Prevalence of factor V G1691A (Leiden) and prothrombin G20210A polymorphisms among apparently healthy Jordanians

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Abstract

Factor V Leiden and prothrombin G20210A are related genetic risk factors for venous thromboembolism (VTE). Analysis for both mutations is increasingly being performed on patients exhibiting hypercoagulability. The objective of this study was to determine the prevalence of factor V Leiden (FVL), prothrombin-G20210A (PT-G20210A) polymorphisms and their coexistence among apparently healthy Jordanians.

One thousand apparently healthy individuals from representative regions of Jordan with no previous history of VTE participated in this study. The mean age of participants was 28.5±6.4 years (age range 18-45 years). Two hundred and eighteen subjects were APC resistant with an APC-R mean of 85.52±15.35 seconds; the non-resistant subjects had an APC-R mean of 159.90±26.96 seconds.

A multiplex polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) for the simultaneous detection of FVL and prothrombin G20210A was used to analyze the 218 DNA samples that were APC-R resistant. Both mutations generate HindIII RFLPs and the prothrombin amplicon contains an invariant HindIII recognition sites. The multiplex PCR-RFLP of Factor V for those 218-samples was: 41 wild-type, 169 heterozygous mutant, and eight homozygous mutant individuals. For prothrombin G20210A, the multiplex PCR-RFLP identified 215 wild-type and three heterozygous mutant individuals.

Factor V positive individuals (n=50) had a mean F-V activity of 78.04%±25.81. F-V activity among wild type (n=41), F-V Leiden heterozygous (n=169) and F-V Leiden homozygous (n=8) were 92.93%±16.17, 87.02%±15.21 and 96.14%±12.32, respectively. Factor II positive subjects (n=47) had a mean factor II activity of

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127.96%±21.37. F-II activity among carriers (heterozygous, n=3) and non-carriers (normal, n=215) of PT-G20210A mutation were 107.67%±9.29 and 105.00%±17.79, respectively.
The prevalence of FVL was 21.8% and there is a little likelihood of the co-inheritance of the FVL and PT-G20210A among healthy young adults, since only few cases were found to be carriers for the two alleles.

INTRODUCTION

Thrombophilia is defined as an increased tendency to develop venous thromboembolism (VTE) [1,2]. Its pathogenesis is mostly related to a genetically determined hypercoagulability state, where an inherited defect is associated with a quantitative or qualitative modification of haemostatic blood components [3,4]. However, because of its episodic nature, thrombophilia is described as multifactorial [5-7].

Dahlbäck *et al.* (1993) identified a previously unrecognized mechanism for thrombophilia that is characterized by a poor response of plasma for the anticoagulant activity mediated by activated protein c (APC) [8]. Later, Bertina *et al.* (1999) identified a certain phenotype of FV in the majority of APC-resistant VTE patients [9]. This phenotype (Factor V Leiden) is characterized by the presence of a Glutamine residue at the 506 position instead of Arginine. This phenotype predicts the presence of a FV-polymorphism with a single Guanine to Adenine base substitution at the nucleotide position 1691 of FV gene. APC resistance associated with VTE, increases VTE tendency for about 3 to 10-folds and 80 to 90-folds among heterozygous and homozygous carriers, respectively [11-13].

Poort *et al.* (1996) identified a genetic variant in the 3'-untranslated region (3'-UTR) of the Prothrombin gene. That variant is characterized by a single base Guanine to Adenine transition at position 20210 of that region. Prothrombin G20210A (PT-G20210A) is associated with a hyperprothrombinemia and a 2 to 4 folds increased risk for VTE [14].

The ethnic and geographic distribution prevalence of FVL and PT-G20210A among general population ranges from 3% to 15% and 1% to 4%, respectively [16-22].

This study was designed to determine the prevalence of F-V Leiden (FVL), prothrombin-G20210A (PT-G2021A) polymorphisms and their coexistence among apparently healthy Jordanians.

MATERIAL AND METHODS

One thousand apparently healthy individuals with no previous history of VTE were randomly selected blood donors from representative regions of Jordan. Two blood samples for each subject were collected; an EDTA blood sample that was used for DNA extraction and a 3.2% trisodium citrated blood sample from which platelets free plasma were obtained by centrifugation for 15 minutes at 4000 rpm. Isolated plasma was aliquoted into 1.5 ml eppindorff tubes and stored at -30 °C for one month.

Genomic DNA was extracted from blood samples, using QIAamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Use of residual human genomic DNAs in this study was approved by both Review Boards at University of Jordan and at Jordan University of Science and Technology.

Activated protein C resistance, factor V and prothrombin activity levels were assessed for each sample using commercially available kits and fully automated system (STACLOT system, Diagnostica stago/France). Manufacturer instructions were followed.

A multiplex polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used for the simultaneous detection of FVL and prothrombin G20210A mutations. Conditions for multiplex reaction were: 300 ng of genomic DNA that was added to a PCR buffer (7.7 mM Tris-HCl ,38.5 mM KCl, and 1.5 mM $MgCl_2$), 0.2 mM of each dNTP, 1µmol/L factor V primers, 0.1 µmol/L prothrombin G20210A primers, and 2.5 U Taq DNA polymerase (promega) in a final volume of 50 µl. DNA was amplified in a thermal cycling conditions consisting of 10 minutes enzyme activation at 95 °C followed by 40 cycles of 94 °C for 1 minute, 55 °C for 1 minute, 72 °C for 1 minute with a final 5 minutes extension at 72 °C [23,24]. The bands before restriction were 241 bp for factor V gene and a 506 bp for prothrombin G20210A.

Twenty five µl of the PCR products were then digested with 2 µl HindIII enzyme (10 u/µl, Promega) and 3 µl of 10X enzyme buffer at 37 °C for either 2 hours or overnight. Digested PCR products were separated on a 3% agarose gel and stained with ethidium bromide.

Data were described using relative frequencies, means, and standard deviations. Independent t test was used to compare means and Pearson's chi-square was used to compare proportions. Data were analyzed using Statistical Package for Social Sciences software (SPSS, version 11.5). A p-value of less than 0.05 was considered statistically significant

RESULTS

One thousand healthy individuals were investigated. The mean (±SD) age of investigated subjects was 28.5±6.4 years old (range 18-45 years). Seven hundred eighty (78.0%) of those were males and 220 (22.0%) were females. A total of 218 (21.8%) were APC resistant (APC-R), 47 (4.7%) were FII positive and 50 (5.0%) were FV positive. FII positive and FV positive coexisted only in 10 (1.0%) subjects. Twenty (9.2%) of APC-resistant and 30 (3.8%) of APC-non resistant were FII positive (p=0.001) and 41 (18.8%) of APC-resistant and 6 (0.8%) of APC-non resistant were FV positive (p<0.005).

The means of APCR, FII and FV according to gender are shown in Table 1. While the mean of APCR was sig-

Table 1. Means (±SD) of APC, F-II, and F-V according to gender.

	N	Men Mean±SD	Women Mean±SD	Total Mean±SD	p-value
APC		148.03±39.37	128.28±36.13	143.68±39.52	<0.005
APC Resistant	218	86.48±14.46	83.34±17.09	85.52±15.35	0.164
APC Non resistant	782	162.8±27.27	147.96±21.77	159.90±26.92	<0.005
F-II		97.52±13.26	107.68±14.40	99.76±14.16	<0.005
F-II Positive	47	120.82±27.45	134.79±9.65	127.96±21.37	0.023
F-II Negative	953	96.81±11.93	104.36±10.98	98.36±12.13	<0.005
F-V		83.82±11.94	90.79±14.70	88.35±12.92	<0.005
F-V Positive	50	70.69±20.12	96.93±29.79	78.04±25.81	0.001
F-V Negative	950	84.46±11.03	90.37±13.11	85.74±11.76	<0.005

nificantly higher among men when compared to women, the mean of FII and FV were significantly higher among women. Table 2 describes the means of FII and FV for APC non-resistant and APC resistant subjects.

Digestion of the amplicons with HindIII restriction enzyme were as follow: factor V wild-type yielded a 241 bp fragment, factor II wild-type gave 407 bp and 99 bp fragments, FVL heterozygous resulted in 241, 209, and 32 bp fragments, prothrombin G20210A heterozygous yielded 407, 384, 99, and 23 bp fragments, Factor V homozygous yielded 209 and 32 bp fragments and prothrombin G20210A homozygous yielded 384, 99, and 23 bp fragments [24].

Multiplex PCR-RFLP was used to analyze the 218 human genomic DNA samples that were APC-R resistant. The multiplex PCR-RFLP of Factor V for the 218-samples identified: 41 wild-type, 169 heterozygous alleles, and eight homozygous alleles. Whereas, for prothrombin G20210A: 215 wild-type and three heterozygous alleles. There were no homozygous prothrombin G20210A alleles among the analyzed samples. Figure 1 shows the multiplex PCR-RFLP for the simultaneous detection of FVL and prothrombin G20210A. The means of FII activity among carriers (n=3) and non-carriers (n=215) of the PT-G20210A allele were 107.67%±9.29 and 105.00%±17.79, respectively. The means of FV activity for the wild-type (n=41), heterozygous (n=169) and homozygous (n=8) FVL were 92.93±16.17, 87.02±15.12 and 96.14±12.32, respectively. The differences between the means for both FII and FV were statistically insignificant.

Table 3 demonstrates the results of APC resistance test by biochemical and by DNA techniques. There were a total of 218 (21.8%) individuals who were positive for the biochemical test. However, when these subjects were double tested with the DNA method, 177 (17.7%) turned to be positive (130 males and 47 females) and 41 had the wild type alleles. Of those who were positive, 8 were homozygous (5 males and 3 females), 169 where heterozygous (125 males and 44 females).

DISCUSSION

Factor V Leiden has been shown to be a strong and common risk factor for venous thrombosis [25].

The prevalence of FVL was 21.8%. This prevalence is higher than that reported by Eid *et al*. and Awidi *et al*., who reported a prevalence of 15% and 13%, respectively [26,27]. The lower prevalence reported by those investigators is probably due to the small sample number investigated. These results, in comparison with other studies, may suggest that FVL is not a rare condition outside Europe [28-31].

The expected hypercoagulability in FVL carriers is associated with an APC resistance status. The subjects who were carriers for FVL were identified by resistant

Table 2. Means (±SD) of F-II and F-V for APC-non resistant and APC-resistant subjects.

	APC		p-value
	Non resistant (n=782)	**Resistant** (n=218)	
F-II	98.13±12.43	105.60±17.97	<0.0005
F-V	84.36±11.75	88.92±15.95	<0.005

Table 3. APC resistance by biochemical and DNA methods in 1000 individuals.

Test	Total positive (%)	Male (%)	Female (%)
APCR Biochemical	218 (21.8)	152 (15.2)	66 (6.6)
Only DNA	177 (17.7)	130 (13)	47 (4.7)
Homo	8 (0.8)	5 (0.5)	3 (0.3)
Hetero	169 (16.9)	125 (12.5)	44 (4.4)
Biochemical + DNA	177 (17.7)	130 (13)	47 (4.7)

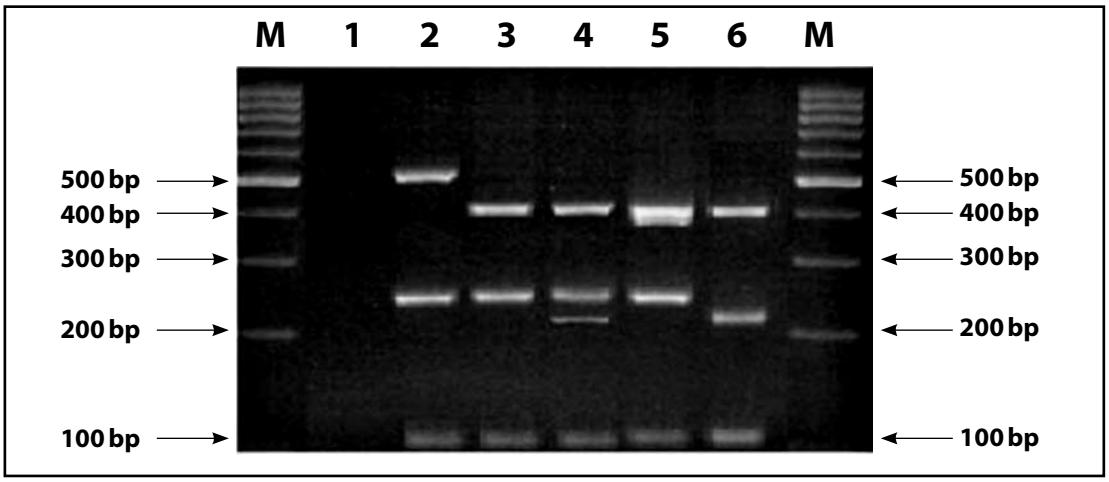

Figure 1. A multiplex PCR-RFLP for the simultaneous detection of FVL and prothrombin G20210A. Three percent agarose gel stained with Ethidium bromide showing: Lanes M: DNA molecular weight markers. Lane 1: Negative control (distilled water). Lane 2: Undigested amplicons of prothrombin G20210 (506 bp) and factor V Leiden (241 bp). Lane 3: Digested products of the wild-type prothrombin and the wild-type factor V with HindIII (407, 241, and 99 bp). Lane 4: Digestion products of the wild type prothrombin (407 and 99 bp), and the digested products of the heterozygous factor V (241, 209 and 32 bp). Lane 5: Digestion products of the heterozygous prothrombin (407, 384, 99 and 23 bp) and the wild type factor V (241 bp). Lane 6: Digestion products of the wild type prothrombin (407 and 100 bp) and a homozygous factor V (209 and 32 bp).

plasma for the APC-anticoagulant activity [10,13,32,33]. In our study, this was represented by a shorter time for clot formation with APC-R mean of 85.52±15.35 sec, while those having the wild type alleles for FV showed normal coagulation time (159.90±26.92 sec).

When considering sex differences, statistically significant ($p<0.005$) difference in APC-R value between APC-nonresistant males and females were found, where the mean value was higher among males than females. Nonetheless, among resistant subjects the difference between the two sexes was not significant ($p=0.164$). These results may be explained by the hormonal influence of APC-R which is related to FVL allele as suggested by Henkens *et al.* [34].

Allele frequency of prothrombin G20210A was found to be 1.8%, where all the three subjects were heterozygous carriers. This prevalence is also one of the highest reported world-wide and it is in an agreement with what had been reported Eid *et al.* [26]. The mean level for F-II activity was 105.00±17.79 and 107±9.29 for subjects with the wild type prothrombin and PT-G20210A polymorphism, respectively. The difference between those two means not statistically significant ($p=0.80$). Swibertus *et al.* suggested that factor II activity level assay is not a sensitive or accurate marker for PT-G20210A polymorphism which is in an agreement with our findings [14].

The correlation between activated protein C resistance and the prothrombin activity level showed that there is a strong and significant negative correlation between these two variables ($p<0.005$). This correlation suggests that as APC-R value decreases (more resistance), prothrombin activity level increases. This certainly supports the fact that APC acts as an inhibitor regulator for the coagulation process where prothrombin activation is an intermediate step [8,32). Gender and age were excluded due to their low and non-significant effect on Factor II activity.

Our results suggest that there is a little possibility of the co-inheritance of the FVL and PT-G20210A among healthy young adults, since only few cases were found to be carriers for the two alleles.

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