Novel single nucleotide mutations in exon-10 of human coagulation Factor V gene in patients with pulmonary thromboembolism

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Introduction

The presentation of acute pulmonary thromboembolism (PTE) has wide spectrum and has variable prognosis. Factor V Leiden (FVL) is the most common inherited thrombophilia, with a prevalence of 3%-7% in the general US population, approximately 5% in Whites, 2.2% in Hispanics and 1.2% in Blacks; PTE most commonly originates from venous thrombosis. The occurrence of venous thromboembolism is a culmination of environmental and genetic risk factors. The current study was sought to identify the mutations in exon-10 of FV gene in patients with PTE.

Methods: Sixty cases diagnosed with PTE and 50 healthy controls were enrolled in the present study. Mutation studies in exon-10 of Factor V gene included PCR-DNA sequencing method.

Results: Of 60 patients, we found two novel transition type point mutations: c.1538 G>A and c.1601 G>A in exon-10 of Factor V which is responsible for the cleavage site for aPC. These point mutations resulted in single amino acid change in protein sequence at p.Arg513Lys and p.Arg534Gln respectively. These mutations prevent efficient inactivation of Factor V and Factor V remains active which facilitates over production of thrombin leading to generation of excess fibrin and excess coagulation which results in deep vein thrombosis and PTE.

Conclusion: We report two novel point mutations (c.1538 G>A and c.1601 G>A) in exon-10 of Factor V gene in Indian patients with PTE.
**Factor V gene mutations in PTE**

[confirmed on CTPA] and admitted in the department of Cardiology, SVIMS for treatment.

**Controls**

Fifty healthy voluntary subjects without any documented signs and symptoms for PTE and other coagulable disease were studied for single nucleotide mutations in exon-10 of FV.

On admission patients were assessed for medical history, clinical presentation, risk factors, vitals, serum creatinine. All the patients underwent electrocardiogram, echocardiogram and lower limb venous ultrasound.

**Genetic analysis**

Five milliliters of peripheral venous blood sample was collected from all the study patients. QIAamp DNA Mini spin-column [Qiagen] DNA extraction kit was used for the isolation of genomic DNA from the blood samples, and the extracted samples were analyzed on 1% agarose gel through electrophoresis method.\(^{12,13}\)

**Polymerase chain reaction procedure**

Polymerase chain reaction (PCR) amplification was done using Eppendorf Mastercycler nexus gradient-flexid model, Hamburg, Germany. The following Oligonucleotide primers were designed using Oligo-6, NCBI blast and Primer-3 software packages and synthesized at Eurofins genomics India Pvt. Ltd. Bengaluru, India (see Table 1).\(^{14}\) PCR reaction conditions are summarized in Table 2.

The amplified PCR products were analyzed on 2% agarose gel in 1X TAE [Tris-Acetate-EDTA, pH: 7.8] to confirm the targeted amplification.\(^{12}\) The purification of PCR products resolved in 2% agarose gel was done by electro elution method with NucleoSpin® PCR (NP-PCR) Purification kit (Taurus Scientific, USA).\(^{12}\) The amplified PCR products were sequenced at Eurofins Genomics India Pvt Ltd., Bengaluru, India.

**Sequence analysis**

Multiple sequence alignment was performed using ClustalX tool (Version 1.83, National Center for Biotechnology Information, Bethesda, MD) to compare the sequences and the mutations were noted. Nucleotide sequences were translated into amino acid sequences using Expert Protein Analysis System (ExPASy) analysis and the changes in amino acid sequences were noted for each sequence. Schematic representation of various steps of genetic analysis is shown in Figure 1.

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**Statistical analysis**

Data was collected on MS-Excel spread sheets. Mean with standard deviation (SD) and frequencies with percentages were calculated for continuous and categorical data. SPSS version 20.0 (IBM Corp., Armonk, NY: USA) was used for the analysis.

**Results**

Sixty consecutive acute PTE patients were studied. Baseline characteristics of the study population are shown in Table 3. Mean age of the study patients was 41.2±12.9 years (range: 21-76 years). Forty-eight (80%) were male and 12 (20%) were female. The principle symptoms were dyspnea (100%), chest pain (33.3%), syncope (20%), hemoptysis (16.7%), and altered sensorium (3.3%). Frequencies of different risk factors were - 43.3% dyslipidemia, 30% smoking, 6.7% cancer, 13.3% hypertension, 10% diabetes, 3.3% stroke and 6.7% coronary artery disease. Mean heart rate was 118±15 beats per minute. Mean systolic blood pressure (SBP) was 114.3 ± 16.6 mm Hg and diastolic blood pressure (DBP) was 73.2 ± 9.3 mm Hg.

We found two novel transition type point mutations: c.1538 G>A and c.1601 G>A in exon-10 of FV which

| Table 1. Details of Oligonucleotide primers & PCR reaction mixture |
|------------------|------------------|
| Oligonucleotide Primers | PCR reaction mixture |
| Forward Primer: 5’-ACCCACAGAAAATGATGCCCAG-3’ | A total volume of 50 µL reaction mixture comprising of the below components was prepared in Milli Q water-1x assay buffer, 1.5 mmol MgCl\(_2\), 500 ng template DNA, 50 pico mole forward primer, 50 pico mole reverse primer, 100 µmol dNTPs mix and 1U of Taq polymerase. |
| Reverse Primer: 5’-TGCCCCATTATTTAGCCAGGAG-3’ |

MgCl\(_2\): magnesium chloride; µL: micro liter; mmol: milli mole; ng: nanogram; µmol: micro mole; dNTP: deoxy-ribo nucleotide triphosphate; U: unit; Taq: *Thermas aquaticus*. 

| Table 2. PCR reaction conditions |
|------------------|------------------|
| Phase of PCR | Temperature | Duration | No. of Cycles |
| Initial denaturation | 94°C | 10 minutes |
| Denaturation | 94°C | 60 seconds |
| Annealing | 61°C | 45 seconds |
| Extension | 72°C | 60 seconds |
| Final extension | 72°C | 10 minutes |

PCR, polymerase chain reaction.
is responsible for the cleavage site for aPC (Figure 2). ExPASy analysis revealed that these point mutations ensued a change of single amino acid in protein sequence at p.Arg513Lys and p.Arg534Gln, respectively. We have not find any mutation in exon-10 of FV among healthy controls.

**Structural analysis**

The built mutant FV structure when superimposed with the control FV structure, exhibited variations with an RMSD values in both domain and non-domain regions, with variable number of strands, helices, helix-helix interactions, β-α-β units, hairpins, β-bulges, β-turns, and γ-turns which can explain the major conformational changes in the mutant FV structure (Figure 3). Mutations in FV gene causes Factor Va resistant to aPC degradation, that can further increase the risk of venous thromboembolism in these patients.

**Discussion**

FVL and prothrombin mutations, among the several, are thought to be the most frequent causative factors for inherited thrombophilia. However, contribution of these factors varies from population to population.

Factor Va and factor VIIIa were cleaved at conserved arginine (R) residues by aPC to inhibit coagulation at positions R306, R506, and less importantly, at R679. FVL is a missense mutation in the FV gene at position G1691A, which can result in change of amino acids from arginine to glutamine (R506Q)\(^7,15,16\) that slows the inactivation of factor Va by aPC and thus producing a genetic risk factor in association with environmental risk factors which causes an increased risk for venous thrombosis.

Other, less common FV mutations also affect aPC resistance, with differing prothrombotic risks. Of these, one of the more important is FV\(_R2\) (H1299R) which is tightly associated to several other polymorphisms and collectively named the R2 haplotype.\(^17\)

FV\(_{Liverpool}\) (I359T), another mutation, which has been reported in a family in which it was alone asymptomatic, changes in the mutant FV structure (Figure 3).

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**Table 3. Baseline characteristics of study population**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Study group (n=60)</th>
<th>Control group (n=50)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>41.2 ± 12.9</td>
<td>40.5 ± 10.3</td>
<td>NS</td>
</tr>
<tr>
<td>Male</td>
<td>48 (80%)</td>
<td>40 (80%)</td>
<td>NS</td>
</tr>
<tr>
<td>Chest Pain</td>
<td>20 (33.3%)</td>
<td>00</td>
<td>NA</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NYHA Class-1</td>
<td>2 (3.3%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>NYHA Class-2</td>
<td>14 (23.3%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>NYHA Class-3</td>
<td>26 (43.3%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>NYHA Class-4</td>
<td>18 (30.0%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td>4 (6.7%)</td>
<td>00</td>
<td>NA</td>
</tr>
<tr>
<td>Stroke</td>
<td>2 (3.3%)</td>
<td>00</td>
<td>NA</td>
</tr>
<tr>
<td>CAD</td>
<td>4 (6.7%)</td>
<td>00</td>
<td>NA</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>26 (43.3%)</td>
<td>00</td>
<td>NA</td>
</tr>
<tr>
<td>Smoking</td>
<td>18 (30%)</td>
<td>00</td>
<td>NA</td>
</tr>
<tr>
<td>Hypertension</td>
<td>8 (13.3%)</td>
<td>00</td>
<td>NA</td>
</tr>
<tr>
<td>Diabetes</td>
<td>6 (10%)</td>
<td>00</td>
<td>NA</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>118.5 ± 15.6</td>
<td>119.4 ± 11.8</td>
<td>NS</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>114.3 ± 16.6</td>
<td>111.1 ± 14.3</td>
<td>NS</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>73.2 ± 9.3</td>
<td>75.6 ± 7.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

NYHA: New York Heart Association; CAD: coronary artery disease; HR: heart rate; SBP: systolic blood pressure; DBP: diastolic blood pressure, NS: not significant; NA: not applicable.
but the same with mutation on the other FV allele (a premature stop codon) was showed association with low FV levels, aPC resistance, and increased thrombotic risk.\textsuperscript{18} \textit{FV}\textsubscript{Cambridge} (R306T) and \textit{FV}\textsubscript{Hong Kong} (R306G) are the two other rare mutations which exhibit insignificant aPC resistance and slightly reduced aPC cofactor activity in vitro. However, no association of R306 mutations with increased risk of thrombosis was exhibited in vivo.\textsuperscript{19,20}

\textit{FVL} prevalence in Indian population is variable from north to south and low occurrence of \textit{FVL} was reported in northern states. Whereas, its relevance in southern part is insignificant. In concurrence with the findings reported by Himabindu et al\textsuperscript{21} in the present study we have not find \textit{FVL} mutation in our study cohort.

In the current study, we found two novel transition type point mutations, i.e., c.1538 G>A and c.1601 G>A in exon-10 of FV which are responsible for the cleavage site for aPC. These point mutations resulted in single amino acid change in protein sequence at p.Arg513Lys and p.Arg534Gln respectively.

These mutations may prevent efficient inactivation of FV by aPC and remains active which facilitates over production of thrombin leading to generation of excess fibrin and excess clotting which results in DVT and PTE. The present study findings require further validation with larger sample size to ascertain the precise impact of these mutations in pathophysiology of PTE.

\textbf{Limitations}

This is a single center study and only South-Indian ethnic population was included. Further multi-center studies with large sample size and different ethnic populations are required to confirm these findings. The methods used for DNA sequencing in the present study are relatively time-consuming and expensive.

\textbf{Conclusion}

We report two novel point mutations (c.1538 G>A and c.1601 G>A) in exon-10 of Factor V gene in Indian patients with PTE. Occurrence of novel insertion mutations G>A at 1538 and 1601 in exon 10 of FV gene which were identified in this study may not be significant. However, if the same study is extended to a larger population may reveal new insights into thromboembolic factors in PTE.

\textbf{Competing interests}

None.

\textbf{Ethical approval}

This study was approved by the Institutional Ethics Committee of our institute with IEC No. 21.

\textbf{Funding sources}

None.

\textbf{Acknowledgments}

We would like to thank Dr. Usha Kalawat, Nodal Officer, ICMR-VRDL, Department of Microbiology, SVIMS, Tirupati for permitting us to utilize PCR services.

\textbf{References}


