

VAL34LEU POLYMORPHISM DETECTION BY REAL TIME PCR ASSAY USING FLUORESCENCE RESONANCE ENERGY TRANSFER ON ROTOR-GENE 6000

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ABSTRACT: Factor XIII (FXIII) Val34Leu is the most important polymorphism of the A subunit in factor XIII gene. The aim of the present study was to develop an efficient method based on real time PCR with Fluorescence resonance energy transfer (FRET) detection and melting curve analysis for the detection of Val34Leu polymorphism.

Specific primers were used to amplify the relevant fragment of the factor XIII-A gene and fluorescence resonance energy transfer hybridization probes were used for detection in a Rotor-Gene Q 5Plex HRM platform.

Melting temperature (*Tm*) for the wild type was at 68.8, while *Tm* for homozygote mutant was at 63.8; heterozygote demonstrated both peaks.

Our results showed that primers and probes proposed in this study demonstrated a high specificity to identify wild type, heterozygous and homozygous mutant genotypes.

Due to the increasing molecular diagnosis in developing countries and the importance of identifying polymorphisms, this real time PCR assay is of great importance. An important advantage of this approach is the high sensitivity and specificity.

Key words: factor XIII, val34leu, Fluorescence resonance energy transfer, real time PCR

1. INTRODUCTION

Blood coagulation factor XIII (FXIII) circulates as a protransglutaminase of two A and two B subunits (FXIII-A2B2) in plasma [1,2,3]. It consists of two potentially active A (FXIII-A) and two inhibitory/carrier (FXIII-B) subunits [4]. The gene coding for the FXIII-A (F13A) is located on chromosome 6p 24-25, it consists of 15 exons and 14 introns [5,6]. The gene of FXIII-B subunit (F13B) is located at position 1q31-32.1. The B subunit of FXIII is composed of 10 tandem repeats called sushi domains [6,7,8,9].

Five common polymorphism have been identified in the FXIII-A subunit, Val34Leu is the most important polymorphism [10,11]. This polymorphism is characterized by a → G → T transition at nucleotide position 100 leading to a Valine Leucine exchange at amino acid position 34 [12]. The FXIII activated by thrombin and Ca 2+ via cleavage the Arg37-Gly38 peptide bond of FXIII-A

subunit. Activated FXIII (FXIIIa) play a fundamental role in the final stage of coagulation cascade and stabilizes fibrin clots by forming fibrin γ -chain dimers that lead to increased rigidity of the thrombus [13].

Various studies have been conducted to discover the link between Val34Leu Polymorphism and venous Thrombosis, some studies have been shown that the Leu34 variant was associated with a decreased risk of venous thrombosis [14,15] but other studies have been shown conflicting results [13,16,17,18].

Several assays have been developed for detecting Val34Leu Polymorphism including single-stranded conformation polymorphism (SSCP) assay [12,14,19], amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) technique [20], allele-specific PCR [21] and restriction fragment length polymorphism (RFLP) [17,22]. But all these methods are labor-intensive and costly because post-PCR processing of PCR product is necessary. Our aim was to develop an efficient method for rapid detection of Val34Leu Polymorphism using Fluorescence resonance energy transfer on rotor-gene 6000.

2. METHODS

The study population consisted of 179 subjects who have been referred to Dr. Ashtiani's laboratory between Jan 2015 until Jan 2016. Genomic DNA was extracted from blood using Geno PlusTM Genomic DNA Extraction miniprep system (Viogene, China) according to the manufacturer's instruction. The prepared DNA was assayed immediately or stored at -20°C until being used.

2.1. The Primers and Probes

The primers and probes were designed with Oligoanalyzer version 2.1 software summarized in table 1. The sensor probe covering the polymorphic region was labelled with fluorescein at the 3' end. A t->G mismatch was introduced in the sensor to decrease the melting temperature. The anchor probe was labeled with the Cy5 fluorescent dye at the 5' end and blocked with phosphate at the 3' end to prevent extension during PCR. The gap between the two probes were two nucleotides.

Table 1. Primers and Probes used for Genotyping the FXIII-A Val34Leu Polymorphism

Primers/Probes	Sequence
Forward primer	5'-TCT AAT GCA GCG GAA GAT GAC-3'
Reverse primer	5'-CCC AGT GGA GAC AGA GGA TG-3'
Sensor	5'-GTGGAGCTTCAGGGCTTGGT-FAM-3'
Anchor	5'-Cy5-CCGGGGCGTCAATTGCAAGGTATGAGC (P)- 3'

2.2. FRET Real-Time PCR

PCR reaction were performed in a final volume of 25 μ l in each reaction containing 500 ng of genomic DNA, 0.8 μ mol/l of each primer, 0.16 μ mol/l of anchor probe, 0.16 μ mol/l of sensor probe, 2

U GoTaq DNA Polymerase (Promega), 2.5 μ L of 10 \times buffer, 0.5 mM of dNTP and 3 mM of MgSO₄. One sample without DNA, as a negative control, was included in all assay series.

3. RESULTS AND FINDINGS

In this study, the proposed protocol was performed on 179 individuals with a clinical History of thrombosis. The melting temperature (T_m) of the Val/Val was 68.8 (wild type), while T_m for the Leu/Leu was 63.8 (Homozygous mutant). Individuals with Val/Leu Genotype of the Heterozygous mutant showed both peaks (figure 1). One hundred and eight outof 179 DNA samples were wild type, 9 samples were heterozugous mutant and 1 sample was homozygous mutatnt. Our results showed that primers and probes propoed in this study demonstrated a high specificity to identify natural homozygous genotypes. Heterozygous and homozygous mutant.

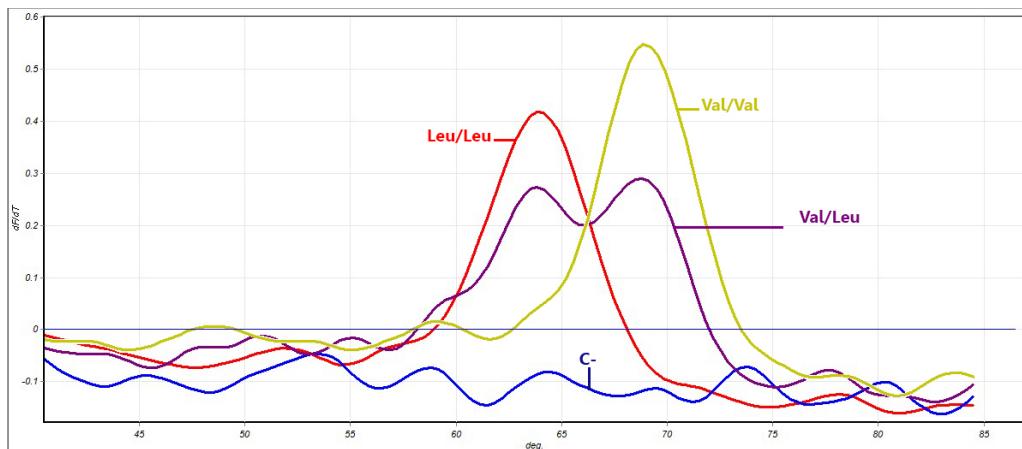


Figure 1. Melting curves

4. CONCLUSION

A FRET Reat-Time PCR method using primes and probes for the identification of Val34Leu polymorphism was developed. Many techniques have been developed to detection Val34Leu polymorphism. Early methods, such as single-stranded conformation polymorphism (SSCP), amplification refractory mutation system-polymerase chain reaction (ARMS-PCR), allele-specific PCR and restriction fragment length polymorphism (RFLP) are too time consuming, labor intensive and require considerable technical skills.

In this study Genotyping was performed with Rotor gene system. An advantage of FRET hybridization assays includes a high level of specificity and the ability to perform melting curve analysis. This method is a high-throughput, efficient, accurate and rapid assay, with this method there is no need for processing after amplification, thus eliminating PCR contamination concerns.

5. REFERENCES

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