTGTAACCTTGATACCAACC-3'	rs1135071	310	64
FSC(36/37)-T	F:5'- ACCTCACCTGTGGAGCCAC-3' RM:5' - GGTAAGGACTCAAAGAACCTCTGGGTCCAG-3' RN: 5' - GGTAAGGACTCAAAGAACCTCTGGGTCCAA-3'	rs63750532	430	65
IVS-2(G-A)1	F:5' -TCTGTCCACTCCTGATGCTG-3' R:5' -TCAAGCGTCCCATAGACTCA-3'	rs33945777	193	58
IVS-1(G-A)110	F:5' - GAAACTGGGCATGTGGAGAC-3' R:5' - CAGCATCAGGAGTGGACAGA-3'	rs35004220	160	61

F, Forward primer; RM, reverse mutant primer; RN, reverse normal primer.

RESULTS

Molecular basis of 120 TM patients in Qazvin province of Iran

In this study, 120 Iranian individuals were enrolled to characterize the molecular basis of β -thalassemia major in northern Iran. Their clinical and hematological information such as blood transfusions, thalassemia appearance and age at diagnosis, hepatosplenomegaly and splenectomy was obtained by retrospective data. According to their inherited mutations genotype, two types were found: β -thalassemia homozygotes ($n = 61$) and compound heterozygotes for β -thalassemia and other β -globin defects ($n = 22$) who inherited two deficient β -globin alleles were also found. Statistical comparison showed that between the studied subjects, IVS-II-I(G-A) (25.4%) (G to A alteration) was the most common mutation. Table 2 shows the other known mutation in order of frequency; IVS-I-110 (G-A) (15.4%), IVS-I-5 (G-C) (13.3%), FSC-8/9 (+G) (5.8%), FSC-36/37(-T) (4.6%), Codon 30 (G-C) (2.5%), IVS-I-6 (T-C) (2.1%) and IVS-I-1(G-A) (0.8%). The first three mutations IVS-II-1(G-A), IVS-I-110 (G-A) and IVS-I-5 (G-C) accounted for about 54.2% of all of the mutations. We Genotypic analysis showed 70 patients

with homozygous mutations TM (40 β^0/β^0 and 30 β^+/β^+) and 15 with compound heterozygous TM (14 β^+/β^0 and 1 $\beta^0/\text{Hb Variant}$).

HRM analysis

The results were analyzed according to the normalized and difference plot. Generally, melting curve aberrations indicate the presence of a mutation. Melting curve shift was found for G to A and T to C conversion with higher resolution in comparison with G to C conversion, as EvaGreen™ dye was used in this research. Figures 1A and 2A depict a temperature shift for the mutant samples in comparison to wild-type sample, which have demonstrated the existence of homozygous allelic variant for mutations IVS-II-I (G-A) and IVS-I-5 (G-C). Figures 1B and 2B show difference plot against one of the control wild types that easily differentiated the mutant samples from the wild types; as the spread of wild-type curves increased, the sensitivity of mutation detection decreased. In contrast to Figure 1B, Figure 2B shows higher sensitivity, because there was no spread between the two wild-type samples. Figures 3 to 6 show other common mutations which were detected by analysis of the

Table 2. Allele frequencies and location of β -globin mutations identified in 120 patients (240 chromosomes).

Mutation	Types of Thalassemia	Location	Number of chromosome (homozygote)	Number of chromosome (compound heterozygote)	Allele frequency
IVS-II-I (G-A)	β^0	intron	42	19	61 (25.4%)
IVS-I-110(G-A)	β^+	intron	26	11	37 (15.4%)
IVS-I-5(G-C)	β^+	intron	28	4	32 (13.4%)
FSC-8/9	β^0	exon	10	4	14 (5.8%)
FSC-36/37(-T)	β^0	exon	10	1	11 (4.6%)
Codon 30(G-C)	β^0	exon	2	4	6 (2.5%)
IVS-I-6(T-C)	β^+	intron	4	1	5 (2.1%)
IVS-I-1(G-A)	β^0	intron	0	2	2 (0.8%)
Codon 8	β^0	exon	0	1	1 (0.4%)
Hb Monroe	Hb Var	exon	0	0	1 (0.4%)
unknown	-	-	-	-	70 (29.2%)
Total number of chromosome			122	48	170 + 70 = 240 (100%)

*unknown: the alleles was not detected with 8 common primer

normalized and difference plot.

A wild-type sample was chosen as the normalization sample for the construction of the normalized and difference plots to establish protocols for the interpretation. Moreover, a known β -thalassemia genotype sample that was realized previously was used in each assay to confirm the presence of the particular mutation. Nevertheless, all the PCR amplicons were also subjected to gel electrophoresis after HRM analysis to confirm the expected size of each amplicon. The results of the real time-PCR HRM assay were verified either with direct sequencing.

DISCUSSION

HRM is a novel methodology that represents a significant progress in mutation detection. Different base substitutions produce slight differences in melting behavior and the resolution of these melting differences requires an appropriate intercalating dye. SYBR green, LC Green, LC Green PLUS and SYTO 9 are the most widely used intercalating dye for monitoring PCR, but SYBR green is not suitable for HRM analysis, since the phenomenon of dye redistribution can hide the small differences in melting behaviour (Giglio et al., 2003; Monis et al., 2005; Herrmann et al., 2006). The third generation intercalating dyes such as Eva Green™ (Biotium Inc, Hayward, CA) have low toxicity in an amplification reaction; also, there is less dynamic dye redistribution to non-denatured regions of the nucleic strand during melting (Wittwer et al., 2003). In this study, EvaGreen™ was selected as a suitable dye; it was able to show β -globin gene mutations with higher resolution.

The study was initially conceived in order to validate a

new method for detection of single nucleotide polymorphisms whereby genetic information could be utilized to predict and detect β -thalassemia disease. In recent years, few investigations have been done in contribution to this method. In one study, using the real-time gap-PCR with SYBR Green1 and HRM analysis used for diagnosis of β -thalassemia 3.5 kb deletion, it was found that mutation could be directly genotyped from the melting curve shapes (Prathomtanapong et al., 2008). Shih et al. (2009) showed β -globin gene mutation using high-resolution melting analysis, they have to redesign their primers set because of SNP interference in identification of β -globin mutation. In our research, first, we have obtained a clear understanding of the sequence targeted, and then we checked the designed primers for species homology, intron-exon boundaries, splice sites and known SNPs to prevent any variation and SNP interference.

Real time PCR HRM machine allows assessment of amplification for all samples before HRM analysis as a quality control measure. Based on our knowledge, samples with poor or late amplification must be interpreted cautiously in HRM analysis. We observed a degree of variation of the melting plots within wild-type samples (Figure 1B). The presence of small amounts of non-specific amplicons and differences in PCR amplification between samples may cause the variation. Difference plots are visually the best way to compare melting profiles. The presence of mutated sequence, even in low ratio, produced difference plots that were easily discriminated from wild-type samples. According to our knowledge and literature research, it was demonstrated that difference plot showed different amount of fluorescence which was due to the different concentration of template DNA (Figures 1B, 2B, 3B, 4B,

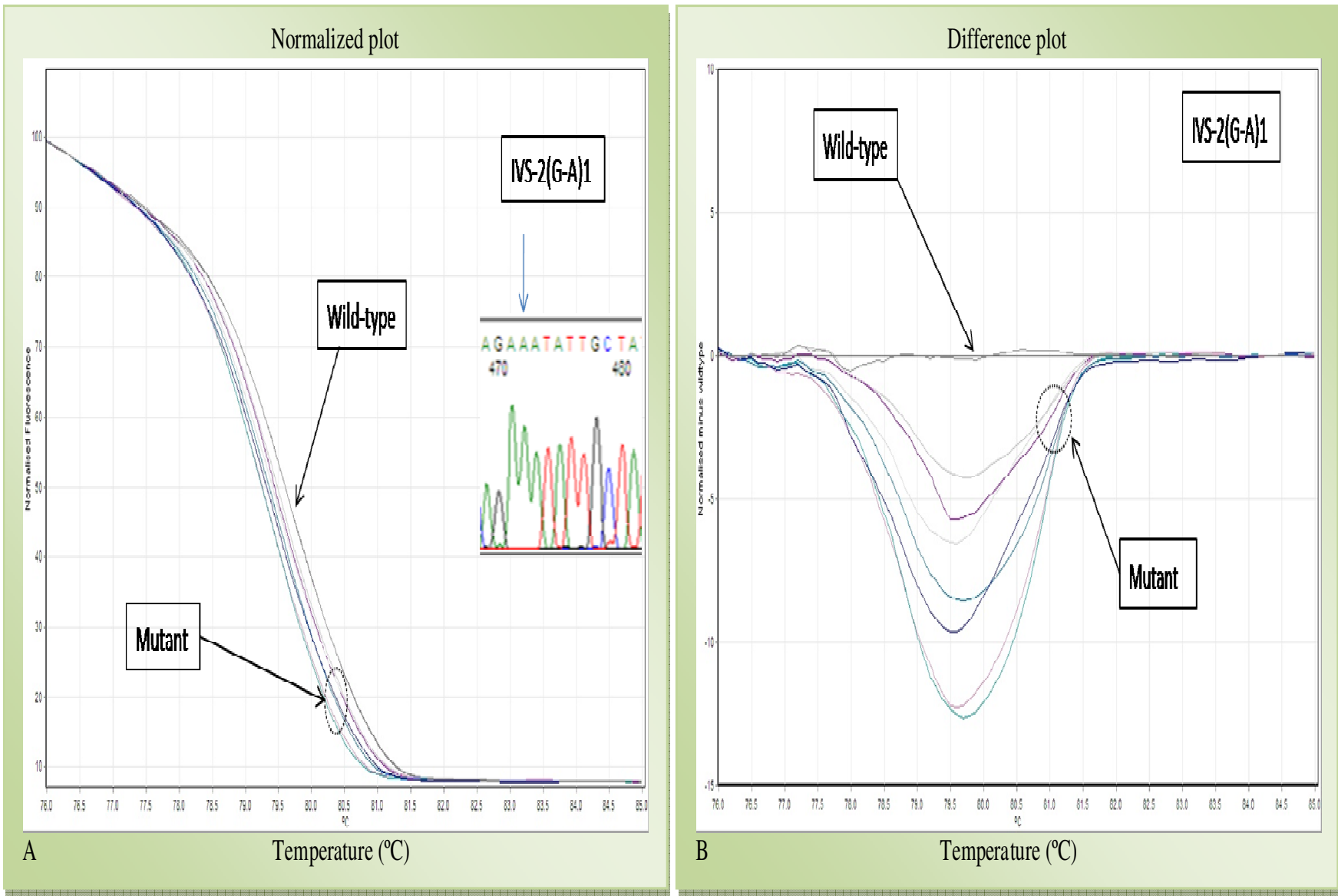


Figure 1. Screening of the β -globin gene mutations for IVS-II-1 (G-A). A, Displays the normalized melt curves of wildtype and mutant samples that differentiated primarily by a shift in the curve on the temperature axis (T_m shift), and also shows the electropherogram of sequencing analysis. B, Samples viewed as a difference plot against one of the controls wildtype.

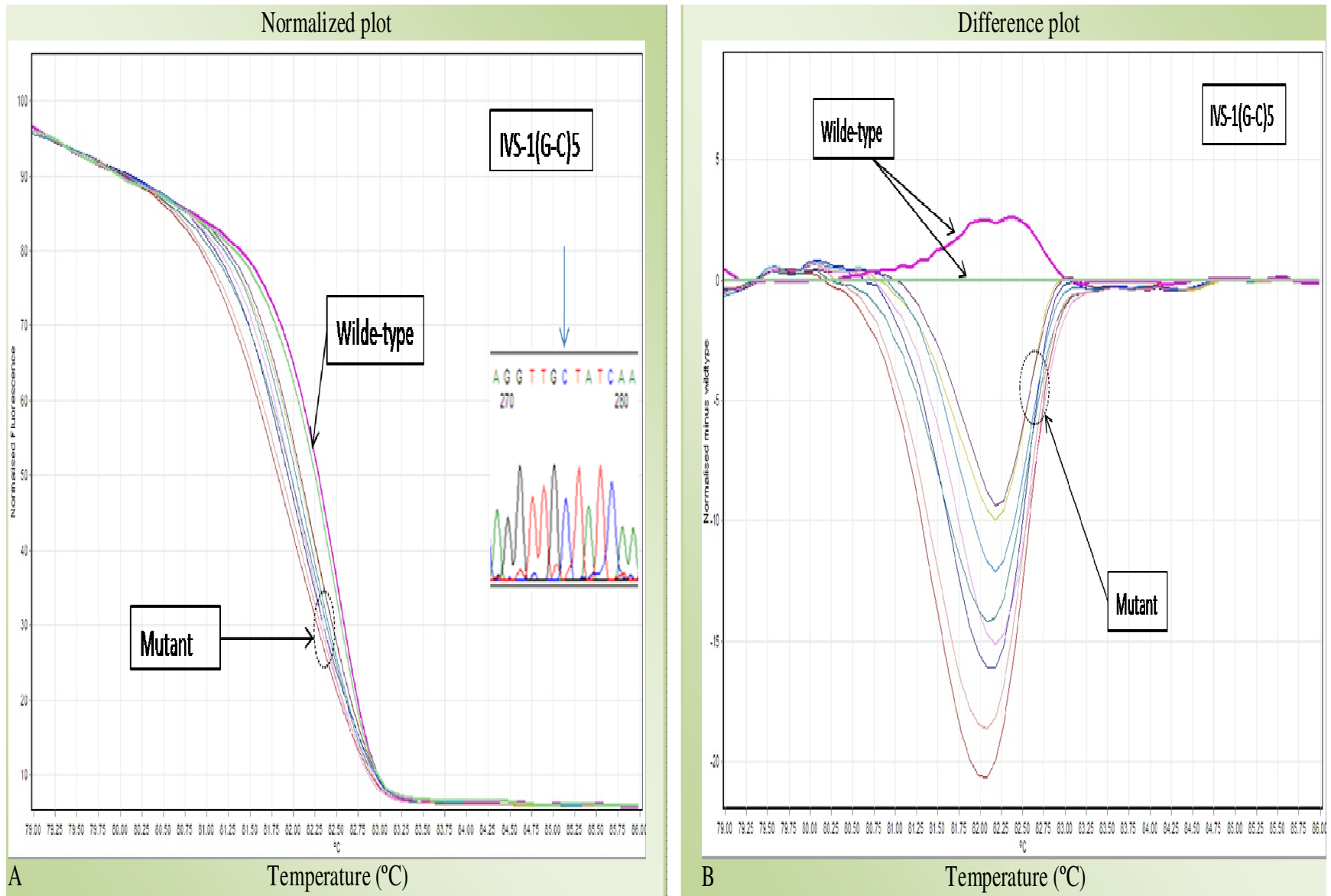


Figure 2. Screening of the β -globin gene mutations for IVS-I-5 (G-C). A: Displays the normalized melt curves of wildtype and mutant samples that differentiated primarily by a shift in the curve on the temperature axis (T_M shift), and also shows the electropherogram of sequencing analysis. B: Samples viewed as a difference plot against one of the controls wildtype.

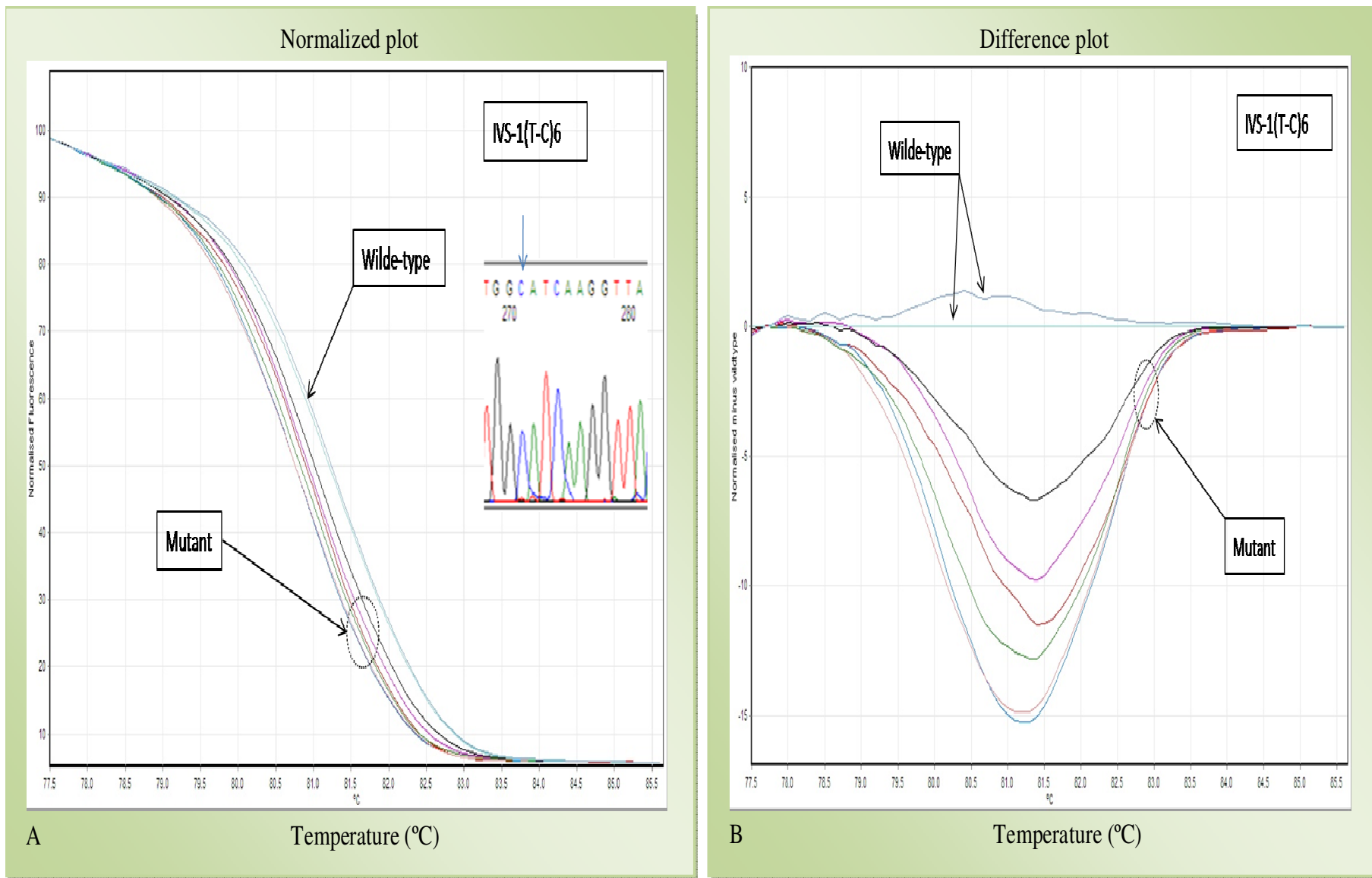


Figure 3. Screening of the β -globin gene mutations for IVS-I-6 (T-C). A: Displays the normalized melt curves of wildtype and mutant samples that differentiated primarily by a shift in the curve on the temperature axis (TM shift), and also shows the electropherogram of sequencing analysis. B: Samples viewed as a difference plot against one of the controls wildtype.

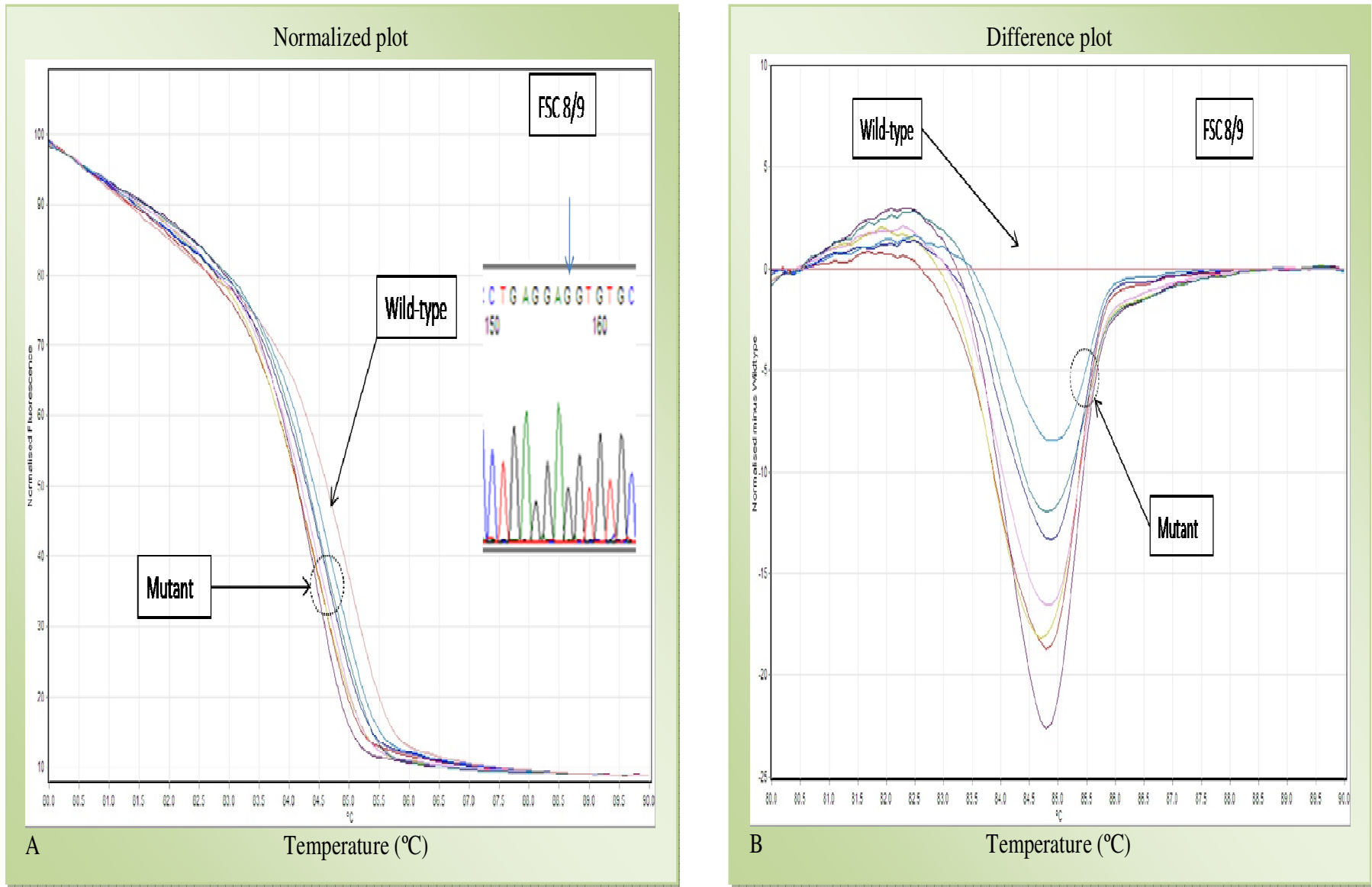


Figure 4. Screening of the β -globin gene mutations for FSC 8/9 (+G). A: displays the normalized melt curves of wildtype and mutant samples that differentiated primarily by a shift in the curve on the temperature axis (TM shift), and also shows the Electropherogram of sequencing analysis. B: Samples viewed as a difference plot against one of the controls wildtype.

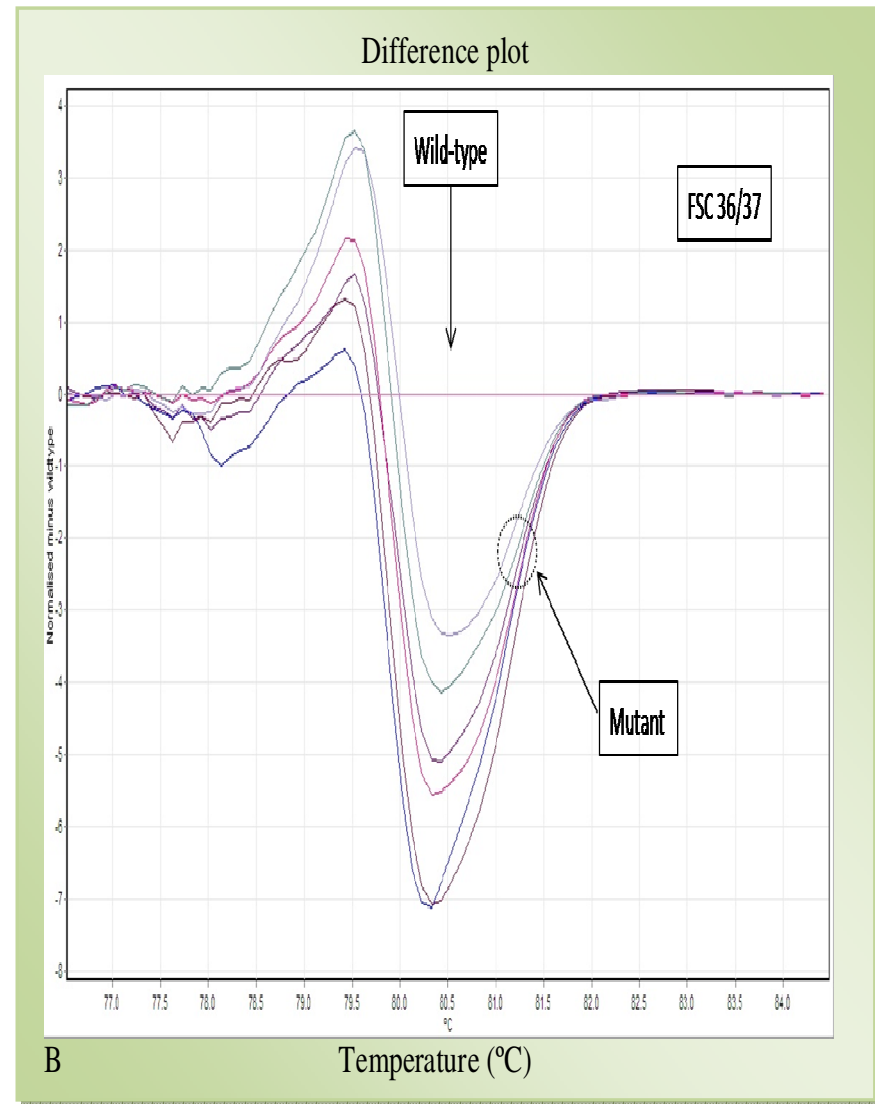
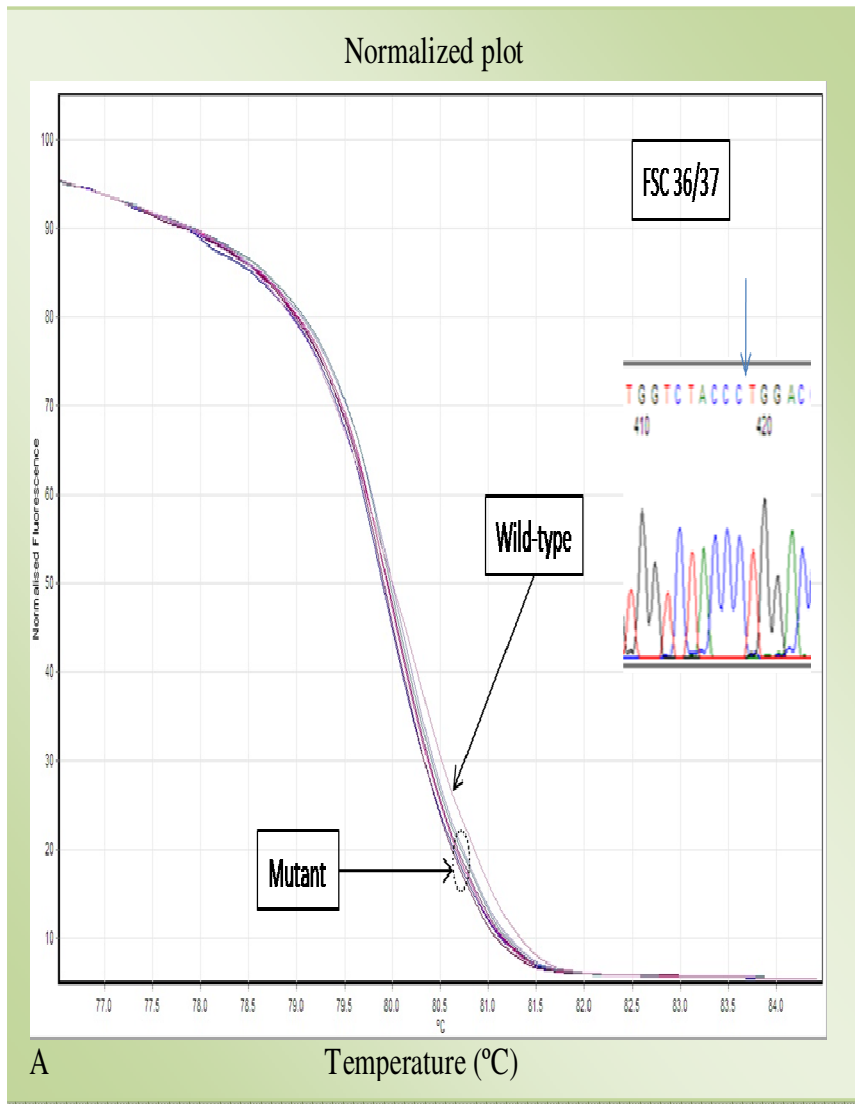


Figure 5. Screening of the β -globin gene mutations for FSC 36/37(-T). A: displays the normalized melt curves of wildtype and mutant samples that differentiated primarily by a shift in the curve on the temperature axis (TM shift), and also shows the electropherogram of sequencing analysis. B: Samples viewed as a difference plot against one of the controls wildtype.

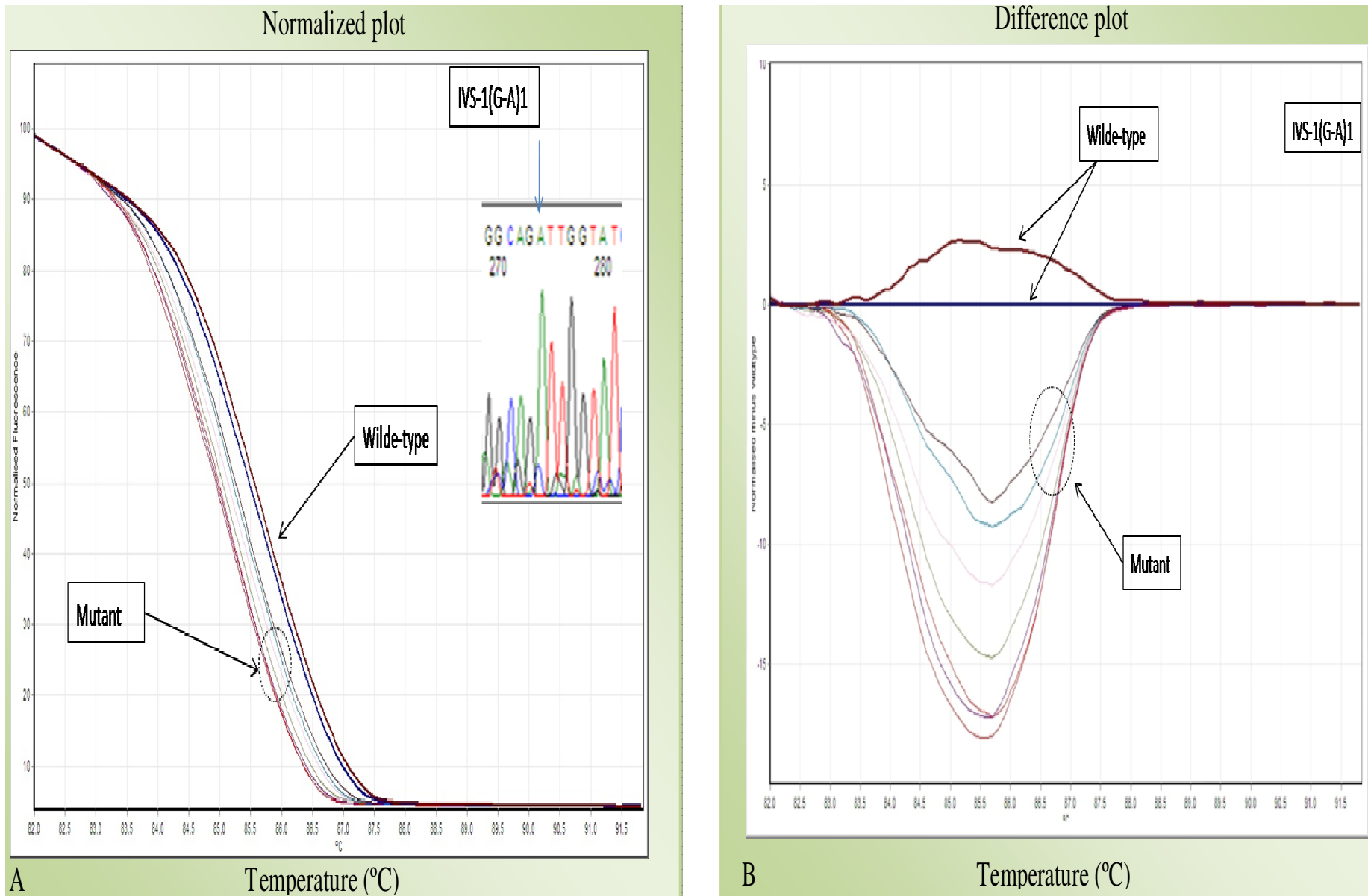


Figure 6. Screening of the β -globin gene mutations for IVS-1-I (G-A). A: Displays the normalized melt curves of wildtype and mutant samples that differentiated primarily by a shift in the curve on the temperature axis (TM shift), also shows the Electropherogram of sequencing analysis. B: Samples viewed as a difference plot against one of the controls wildtype.

5B and 6B).

Several studies on the frequency of mutations associated with β -thalassemia in Iran were carried out previously with different methods (Najmabadi et al., 2002; Habibi Roudknar et al., 2003; Derakhshandeh-Peykar et al., 2007). Each country or area was found to have only 3 to 4 common mutations regarding 70% or more of the beta thalassemia alleles (Weatherall et al., 2001). In this study, finally, three mutations [IVS II (G-A)₁, IVS I (G-A)₁₁₀ and IVS I(G-C)₅] of β -globin gene, were identified in Qazvin province which embraced approximately 54.1% of all the mutations. Sarookhani et al. (2009) showed three abundant alleles; IVS II-1, IVS-I-110 and FSC 8/9 in order of frequency, which was done using ARMS PCR. On the other hand, in contradiction to the findings of Sarookhani et al. (2009), we found IVS I nucleotide 5 in the third position that appeared with lower frequency in his research. Some other notable differences in the regional frequency of some mutations were also found. This was probably due to the bigger scope of this study as well as using high resolution melting analysis as a highly sensitive method.

An essential step in the development of HRM assays for mutation detection is sensitivity testing. Previously, the role of the size of the PCR product in SNP genotyping was examined and it was found that smaller amplicons gave better differentiation between genotypes (Liew et al., 2004). Consistent with those results, we found that using smaller amplicons (193 and 160 bp PCR amplicons) increased the sensitivity of mutation detection for β -globin gene mutations.

Conclusions

Thalassemia major patients showed a degree of heterogeneity in both phenotypic and genotypic aspects. Some cases (29.2%) could not be detected with known primers, so they remain unknown until further investigation. Consequently, we demonstrated a robust assay for screening the β -globin gene mutations that is applicable to clinical samples. The main limitation of HRM is that the precise mutation cannot be readily identified and it thus needs to be used in combination with a sequencing method. Nevertheless, because HRM is sensitive, inexpensive and has high throughput, we believe that HRM is now the method of choice for screening β -globin gene mutations in the β -thalassemia patients sample prior to sequencing, especially when the mutations are likely to occur at low frequency. This could be highly useful for carrier detection and prenatal diagnosis among the population residing in the Qazvin Province of Iran and other provinces of Iran.

ABBREVIATIONS:

HBB, Hemoglobin beta; **HRM**, High-Resolution Melting;

Hb, Hemoglobin; **Tm**, Temperature melting; **PCR**, Polymerase Chain Reaction; **SNP**, Single Nucleotide Polymorphism; **ARMS**, Amplification Refractory Mutation System, ARMS; **IVS**, Intervening sequence; **FSC**, Frame shift; **Cd**, codon; **TM**, Tahalssemia Major

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