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Coagulation Factor V Mutations in Iranian Patients with Venous Thrombosis

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Abstract: Human coagulation factor V serves as a cofactor for factor X in thrombin formation. Generation of thrombin leads to fibrin and clot formation. In this study 65 patients were selected with venous thrombosis. Patients with a positive Protein C resistance were chosen and screened genetically by Conformation Sensitive Gel Electrophoresis (CSGE) for the presence of factor V mutations, the suspected mutations were sequenced. Three new mutations were found as follows, Met 385 thr, Met 1736 Val and Lys 386 Lys. Present study shows factor V mutations leading to thrombosis that have been never reported in Iran

Key words: venous thrombosis, factor V Leiden, activated Protein C resistance, Iran

INTRODUCTION

Human coagulation factor V acts as a cofactor in converting prothrombin to thrombin. In the coagulation process factor V is first converted to its active form, factor V_a in plasma, this is accomplished by factors X_a and/or α-thrombin[1]. Factor V is a single polypeptide chain molecule composed of 25 exons and 2224 amino acids. Upon activation by thrombin or factor X_a, factor V is cleaved into a dimmer composed of a heavy chain and a light chain. Factor V has five structural domains A_1, A_2, B, A_3, C_1 and C_2.

Once activated the B domain coded entirely by exon 13 is degraded. Activated factor V (FV_a) is a cofactor for factor X_a; together they form the prothrombinase complex, which activates prothrombin into thrombin leading to fibrin and clot formation. Factor V_a is quickly inactivated by Activated Protein C (APC) for maintenance of a balance in hemostasis[1]. The inactivation is a sequential process factor V_a is first cleaved at Arg 506 and subsequently at Arg 306 and Arg 679. Mutation in any one of these amino acids will predispose the affected individual to develop thrombosis a state called thrombophilia. APC resistance is present in 3 to 5% of asymptomatic Caucasians and is found in approximately 20% of unselected patients with venous thrombosis[2,3]. In at least 95% of cases resistance to APC is caused by a single point mutation of Arg 506 in the factor V gene[4]. A transition (G to A) at nucleotide 1691 in exon 10 results in the synthesis of a variant factor V molecule (Factor V Leiden) with the substitution Arg → Gln at amino acid position 506[5-7]. Patients with this genetic mutation may experience recurrent thrombotic episodes, throughout their lives. A heterozygous state for this mutation is associated with a 5 to 10 fold increase in the risk of thrombosis and a 50 to 100 fold increase for the homozygous state[8-10]. In approximately 5% of all patients with phenotypic APC Resistance, the Arg 506 Gln was absent and there seems to be other mutations involved that can lead to thrombosis. Other possible factor V mutations have never been evaluated in the Iranian Thrombosis patients.

The aim of this study was to evaluate the presence of other Factor V mutations in Iranian patients with venous thrombosis.

MATERIALS AND METHODS

Patients: APC resistance was measured on plasma samples from 65 patients by modified APC resistance assay after a diagnosis of deep vein thrombosis or pulmonary embolism was made. Deep vein thrombosis was diagnosed by ultrasound and venography and pulmonary embolism by ventilation-perfusion lung scanning. Sample number was obtained by the statistical formula for estimated sample number.

Modified APC resistance assay: APC resistance in the presence of factor V-depleted plasma was assessed using the Coatest APC resistance - C kit and factor V - depleted

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plasma (Chromogenix)[11]. Plasma was prediluted 1 in 5 with factor V-depleted plasma and APC sensitivity ratios were determined as in standard assay. Modified APC sensitivity was less than 120 sec in patients and greater than 120 sec in normal individuals. Patients suspected of having the mutation by this coagulation test were analyzed genetically for the presence of factor V mutation.

Factor V exons amplification: Genomic DNA was extracted from whole blood in each patient and six different exons including exons 4, 7, 8, 13, 16 and 25 were amplified by PCR in every patient, these exons had the highest chance of finding mutations according to literature. The amplification primers were designed to include 50 bp upstream and downstream of each exon by this possible mutations in the exonic sequence will be definitely detected by conformation sensitive gel electrophoresis. Each amplification reaction (50 μL) contained 0.5 μg of genomic DNA, 250 μmol L^{-1} of each deoxynucleotide triphosphate, 20 pmol of each amplification primer and 0.25 U of taq DNA polymerase (Cinnagen, Tehran) in 10 mM Tris - HCl, 50 mM KCl, pH 8.3 and 1.5 mM mgCl$_2$. The amplification was performed with an Initial denaturation for 5 min at 94x°C, followed by 30 cycles including denaturation at 94°C for 30 sec annealing at 56°C for 40 sec and extension at 72 for 40 sec with a final extension for 5 minutes at 74°C[12]. At the end, 10 μL of the reaction mixture was analyzed by electrophoresis on a 2% agarose gel.

Scanning of the PCR products by CSGE: For heteroduplex analysis by Conformation Sensitive Gel Electrophoresis (CSGE)[13], PCR products were electrophoresed in a 1 mm thick gel with 22-well comb prepared with 10% polyacrylamide, 99:1 ratio of acrylamide (Sigma) to 1, 4-bis(acryloyl) piperazine (Fluka), 10% ethylene glycol (Sigma), 15% formamide (GIBCO), 0.1% ammonium persulfate (Sigma) and 0.07% N, N, N', N'-tetramethylethylenediamine (Sigma) in 0.53 TTE buffer (44 mM Tris14.5 mM Taurine/ 0.1 mM EDTA buffer, pH 9.0). It was important not to autoclave the TTE buffer to obtain optimal separation of heteroduplexes and homoduplexes. The optimal polymerization time was about 15 min. PCR products containing heteroduplexes were mixed with 5 microliter of stock loading buffer (stock solution of 30% glycero 10.25% bromphenol blue 0.25% xylene cyanol FF). Samples were separated by electrophoresis on a standard DNA sequencing gel apparatus with 37 cm glass plates using 0.5X TTE as the electrode buffer. The gel was pre-electrophoresed for 30 min and then samples were electrophoresed at room temperature using 40 W for 4 h. After electrophoresis, the gel was stained on the glass plate in 1 mg mL^{-1} of ethidium bromide for 10 min followed by destaining in water. A hand-held UV torch was used to visualize the bands. The gel was photographed with either Polaroid camera or high-quality charge-coupled-device camera for gel documentation. This method is basically used for screening unknown mutations

Sequencing the suspected mutations: Briefly the each patient's exon with suspected mutation was sequenced by an automated Pharmacia –Amersham Sequencing machine and the results were analyzed using the Alfwin software

RESULTS

Screening the PCR products by CSGE: Six exons were screened in each patient. Exon 8 in one of the patients and exon 16 in fifteen patients showed heteroduplexes on CSGE gel (Fig. 1).

Sequencing the suspected exons: In exon 8 we found two mutations, first was a missense mutation when a cytosine was transverted to Thymidine causing the replacement of Methionin by Threonin in amino acid 385 [Met 385 Thr(T>C)]. The second mutation was a silent mutation with an Adenine to Guanine transition and no amino acid change [Lys 386 Lys (A>G)].

In Exon 16 we found a missense mutation in fifteen patients. This again was an Adenine to Guanine transition causing the replacement of Methionin by Valine [Met 1736 Val(A>G)]

DISCUSSION

In this study for the first time we have analyzed the presence of factor V mutations in Iranian patients with venous thrombosis. Two of these mutations (Met 385 thr, Met 1736 Val) are part of the HR2 haplotype and are known to be risk factors for thrombosis, they have never been reported in Iran. We also found a silent mutation(Lys 386 Lys) which has never been reported in
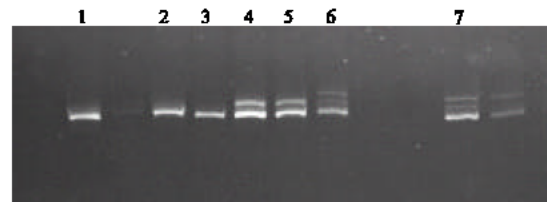

Fig. 1: CSGE analysis of exon 16
Lanes: 1, 3 and 4: Normal patients, Lane 2, 5, 6 and 7 patients with suspected mutations

the world but we still don't know if this could also be a risk factor, future investigations are needed to determine the significance of this mutation. There are other genetic risk factors as well predisposing an individual to develop venous thrombosis. Protein S, protein C and antithrombin deficiency are examples of important genetic risk factors. Acquired risk factor such as obesity, cancer, contraceptive pills, pregnancy and smoking also play a role in the development of venous thrombosis.

It is easy to see that venous thrombosis is a complex multifactorial disease. Several genetic and acquired risk factors must come together and through the interplay of these factors venous thrombosis develops.

Venous thrombosis annually affects 1 in 10000 persons younger than 40 years and 1 in 1000 persons older than 75 years of age causing significant morbidity and mortality[14].

Differences in geographic distribution of factor V mutations have a wide frequency spectrum. In western societies APC resistance by factor V mutations other than the famous Leiden mutation (Arg 506 Gln) are not very common; therefore present aim was to see if factor V low frequency mutations have the same frequency as in western societies. Present report shows the frequency of other factor V mutations to be higher in our patients with venous thrombosis.

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