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Screening for mutations in 10 exons of the 5 coagulation factor gene in Gilan province thrombosis patients

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ABSTRACT

The Coagulation factor Vis a cofactor protein of the coagulation system.Deficiency of this protein leads to predisposition for hemorrhage, while some mutations predispose for thrombosis.Whole blood samples were collected from 46 individuals to thrombosis problems from the Rasht Ashtiani laboratory with medical ethics. After DNA extraction, screening performed for presence FVL and others mutations in 3, 4,6,7,8, 12, 13, 15, 16 and 25exons via Real-time PCR and PCR sequencing.Out of 46 samples, the mutations were identified in 12patients. Among of them, 3 patients were heterozygous for Factor 5 Leiden, 4patients were heterozygous for 19257 G>C missense mutation in 3 exon, 3patients were homozygous for 48571 A>G missense mutation in 13 exon and 2patients were homozygous for 61795 A>G missense mutation in 16exon.Although these mutations have been reportedin the human populationsbut the linkage between these mutations and the predisposition to thrombophilia could be indicative of indirect their role in causing thrombosis.

Keywords: Coagulation factor V, thrombosis, Real-time PCR.

INTRODUCTION

Coagulation factor V is a critical factor of the blood coagulation cascade. This factor circulates in plasma, and is converted to the active form by the release of the activation peptide by thrombin during coagulation [1]. The active factor V is a cofactor that participates with activated coagulation factor X to activate prothrombin to thrombin[2]. Defects in this gene result in either an autosomal recessive hemorrhagic diathesis or an autosomal dominant form of thrombophilia, which is known as activated protein C resistance[3,4].The gene for factor V is located on the 1q23chromosome. The gene spans 70 kb, consists of 25 exons, and the resulting protein has a relative molecular mass of approximately 330kDa[5].According to large size of the Factor 5gene, many mutations have been identified in the human population, However not all of them are associated with thrombophilia[6].The factor 5 gene mutations that cause resistance to C protease and associated with thrombophilia, can be cited the following mutations: Arg306Gly (Hong Kong), Ile359Thr (Liverpool), Ile387Thr, Cys613Arg, Arg334Thr, Arg679Gln, Arg506Gln (Leiden), Arg306Thr (Cambridge)[7].In this study, we evaluated the presence mutations in several main exons of factor V gene in individuals which due to thrombosis problems admitted to laboratories.

MATERIALS AND METHODS

Patients

46 Blood samples from individuals with thrombosis problems (4 males and 42 females) were collected in a case report study. They were referring to the Rasht Ashtiani laboratory in Gilan (Iran)for the 6-monthperiod. These individualsthe ethnically were belonging to different regions of Gilan and were with the mean age 38 ± 5 years old rangedfrom 30 to 60 years old.The Ethic clearance for thisstudy was acquired from the Ethical Committee of Faculty of Medicine, Tonekabon Islamic Azad University, Iran; and informed consent was obtainedfrom all participants.

Isolation of Genomic DNA

One milliliter of peripheral blood samples intoEDTA anticoagulant were collected from all participantsand were immediately stored at -20°C. DNA was isolated from Whole blood using the High Pure PCR Template Preparation Kit (Roche, Germany) according to themanufacturer's instructions.The quality and quantity ofextracted DNAs were analyzed by Agarose gel electrophoresis and Biophotometer (Eppendorf, Germany).

Detection of Factor V Leiden (G1691A)

For the detection of Factor V Leiden mutationwere used of the commercial kitPZP Institute (Iran).This technique is based on the FRET technology and requires to Rotor Gene instrument (Qiagen, US).This test performed according to the manufacturer's instructions.

Investigations on mutations of the other exons

Identification of nucleotide changes in 3, 4, 6, 7, 8, 12, 13, 15, 16 and 25exons performedin all sampleswith the primers used in the Previously study Chegeni and Van Wijk[8,9] via Real Time PCR and High Resolution Melting**.**(Table 1).Each PCR reaction tube contained 50-100ng of each DNA template, 2.5µl 10X buffer, MgCl2 1.5mM, dNTPs0.2mM, 10pmol of each primer, 2.5 U of Taq DNA polymerase (Fermentas, Burlington, Canada) and 2mMSYTO-9 (Invitrogen, USA) in a total volume of 25 µl. HRM-Real-Time PCR conditions consisted of a pre-denaturation at 95ºC for 4min followed by 35 cycles of 95ºC for30sec, 51 to 59 ºC for 40sec, 72ºC for 40sec and a final extension of 72ºC for 5 min. HRM was analyzed in 70ºC-90ºC witha 0.2ºC temperature shift in 5 second and absorption in Green Channel. The PCR products were analyzed on a 2%agarose gel and confirmed by presence of PCR band.

Table 1- FV gene amplicon size and primer sequences

Primer	Sequence 5' to 3'	Size(bp)	Annealing (C°)	Reference
Fve3-f Fve3-R	GATGACCCTGAATACAGACATAG GATGCTGGTATTAAAGACTTAGAC	228	59	9
Fve4-F Fve4-R	ACTGCCCACATGTCTTGATGG TGACAGAACTCCTGACCATTCC	311	58	9
Fve6-F Fve6-R	GCCTAATCCTTTAGCAATCCCTG CATTGAGAAGCAAGACTGTCAGG	548	58	9
Fve7-F Fve7-R	GAGTTATTTCATTGTCTTTCTGTCC GTCTTGAACCTTTGCCCAG	241	58	9
Fve8-F Fve8-R	GCAGAATGTTTAAGCACAAGG CTATGTAATTTCTCCCATGATTCTG	306	56	9
Fve12-F Fve12-R	CATAGACTTGGAATTTTAACAG CAAGCTTCCTCTGTGAGTGTC	286	50	9
Fve13a-1F Fve13a-1R	GTCTTTTCCCAGACTTCCAG TGTTCTGGTAATCATAGTCAGC	255	58	8
Fve13b-2F Fve13b-2R	CCAACAAGCCTGGAAAGC CTTTCTGAAATTTCTGAAAATG	229	57	8
Fve13c-3F Fve13c-3R	ATAGTGGGCCTCAGTAAAG TTTTTTCAGCAGTAATGG	317	51	8
Fve15-F Fve15-R	GGCCATATCTCACAGGATGG GTCATCTGAAGAGCTGCATGG	600	56	9
Fve16-F Fve16-R	TCCTGAGAAAGAGGCAATAC TCTTGTGAATATCTAAGGGC	333	52	8
Fve25-F Fve25-R	AGCCATTTATGTTGTCATTAAAG TAATAGCCATTATCTTACTTACTG	390	55	8

High-resolution melting analysis was performed on the software Rotor-Gene6000 Software (v1.7) and the Difference graph and Normalized graph was used for data analysis and was prepared curve-negative derivative of fluorescence (F) versus temperature (df/dt).

DNA sequence analysis

To confirm the presence and identify the type of modified nucleotide in fragments with different melting curvethan control, PCR reactions were performed on selected samples in MyCcler PCR machine (Bio-RAD, CA, USA). PCRconditions were the same as HRM Real-Time PCR (50µl total volume and 35 cycles) and contained all theingredients except SYTO-9. After qualitative confirmation of each of amplicons, PCR sequencing was performed with both forward and reverse primers (Macrogen, Seoul, Korea).Resulted sequences were covered with DNAman software (Version 5.2.9)(Lynnon Bio Soft) and then aligned with National Center for Biotechnology Information(www.ncbi.nlm.nih.gov) Gene Bank sequences using online SNP flanks software to identify each of SNPs.

RESULTS

Out of the 46 patients,three heterozygous genotypeswere identified for factor V Leiden mutation (G1691A) by PZP Kit.Figure 1 show the FRET Real Time PCR for factor V Leiden mutation. Also the nucleotide screening by HRM technique and then direct sequencingshowed three types of missense mutations in 3, 13 and 16 exons of factor 5 gene in 9 patients. Figure 2 show the melting graph exon 3 of factor 5 gene.

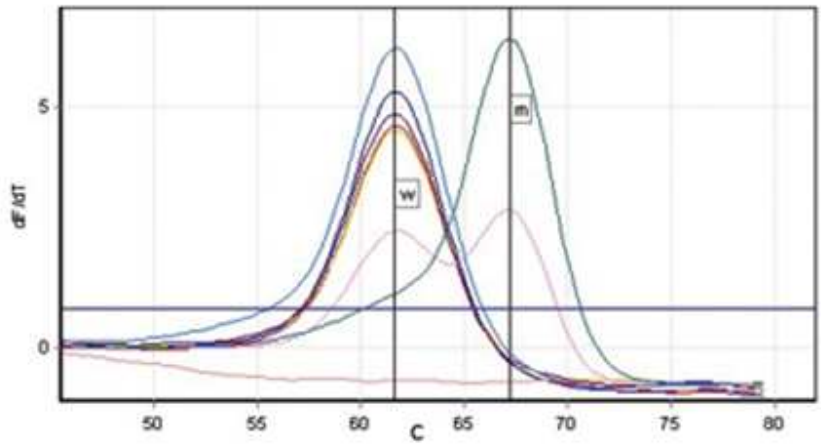

Figure 1. FRET Real Time PCR for factor V Leiden mutation by PZP Kit.The Heterozygote genotype two Peaks are detected in Melt channel FAM at both 62 ± 1°C and 67± 1°C while in homozygote normal and mutant are detected only one peak one of at 62 ± 1°C and other at 67± 1°C respectively

Four out of patients had the missense mutation at codon 107 (Asp107His) (19257 G>C) with heterozygous genotype in exon 3. Three out of patients had the missense mutation at codon 710 (His710Arg) (48571 A>G) with homozygous genotypes and two out of patient had the missense mutation at codon 1764 (Met1764Val) (61795 A>G)with homozygous genotypes.

Also we detected two types of SNPsin 4 patients. Two out of them had A/C substitution(51287 A>C)[rs9332609] with heterozygousgenotype in 13 intron and the remaining two patients had A/C substitution (77008 A>C) [rs2227243] with homozygous genotype in 25 intron.

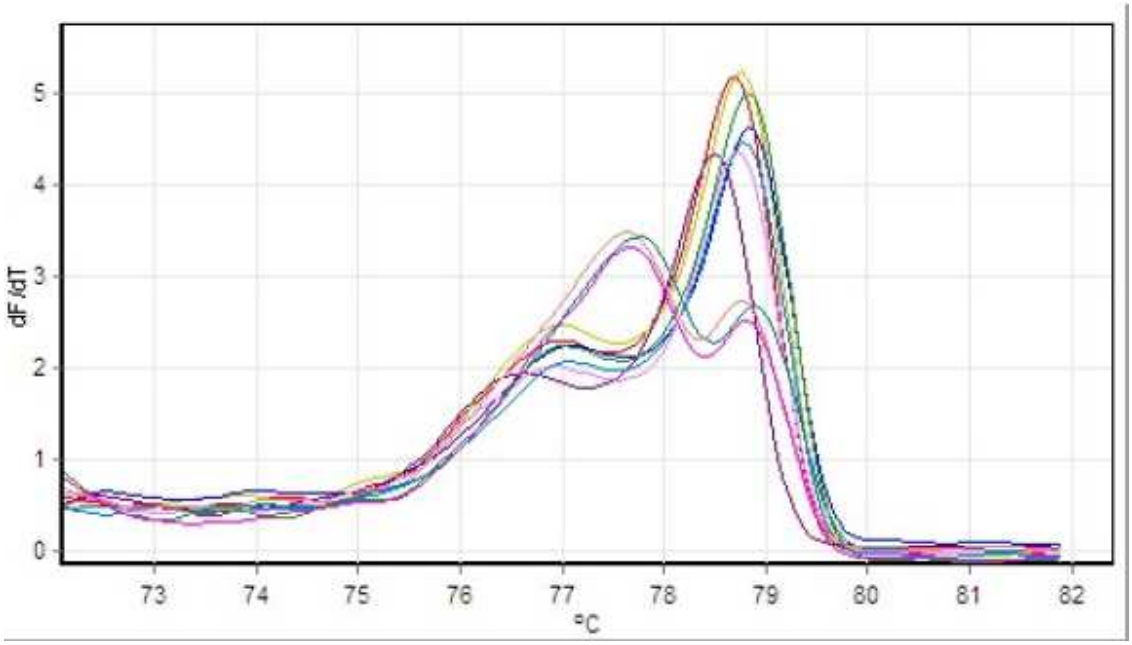

Figure 2.Melting curve analysis obtained for exon 3 in some of samples. Graphs showed two different melting patterns

DISCUSSION

Despite many studies that have been done on the coagulation factor 5geneis stilllittle knowledge about how itinduced the disease.Due to the large size of factor 5 gene,many mutations have been identified in the human population, but not all of them are associated with thrombophilia.In this study, we have detected for the first timethe presence of three unrelated factor V gene mutations in Iranianpatients with predisposition to thrombosis.In our study population, three missense mutations, Asp107His (8.69%), His710Arg (6.52%) and Met1764Val (4.34%) were identified.So far, evidence of the association of these mutations has been reported with a predisposition to thrombophilia. Although these mutations were previously reported in human populations,

However, the high frequency of these mutations in patients with suspected to thrombophilia can be a sign of the relationship between them along with other factors.

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