Prevalence of rare F5 variants in general population from Bosnia and Herzegovina

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Received: 22 April 2021 / Accepted: 25 June 2021 / Published online: 2 July 2021
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Abstract
Human gene F5, encoding coagulation factor V, was previously reported to be highly polymorphic. Apart from FV Leiden, several other rare variants have been detected in clinical practice and associated with thrombotic events, especially in cases when patient’s phenotype and FV Leiden genotype were not in agreement. In this study, the prevalence of 17 rare F5 variants has been studied on a sample of 130 healthy adult individuals from the general Bosnian-Herzegovinian population. DNA was isolated from buccal swab samples, while genotyping was performed using MALDI-TOF MS method. The results have shown that Asp2194Gly and Met2120Thr are polymorphic in the study population with minor allele frequencies of 0.077 and 0.073, respectively. Additionally, these two variants were mutually exclusive with FV Leiden and none of them was positively associated with participants’ family history of cardiovascular or cerebrovascular diseases. While the obtained results are in agreement with previously reported data for the general Caucasian populations, it is worth noting that only two rare F5 variants were detected in the study population, albeit at considerable frequencies. Still, scientific information on rare F5 variants is rather scarce and further studies aiming to assess functional importance of these variants, as well as their role as prothrombotic factors are necessary.

Keywords Bosnia and Herzegovina · Factor V Asp2194Gly · Factor V Leiden · Factor V Met2120Thr · Rare Factor V variants · Thrombophilia

Introduction
Human coagulation is viewed as a cascade in which one coagulation factor leads to activation of another with the final goal of prothrombin (factor II, FII) conversion to activated thrombin. In this process, coagulation factors are viewed as proenzymes or cofactors that must be activated to exert their procoagulant properties. Coagulation cascade is represented as a Y-shaped scheme with intrinsic and extrinsic pathways meeting at the prothrombinase complex which consists of activated factor X (FXa) and activated factor V (FVa) and is in charge of prothrombin activation. The coagulation cascade pathways seem to be non-redundant, as the extrinsic pathway initiated by activated factor VII/
tissue factor (FVIIa/TF) complex works on the surface of TF-expressing cells, while intrinsic pathway beginning with factor XI (FXI) activation works on the surface of activated platelets [1–3].

Coagulation factor V (FV) is encoded by \( F5 \) gene whose mRNA product is 6.8 kb long and has 25 exons, while the corresponding polypeptide is 2224 amino acids long. FV is activated by thrombin due to cleavage at arginine residues 709, 1018 and 1545. Activated FV (FVa) is inactivated in a coagulation cascade by the peptide cleavage at arginine residues 306, 506 and 679 by anticoagulant protein C (APC) through its serine protease activity. While the intermediate deactivation state is produced through rapid reaction at Arg506 site, the reaction taking place at Arg306 site is slower and finalizes deactivation of FVa [4].

FV Leiden (FVL; c.1601G > A, p.Arg506Gln, rs6025) is a pathogenic variant that was first identified in individuals with APC resistance, since it disables APC in recognizing the major cleavage site on FV peptide, causing unchecked continuation of prothrombin activation [5, 6]. FVL is a major predisposing factor for venous thrombosis, since the heterozygous genotype increases the risk of venous thromboembolism (VTE) approximately 5- to tenfold, while homozygosity increases the risk up to 40- to 80-fold [6, 7]. It was also reported that FVL mutation is present in 18–25% of all VTE cases [8, 9].

However, \( F5 \) gene is highly polymorphic, even considering its size. There have been several hypotheses aiming to explain this phenomenon. The most probable one relates the frequency of \( F5 \) DNA variants with its dual role as procoagulant and anticoagulant protein, whereby this gene is usually described as Dr. Jekyll and Mr. Hyde of human coagulation [10]. According to the previously published literature [11], rare \( F5 \) variants have been detected in clinical practice when the results of FVL genotyping and observed patient’s phenotype were not in alignment, thus implying other underlying mutations. An overview of the most significant rare \( F5 \) variants is given in Table 1, along with their clinical significance.

Most of these variants were reported as isolated clinical cases and are usually not systematically studied to determine their prevalence in the general population. Therefore, the aim of this study was to perform genotyping for 17 rare \( F5 \) variants in the general population from Bosnia and Herzegovina.

### Table 1

| Variant       | Nucleotide change | Amino acid change* | dbSNP entry | ClinVar entry | Related phenotype          | ClinVar interpretation | References |
|---------------|-------------------|--------------------|-------------|---------------|-----------------------------|------------------------|------------|
| Hong Kong     | c.1000A > G       | p.Arg334Gly        | rs118203905 | VCV000000643.1 | Mildly reduced APC sensitivity in vitro | Pathogenic            | [4, 11]    |
| Cambridge     | c.1001G > C       | p.Arg334Thr        | rs118203906 | VCV000000644.1 | APC resistance              | Pathogenic            | [11, 14]   |
| Stanford (4 bp Del Ex13) | 2805ATTG           | Frameshift         | N/A         | RCV000000680.3 | Factor V deficiency         | Pathogenic            | [11]       |
| Seoul-1 (8 bp Del Ex7) | 1131AAGAGGGTG       | Frameshift         | N/A         | RCV000000682.3 | Factor V deficiency         | Pathogenic            | [11, 15]   |
| Seoul-2       | c.5189A > G       | p.Tyr1702Cys       | rs118203907 | VCV000000649.2 | Factor V deficiency         | Pathogenic            | [11, 15]   |
| Casablanca    | c.2401C > T       | p.Gln801Ter        | rs118203908 | VCV000000650.1 | Factor V deficiency         | Pathogenic            | [11, 15]   |
| Arg1133Ter    | c.3481C > T       | p.Arg1133Ter       | rs118203909 | VCV000000651.1 | Factor V deficiency         | Pathogenic            | [11]       |
| 2952 Del Ex13 | 2952 T            | Frameshift         | N/A         | RCV000000686.2 | Factor V deficiency         | Pathogenic            | [11]       |
| 5493 Ins Ex16 | 5493G             | Frameshift         | N/A         | RCV000000687.3 | Factor V deficiency         | Pathogenic            | [11]       |
| Arg2074Cys    | c.6304C > T       | p.Arg2074Cys       | rs118203910 | VCV000000654.3 | Factor V deficiency         | Likely pathogenic     | [11]       |
| Liverpool     | c.1160T > C       | p.Ile359Thr        | rs118203911 | VCV000000655.1 | APC resistance              | Pathogenic            | [10, 11]   |
| Glu119Ter     | c.439G > T        | p.Glu119Ter        | rs118203912 | VCV000000645.1 | APC resistance              | Pathogenic            | [11]       |
| Asp2194Gly    | c.6665A > G       | p.Aspartate2194Gly | rs6027      | VCV000255215.2 | Lower factor V levels       | Benign/likely benign  | [10]       |
| Met2120Thr    | c.6443T > C       | p.Met2120Thr       | rs9332701   | VCV000255214.2 | Lower factor V levels       | Benign/likely benign  | [10]       |

*Ter stands for termination codon*
and Herzegovina and identify which variants are present and at what frequency. In addition, it was aimed to assess whether statistically significant correlation between rare F5 variants and family history of cardiovascular diseases can be detected.

Materials and methods

Sample collection and DNA isolation

This study has been performed on 130 buccal swab samples collected from unrelated adult individuals of both sexes from different parts of Bosnia and Herzegovina without previous history of cardiovascular diseases. Sample collection has been performed between August and October 2017. Prior to volunteering to donate a sample, all participants signed an informed consent form. Ethical clearance for conducting this study was obtained from the Ethical Committee of the International Burch University, Sarajevo, Bosnia and Herzegovina (granted on July 6th 2017, document Number 04-172-1/17).

DNA was isolated using modified Miller’s protocol [16] with an overnight incubation step at 60 °C. The quality and quantity of DNA isolates was tested spectrophotometrically using BioSpec-nano spectrophotometer (Shimadzu Corp., Kyoto, Japan).

DNA genotyping

Factor V Leiden mutation was analyzed along with 17 other rare variants in F5 gene. PCR amplification was carried out according to Storm and Darnhofer-Patel [17] in two multiplexes as follows:

1. Multiplex reaction for 14 rare F5 variants: Casa-blanca, Met2120Thr, 2952 DelT Ex13, 4 bp Del Ex13, Arg1133Ter, Tyr1702Cys, Arg2075Cys, Ile359Thr, G > A Ex18, Cambridge, InsA Ex13, Asp2194Gly, 5493 InsG Ex16 and Glu119Ter, as well as Leiden mutation
2. Multiplex reaction for three rare F5 variants: G > A Ex16, Seoul-1 and Hong Kong.

Multiplex reactions were designed and appropriate primers selected in Assay Design Suite v2 (Agena Bioscience, San Diego, CA, USA).

Excess unreacted dNTPs were removed from reaction mixtures via shrimp alkaline phosphatase (SAP) treatment [17], followed by a general iPLEX reaction for SNP genotyping using single-base extension chemistry [17]. Thermal cycling and sample incubation in these three reactions were performed using GeneAmp PCR System 9700 (Applied Biosystems, Waltham, MA, USA) in 384-well plates.

Samples were subsequently treated with Clean Resin to achieve ion exchange conditioning and the removal of excess cations. SNP genotyping was performed using 15-nl samples utilizing MALDI-TOF MS approach through two software packages, namely SpectroACQUIRE for raw data collection and MassARRAY Typer for plate design and accessing genotype data, both manufactured by Agena Bioscience (San Diego, CA, USA) [17].

Statistical analyses

To confirm whether tested loci are in Hardy–Weinberg equilibrium (HWE), we used GenAIEx v6.5 and chi-square statistics at significance level of 0.05 [18, 19]. Linkage disequilibrium (LD) between loci was tested for in pairwise manner using online version of GenePop v4.2 at significance level of 0.05 [20, 21]. In order to test for possible correlation between rare F5 variants and participants’ family history of cardiovascular diseases, IBM SPSS Statistics for Windows v23.2 (IBM Corp., Armonk, NY, USA) was used, whereby Fisher’s exact test was performed at significance level of p < 0.05.

Results

In this study, the prevalence of 17 rare F5 variants, in addition to Factor V Leiden (previously published in [22]), was studied in 130 individuals from the general Bosnian-Herzegovinian population. Genotyping procedure was repeated for Leiden mutation for practical reasons; namely, it was already contained in one of the multiplexes used in the present study. The results of Leiden genotyping reported here are fully concordant with those published in our previous paper [22]. Females accounted for 63.8% of the study population. Mean participants’ age was 33.8 ± 14.3 with the youngest participant being 19 and the oldest 82 years old at the time of sample collection.

Out of 18 investigated loci, 13 of them were defined as SNP changes, along with three deletions and two insertions. Apart from Factor V Leiden, two more loci were found to be polymorphic in Bosnian-Herzegovinian population, namely F5 Asp2194Gly and F5 Met2120Thr (Fig. 1).

In terms of Factor V Leiden, six study participants were heterozygous (4.6%), while the rest of them carried wild-type genotype, meaning that the minor allele frequency (MAF) is 0.023. Heterozygous genotype was found in 16 and variant homozygous in two study participants on locus Asp2194Gly, while at locus Met2120Thr, 15 heterozygous and two variant homozygous genotypes were detected. MAFs for Asp2194Gly and Met2120Thr were 0.077 and 0.073, respectively. It is interesting to note that Factor V Leiden mutation was mutually exclusive with two rare F5
variants. Moreover, one participant was found to be compound heterozygous for Asp2194Gly and Met2120Thr, while the rest of them had mutated allele(s) at only one of these two loci.

Three polymorphic loci are in agreement with Hardy–Weinberg equilibrium with \( p \)-values of 0.788, 0.128 and 0.091 for Factor V Leiden, \( F5 \) Asp2194Gly and \( F5 \) Met2120Thr, respectively. LD analysis at significance level of \( p = 0.05 \) revealed no indication of LD between loci Asp2194Gly and Met2120Thr.

Fisher’s exact test was used to check if variant distribution is correlated with the family history of cardiovascular and cerebrovascular diseases. At \( p = 0.05 \), neither Asp2194Gly nor Met2120Thr were significantly correlated with participants’ family histories.

**Discussion**

Rare \( F5 \) variants were previously detected and studied in human populations worldwide. Furthermore, many \( F5 \) variants have their common names reflecting cities in which they were first detected [14, 15, 23–25]. It is, however, necessary to conduct systematic and more elaborate studies to extend our understanding of the prevalence of \( F5 \) variants, and especially their functional and clinical significance.

In the general Bosnian-Herzegovinian population, two rare \( F5 \) variants were detected, namely Met2120Thr and Asp2194Gly. According to ClinVar [13], these two variants are benign or likely benign, with two out of four stars in the revision status, which makes them the most heavily supported rare \( F5 \) variants in the literature. Two more variants are supported with one star, namely Arg2074Cys and Seoul-2, while the rest of them have zero stars in their review status. The fact that both polymorphic variants in the Bosnian-Herzegovinian population are benign or likely benign implies that the mutational status of the population is rather positive with no pathogenic variants being detected, but also that the variants detected in the current study population are fairly common when compared to the rest of the dataset and, therefore, reported in ClinVar more frequently than the others.

The importance of Met2120Thr SNP has been demonstrated using recombinant FV protein. It has been shown that this variant is significantly associated with decreased FV levels in vivo and in vitro. MAF for Met2120Thr is 3% in Caucasians and it seems to be restricted to this population only since African and East Asian populations did not have this variant in previously published studies [10]. The present study identified higher MAF of 7.3% with two individuals being homozygous carriers of 2120Thr allele.

On the other hand, Asp2194Gly variant constitutes HR2 haplotype that was first described in 1998 in terms of four linked missense variants with His1299Arg being functionally the most significant. It was immediately associated with lower FV levels in plasma [10, 26]. This haplotype was later characterized in more detail and is now believed to consist of 13 linked polymorphisms in exons 8 through 25 of \( F5 \) gene with seven of them being amino acid changes related to functional modifications of the protein [4, 26, 27]. The function of this haplotype is now mainly associated with an increased factor V1 to V2 ratio, thus increasing prothrombotic activity, as well as with decrease in APC-mediated degradation of FV, including individuals with wild-type Leiden genotype [26, 27]. Asp2194Gly is described as one of the major functional determinants of FVHR2 haplotype [28], interfering with the transport of mutant protein from ER to Golgi complex [29].
compared to other rare variants, HR2 is relatively common with MAF of 5 to 17% in Europe, Asia and Africa, reaching its peak value of 50% in Indian tribes from Costa Rica [26].

There has been an ongoing debate of the role of HR2 haplotype in predisposition to thrombotic events with previous studies reporting opposing results. In their meta-analysis, Castaman and colleagues [26] found statistically significant differences in VTE frequency in FV Leiden carriers alone when compared to FV Leiden/HR2 compound carriers. The authors have also concluded that HR2 haplotype can only be regarded as a mild prothrombotic factor since this variant alone did not significantly increase the risk of VTE compared to wild-type individuals [26]. On the other hand, in one of the most extensive and detailed meta-analyses on this topic, Gohil and colleagues [30] identified, among others, F5 variants representing risk factors for VTE. FVHR2 haplotype was analyzed by comparing the results of 14 studies in Caucasian populations. Population attributable risk (PAR) for VTE was 2.6% in the presence of this variant, while MAF was 6% in both patients and healthy controls. This meta-analysis identified a significant risk of VTE in the presence of HR2 haplotype mutant allele [30].

Asp2194Gly SNP alone corroborates lower FV levels in vivo, which was shown to contribute to increased APC resistance and, therefore, increased probability of thrombotic event. However, it is expected that this variant has a prothrombotic character if accompanied by FV Leiden mutation. MAF for this SNP is 0%, 7% and 5% in Africans, Caucasians and Asians, respectively [10]. This finding is in agreement with the present study, in which 2194Gly allele had frequency of 7.7%.

In the most detailed study of Met2120Thr and Asp2194Gly variants in Caucasian populations so far, 1013 Italians were genotyped and variant alleles were detected for both polymorphisms. The results report that 4.4% and 13.2% participants were Met2120Thr and Asp2194Gly carriers, respectively [28]. The same study offers extensive information on functional changes associated with these two variants. FV Leiden/Met2120Thr compound heterozygotes had significantly decreased APC ratios, while this difference was not significant for FV Leiden/Asp2194Gly compound heterozygotes. The prevalence of FV Leiden/Asp2194Gly genotype was significantly higher in symptomatic VTE patients than in asymptomatic controls. Higher frequency of FV Leiden/Met2120Thr genotype was also observed in symptomatic patients than in controls, but the difference was not statistically significant. Additional analysis confirmed that both variants led to a decrease in recombinant FV activity and antigen levels in vitro and protein levels in plasma in vivo. In heterozygous state, Met2120Thr decreased Factor V levels by 25% in vivo and 34% in vitro in expression studies. Comparable to this, heterozygous Asp2194Gly decreased FV levels by 20% and 45% in vivo and in vitro, respectively [28].

Human coagulation cascade is a delicate and fine-tuned pathway, while thrombophilia is a heterogenous condition being associated with many acquired and hereditary variants. Aside from FV Leiden, several other variants in F5 gene have been detected in clinical practice. We have shown here that Met2120Thr and Asp2194Gly are present in healthy population from Bosnia and Herzegovina and that the variant distribution is not significantly correlated with participants’ family history of cardiovascular and cerebrovascular diseases. In order to fully assess the clinical significance of these and other F5 variants, it is necessary to design and implement studies that will aim at comparing general and patient populations and assessing potential prothrombotic nature of rare F5 variants. Furthermore, functional consequences of rare F5 variants, including varying FV levels in blood and impaired protein secretion are to be investigated in order to get better understanding of their roles.

Author contributions AA performed sample collection, experimental work, data analysis and participated in manuscript writing. RS and NS performed experimental work and participated in manuscript preparation. SD performed statistical analyses and participated in manuscript drafting. WH and DM supervised the project and critically revised the manuscript. DP designed and supervised the project and critically revised the manuscript. All authors approved the final version of the manuscript for publication and agree to be held accountable for all aspects of the work.

Data availability The datasets generated and/or analyzed during the study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no competing interests to disclose that are relevant to the contents of this article.

Ethical approval Ethical clearance for conducting this study was obtained from the Ethical Committee of the International Burch University (Sarajevo, Bosnia and Herzegovina), protocol Number 04-172-1/17. The study was conducted according to the 1964 Helsinki Declaration.

Informed consent Informed consent was obtained from all individual participants included in the study prior to sample collection.

Consent for publication All participants signed an informed consent agreeing to publication of anonymized genotype data.
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