Prevalence of the Factor V G1691A and the Factor II/prothrombin G20210A gene polymorphisms among Tamilians

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Abstract

We have investigated the prevalence of the Factor II G20210A and Factor V G1691A single nucleotide polymorphisms (SNPs) in a South Indian-Tamil Nadu population. The SNP genotyping was performed using a polymerase chain reaction (PCR)/restriction fragment length polymorphism analysis and by a recently FDA-approved LightCycler real-time PCR assay. Of 72 samples that were genotyped, 4 (5.5%) patients were heterozygous for the Factor V SNP and no homozygous mutant patients were identified. None of the patients were shown to be either heterozygous or homozygous mutant for the Factor II SNP. All samples showed 100% concordance between the PCR/RFLP assay and the LightCycler assay. While this study identified the prevalence of the Factor V SNP to be similar to that of other reported populations, the absence of the Factor II allele is consistent with reports in more isolated populations. In addition, the results of this study do not support a role for these SNPs in acute myocardial infarction in the Tamilian population.

Keywords: Factor V G1691A; Single nucleotide polymorphisms; Tamilians

Introduction

Cardiovascular disease is the leading cause of death in most developed countries of the world. Apart from the traditional risk factors that contribute to cardiovascular disease, a number of candidate genes and genetic abnormalities may be involved in the pathophysiology of the disease. Among these variants, single nucleotide polymorphisms (SNPs) in the Factor II or prothrombin and Factor V genes have been shown to play a role in both venous and arterial thrombosis. In addition, it is now accepted that these SNPs are the most common inherited abnormalities in blood coagulation that lead to thrombophilia (van Rooden et al., 2004). To that extent, it is also well known that thrombosis of a disruptive atherosclerotic plaque induces most of the adverse cardiovascular events.

The gene encoding human Factor V is located on chromosome 1q21–25 (Cripe et al., 1992; Jenny et al., 1987; Wang et al., 1988). The Factor V SNP, also referred to as the Factor V Leiden, results in the substitution of guanine by adenine at nucleotide 1691 (G1691A). This genetic change in turn results in the replacement of glutamine for arginine at position 506. The resulting amino acid substitution due to this SNP slows down the proteolytic inactivation of Factor Va by activated protein C (activated protein C resistance), thereby increasing the generation of thrombin. This reduced anticoagulant effect of activated protein C leads to an increased tendency toward thrombosis (Griffin et al., 1993; Samaha et al., 1995; Seligsohn and Lubetsky, 2001).

Similarly, another genetic variation has been identified in the 3'-untranslated (UT) region of the prothrombin gene which is also associated with increased risk of venous
thrombosis (Poort et al., 1996). Prothrombin is the precursor to thrombin in the coagulation pathway which leads to the formation of fibrin. The prothrombin gene consists of 14 exons spanning approximately 21 kb at position p11–q12 of chromosome 11 (Degen and Davie, 1987; Royle et al., 1987). A single G to A polymorphism at nucleotide position 20210 was found to be associated with increased gene expression levels and can result in an increased risk for thrombophilia (Mandel et al., 1996; Poort et al., 1996; Rosendaal et al., 1997; Seligsohn and Lubetsky, 2001).

The prevalence of these two SNPs varies depending on the geographical location and the ethnic background of the population (Franco et al., 1998). In the present study, we compared a PCR-mediated restriction fragment length assay and a real-time PCR assay in the investigation of the frequency of these SNPs in acute myocardial infarction patients and healthy individuals among Tamilians. To the best of our knowledge, this is the first study which assesses the SNP frequency in this Tamilian patient population.

**Materials and methods**

**Patient specimens**

Whole blood was collected into EDTA-anticoagulated vacutainer tubes. We studied 72 individuals including 52 with acute myocardial infarction and 20 healthy individuals with no documented history of heart disease.

**DNA extraction**

Genomic DNA was isolated from whole blood samples using the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) as per the instructions given by the manufacturer. DNA was extracted from 200 μl of whole blood using the spin columns provided. The isolated DNA was stored at −20°C. The DNA quality was confirmed by electrophoresis using a 0.7% agarose gel and quantity determined using absorbance spectrophotometry.

**PCR/RFLP analysis**

Genomic DNA (approximately 100 ng), isolated as described above, was incubated in a total reaction volume of 100 μl containing both the forward and reverse primers for the Factor V G1691A (0.4 μM) or Factor II G20210A (0.2 μM) SNPs, respectively, 2.5 units AmpliTaq Gold (Perkin-Elmer, Norwalk, CT, USA), 50 μM each deoxynucleotide triphosphate, 4.0 mM MgCl2, 60 mM Tris–HCl (pH 8.5), and 15 mM ammonium sulfate. DNA was initially denatured at 94°C for 1 min prior to amplification in an MJ Research PTC-100 thermocycler. PCR amplification was accomplished using 37 cycles consisting of 30-s denaturation at 94°C, 30-s annealing at 58°C, and 30-s extension at 72°C. The final cycle included a 2-min extension step at 72°C. The Factor V SNP abolishes an MnlII restriction enzyme recognition sequence while the Factor II SNP generates a restriction site for HindIII. These SNPs are thus detected by digestion of PCR amplified products with each of these enzymes. The restriction digestion was performed in a total volume of 30 μl and consisted of 15 μl PCR product, 10.2 μl dH2O, 5 units MnlII enzyme, 10 units HindIII enzyme (New England Biolabs, Beverly, MA, USA), and 3 μl 10× buffer and 0.3 μl 100 × BSA. Samples were then incubated for 3 h at 37°C. Restriction fragment size analysis was performed by visualization of digested PCR products after separation by gel electrophoresis in a 2.5% SFR/TBE (Amresco Inc., Solon, OH, USA) agarose gel and staining with ethidium bromide (Fig. 1).

**Real-time PCR analysis**

Real-time PCR detection of these two SNPs was performed using an FDA-approved LightCycler assay from Roche Diagnostics (Indianapolis, IN, USA). A 222-bp fragment of the Factor V gene and a 165-bp fragment of the Factor II gene were amplified from genomic DNA, and labeled using specific hybridization probes. These probes consist of two different oligonucleotides, one labeled at the 5’-end with LightCycler Red 640, and one labeled at the 3’-end with fluorescein. These probes hybridize to internal sequences within the amplified product. Hybridization to the template DNA results in a fluorescent resonance energy transfer (FRET) between the two fluorophores. The light source of the LightCycler excites the donor fluorophore, fluorescein, and energy is transferred to the acceptor fluorophore, LightCycler Red 640. The emitted fluorescence is measured by the LightCycler instrument.

The genotype was determined by melt curve analysis after the amplification cycles are completed. The fluorescencelabeled probe spans the sequences for both the Factor V and the Factor II SNPs. If an SNP is present, the mismatch of the SNP-specific probe destabilizes the hybrid, and the decrease in fluorescence will occur at a lower temperature (Figs. 2 and 3).

**Results**

In this study, our multiplex PCR/RFLP assay for the detection of the Factor V and Factor II SNPs was performed by digesting the PCR amplicons with MnlI and HindIII. The resulting banding pattern for a homozygous normal or homozygous mutant individual was 163 bp and 200 bp, respectively (Fig. 1). Similarly, the resulting banding pattern for a Factor II homozygous normal or homozygous mutant individual was 283 bp and 259 bp, respectively (Fig. 1).

Each patient sample was also genotyped using the FDA-approved LightCycler assays. These real-time PCR assays using FRET technology to detect SNPs rely on melt curve analysis for determination of genotyype. A Factor V SNP
will decrease the melting temperature from 65°C to 57°C (Fig. 2), and the Factor II SNP will decrease the melting temperature from 59°C to 49°C (Fig. 3). Graphic results for homozygous normal, heterozygous, and homozygous mutant are easily interpretable.

Of the 72 samples that were genotyped, 4 (5.5%) patients were heterozygous for the Factor V SNP and no homozygous mutant patients were identified. Of these four patients, one was a healthy control and three were individuals with an AMI. None of the patients were shown to be either heterozygous or homozygous mutant for the Factor II SNP. All samples showed 100% concordance between the PCR/RFLP assay and the LightCycler assay. The LightCycler assay required less labor and provided a much quicker turn-around time.

Discussion

Although the factor V Leiden and prothrombin gene mutations have been primarily associated with venous thrombosis, other studies have reported increased risk of
myocardial infarction in patients with other cardiovascular risk factors (Doggen et al., 1998). The Factor II (prothrombin) gene SNP, G20210A, is frequently associated with the factor V SNP in thrombophilic patients (approximately 10%) and shows ethnic-specific variation in allele frequency (Franco et al., 1998; Howard et al., 1998). Because of this association and the fact that these SNPs may play a role in arterial thrombosis associated with myocardial infarction, we examined a Tamilian patient population in which these SNPs have not been described previously.

In this study, we used two molecular genotyping methods to assess the prevalence of the Factor V and Factor II gene SNPs in a Tamilian patient population. When we analyzed the Factor II G20210 SNP, among the 52 AMI and 20 control Tamilian individuals, we observed that the Factor II SNP (A allele) was completely absent among the study subjects. The absence of this genetic risk factor for myocardial infarction among Tamilians may be due to the difference in the geographical location and ethnic background which contributes to the risk of coronary artery disease. This result is in accordance to the findings of a previous study conducted among different ethnic groups including Whites, African and Brazilian Blacks, Asians and Amerindians which also showed the absence of this novel genetic risk factor for arterial thrombotic disease (Franco et al., 1998). The prevalence of the Factor V and Factor II SNPs has also been studied in several genetically isolated populations including the Inuit and Pima Indians (de Maat et al., 1998; Kohler et al., 1997). Similar to our results in Tamilians, these studies did not identify the Factor II SNP in either of these populations (Inuit and Pima Indians).

Analysis for the Factor V G1691A SNP in our Tamilian population identified one individual in the control (5%) group and three (5.8%) individuals in the AMI patient group to be heterozygous for the SNP (Table 1). While no clinical association appears to be present among the AMI and control groups, we did identify the prevalence of Factor V mutant alleles to be 5.5% in the Tamilian population. This frequency is similar to that of the European and American populations where it is reported to be 5–10% (Beauchamp et al., 1994). The mutant allele for Factor V gene from various healthy populations have been studied and it was found to be completely absent in Africans, Koreans, Peruvian Indians, Chinese, Japanese, Mongolians, and Taiwanese (Bertina et al., 1994; Flint et al., 1993; Helley

<table>
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<tr>
<th>Parameters</th>
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<th>Alleles</th>
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<tr>
<td>Factor V G1691A</td>
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<td>GA</td>
</tr>
<tr>
<td>Controls (n=20)</td>
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<td>1</td>
</tr>
<tr>
<td>AMI patients (n=52)</td>
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<td>3</td>
</tr>
<tr>
<td>Factor II G20210A</td>
<td>Controls (n=20)</td>
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Fig. 3. LightCycler melt curve analysis demonstrating normal control Factor II from heterozygous control (two peaks) patterns.
et al., 1996; Hong, 2003; Ishida et al., 1995; Pepe et al., 1997; Rosendaal et al., 1995). The prevalence of the mutant Factor V ‘A’ allele among the Tamilians was found to be 0.03, which is very low when compared to a Canadian population that have a maximum mutant allele frequency of 0.09 (Liu et al., 1995). The heterozygous frequency for Factor V was found to be completely absent in Japanese and Africans while 5–10% of Europeans carry this SNP (Rees et al., 1995; Takamiya et al., 1995).

Our findings showed that the Factor II G20210A SNP is completely absent among Tamilians, indicating the lack of a potential founder effect that may be present in individuals of European ancestry. In addition, our study showed no association between the Factor II G20210A SNP, the Factor V G1691A SNP, and myocardial infarction among Tamilians. These findings, although small in number, are consistent with those reported in other populations and support the complex nature of cardiovascular disease and the many other associated risk factors. Finally, while screening for these two SNPs may be warranted for risk of thrombophilia in Tamilians, it is not indicated for assessing risk of acute myocardial infarction in the Tamilian population.

References