

A Multiplex Allele Specific Polymerase Chain Reaction (MAS-PCR) for the Detection of Factor V Leiden and Prothrombin G20210A

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ABSTRACT

Introduction: In order to determine the frequencies of factor V Leiden and prothrombin G20210A point mutations in the Iranian population with Azeri Turkish origin.

Material and methods: 120 unrelated individuals from general population randomly selected and were examined for factor V Leiden and prothrombin G20210A mutations using a multiplex allele specific polymerase chain reaction (MAS-PCR) assay.

Outcomes: The frequency of prothrombin G20210A mutation was 2.08%, which means 5 chromosomes out of 240 chromosomes had prothrombin G20210A mutation. The distribution of prothrombin 20210 GG, GA, AA genotypes and prothrombin 20210A allele were 37(92.5%), 3(7.5%), 0(0%) and 3(3.75%) in males and 78(97.5%), 2(2.5%), 0(0%) and 2(1.25%) in females, respectively. Factor V Leiden was not found in our tested group (zero chromosomes out of 240 chromosomes). Analysis of the observed frequencies in the studied groups indicates that there is no statistically significant difference between females and males, regarding prothrombin G20210A mutation (p value > 0.05).

Conclusions: This is the first study in its own kind in this population and implies that the frequency of Factor V Leiden G1691A (R506Q, FV-Leiden) allele is extremely low but the prothrombin G20210A mutation is more frequent in the tested group.

Keywords: factor V Leiden, prothrombin G20210A, normal population

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The heterogeneity of human diseases informs that the role of polymorphisms and mutations is a complicating factor for determining the molecular basis of diseases in different ethnic groups (1). In the most of cases, human diseases are caused by substitutions of a single base or insertions/deletions of a unique element in a gene (1). Thrombophilia or hypercoagulability is a group of inherited human disease which promotes blood clotting, giving a problem including abnormal thrombosis or increases in blood clotting factors (I, II, VII, VIII, IX, and XII) as well as decreasing in protein S levels and reduction in the activated protein C activity (2-9). Several mutations within thrombophilic genes have been identified and the most important includes the factor V G1691A mutation (FV Leiden) (10-12) and prothrombin G20210A mutation (10-12). The association between thrombophilic biomarkers and human disorders such as factor V G1691A, prothrombin G20210A mutations and recurrent miscarriage (13-22), angiotensin-1-converting enzyme gene and silicosis (23), factor XIII (V34L), plasminogen activator inhibitor-1 (4G/5G) and myocardial infarction (5,24,25), methylenetetrahydrofolate reductase (MTHFR) (C677T and A1298C) and early first trimester recurrent pregnancy loss (5,25,26) have been reported. But, still there is controversial (27-33). Determining of single nucleotide variation patterns as the most frequent changes of the DNA sequences provide informative information about population genetic background, gene pool, and individual specific biomarkers (34). The aim of the present study was to determine the frequency of Factor V G1691A and prothrombin G20210A mutations in West Azerbaijani normal general population. □

METHODS AND MATERIALS

The Ethics Committee of the Urmia University of Medical Sciences approved the project. 120 unrelated individuals were recruited to the present investigation for the study of Factor V G1691A and prothrombin G20210A mutations in West Azerbaijani normal general population. Normal individuals were included among participants in genetic counseling sessions taken place in genetic center of Urmia University of Medical Sciences during the period March 2008 through September 2010. All healthy individuals were selected randomly regarding their past medical history and exclusion of any specific disorders (e.g., cardiovascular and thrombophilia). Informed written consent was obtained from participants. DNA was isolated from 5 ml blood samples from each person by “salting out” method (35). Multiplex Allele Specific Polymerase Chain Reaction (MAS-PCR) amplification was carried out with Factor V Leiden G1691A and FII G20210A mutations sequence-specific primers at the same reaction. MAS-PCR protocol and sequences of primers have been described previously (36) (see table 1). Genomic DNA was amplified using normal or mutant specific primers as forward primer and a common reverse primer. Amplified PCR products were separated on 2% agarose gel electrophoresis. Gels were stained with ethidium bromide. Presence or absence of PCR products were visualized by UV transilluminator on UV gel documentation. Factor V Leiden G1691A and FII G20210A mutations give a PCR product of 150 and 340 bp respectively. □

Mutations	Sequences of primers	PCR products	PCR Thermal profile
FV G1691A	(C): 5'-gga cta ctt gac aat tac tgt tct ctt g-3'	150bp	initial denaturation: 95°C for 10 min
	(N): 5'-gca gat ccc tgg aca gac g-3'		10 cycles:
	(M): 5'-gca gat ccc tgg aca gac a-3'		denaturation: 94°C for 30 sec,
Prothrombin G20210A	(C): 5'-tct aga aac agt tgc ctg gca g-3'	340bp	annealing: 60°C for 30 sec,
	(N): 5'-gca ctg gga gca ttg agg atc-3'		extension: 72°C for 1 min;
	(M): 5'-gca ctg gga gca ttg agg att-3'		and 25 cycles:
			denaturation: 94°C for 30 sec,
			annealing: 55°C for 30 sec,
			extension: 72°C for 1 min

TABLE 1. Sequences of primers, PCR thermal profile and PCR products used for identifying the Factor V G1691A and Prothrombin G20210A mutations

C = common; N = normal; M = mutant.

OUTCOMES

The allele frequency and observed/expected frequencies of different genotypes were computed for FV Leiden G1691A (R506Q, FV-Leiden) and FII G20210A mutations in males, females, and males& females groups. All observed/expected allelic/genotypic frequencies are reported in table 2. Comparison analysis between observed and expected frequencies indicate that genotypic distributions were in Hardy-Weinberg equilibrium, regarding of Factor V Leiden G1691A and FII G20210A mutations in males, females, and males& females groups (see table 2). The prothrombin G20210A mutation was more frequent in our tested group, that is, 3 chromosomes out of 80 chromosomes in males (3.75%), 2 chromosomes out of 160 chromosomes in females (1.25%), and totally, 5 chromosomes out of 240 chromosomes in males+females (2.08%) groups had prothrombin 20210A mutation. Prothrombin 20210AA (homozygote) mutation as well as Factor V Leiden was not found in our groups (zero out of 240 chromosomes). The prothrombin 20210 GA (heterozygote) genotype was 7.5% in males and 2.5% in females. On the other hand, 5 people out of 120 people (3 out of 40 males and 2 out of 80 females) had prothrombin G20210A mutation that means 4.17% were carriers of the prothrombin

20210A mutation in tested general group. Analysis of the prothrombin G20210A mutation profile in the studied groups indicated that there is no statistically significant difference between females and males, regarding observed frequencies. Prevalence of prothrombin G20210A mutations was similar between males and females and differences were not statistically significant regarding prothrombin 20210GA genotype (OR [95% CI]: 3.162[0.50-19.74], $\chi^2 = 1.66$, p-value =0.196) and prothrombin 20210A allele (OR [95% CI]: 3.077 [0.50-18.80], $\chi^2 = 1.63$, p-value =0.201). Prevalence of the prothrombin 20210GG genotypes (OR [95% CI]: 0.316 [0.05-1.97], $\chi^2 = 1.66$, p-value =0.196) and prothrombin 20210G alleles (OR [95% CI]: 0.324 [0.05-1.98], $\chi^2 = 1.63$, p-value =0.201) were not significantly different between males and females. Our results are similar to studies conducted in other populations throughout the world regarding the frequency of FII gene mutation (G20210A). Prothrombin 20210 G>A mutation were detected as 0.7-4.5% in the world (37-43). Factor V Leiden G1691A (GA and AA) and prothrombin 20210AA genotypes were not determined in the present study. However, factor V Leiden mutation is extremely low in the tested group and this is fit to a report with Asian origin and different from some others (44-47). Allelic frequency of factor V

Group	FII G20210A	Observed (%)	frequency	Expected	H.W.E. analysis
Total N=120 ^a	GG	115(95.8)	0.96	115.1	$\chi^2=0.05 < 3.84$
	GA	5(4.17)	0.04	4.896	p-value =0.97 >0.05
	AA	0(0)	0	0.052	df=2
	G	235(97.92)	0.98		
	A	5(2.08)	0.02		
Male N=40 ^b	GG	37(92.5)	0.93	37.06	$\chi^2=0.06 < 3.84$
	GA	3(7.5)	0.08	2.888	p-value =0.97 > 0.05
	AA	0(0)	0	0.056	df=2
	G	77(96.25)	0.96		
	A	3(3.75)	0.04		
Females N=80 ^c	GG	78(97.5)	0.98	78.01	$\chi^2=0.01 < 3.84$
	GA	2(2.5)	0.03	1.975	p-value =0.99 > 0.05
	AA	0(0)	0	0.013	df=2
	G	158(98.75)	0.99		
	A	2(1.25)	0.01		

TABLE 2. FII G20210A genotype and allele distributions in a general population and HWE analysis for studied groups which show that each group (males, females and males+females) is consistency with HWE regarding distributions of genotypes or alleles

a: A allele frequency =0.0208, b: A allele frequency =0.038 and c: A allele frequency = 0.01; H.W.E. : Hardy-Weinberg Equilibrium, df: degree of freedom, N: number.

Leiden has been found to differ around the world, and in Caucasian populations varies between 4% and 7% (42). As shown in figure 1, samples 1, 2 are homozygote normal individuals regarding Factor V Leiden G1691A and FII G20210A mutations and the sample 3 is carrier (heterozygote) for FII G20210A mutation as well as normal for Factor V Leiden G1691A mutation. Factor V Leiden 1691A allele and FII 20210AA (homozygote) genotype were not found in the present examination. □

DISCUSSION

The allelic/genotypic frequencies of the thrombophilic markers such as factor V Leiden and prothrombin G20210A mutations were studied in the present study. Among the trombophilic markers, Factor V Leiden G1691A (R506Q, F V-Leiden) and FII G20210A were selected for study because of high prevalence of these mutations in among Caucasians throughout the world (42,48). However, the prevalence of the Factor V Leiden G1691A and FII G20210A mutations have not been evaluated in a general population with Azeri Turkish origin. Therefore detection of the FV Leiden G1691A (R506Q, FV-Leiden) and FII G20210A mutations distributions in our group could be considered as informative regarding trombophilia state and a basis for cases-control association studies in the future. Factor V Leiden and prothrombin G20210A mutations were analyzed in healthy individuals via MAS-PCR. Genomic amplification of the factor V Leiden and prothrombin G20210A mutation yielded a 150 bp fragment for factor V Leiden and a 340 bp fragment for prothrombin G20210A mutation. Our findings as shown in table 2 indicate that the frequencies of factor V Leiden and prothrombin G20210A mutations were zero and 2.08% respectively. Factor V Leiden 1691 (GA or AA) and prothrombin 20210AA genotypes were not found in our group. The prevalence of prothrombin 20210GA (heterozygote) genotypes was 4.17%. The frequency of factor V Leiden mutation in the present study is lower than some other studies (37,39-42,44-51) (see table 3). As figure 2 shows, the prevalence of factor V Leiden mutation in some studies including Rahimi et al (37), Zeinali et al (39), Karimi et al (40), Finan et al (41), Mahjoub et al (21), Altintas et al (32), Ridker et al 1998 (47), Sottilotta et al (20), Pauer et al (51), Reznikoff-

Etiévan et al (14) is higher than our study (p-value and χ^2 with degree of freedom=2 are shown in figure 2). But, our findings indicate that the frequency of factor V Leiden mutation in the present study are similar to the findings of the other investigations throughout the world such as Behjati et al (38), Souza et al (16), Foka et al (17), Dizon-Townson et al (46), Hashimoto et al (30) (p- value >0.05, for more details see figure 2). However, the prothrombin G20210A mutation was more frequent in our population.

population	Factor V Leiden G1691A	FII G20210A	Reference (no.)
Present study	0	2.5	3
Iran	2.97	1.6	Rahimi et al (37)
	0	3.2	Behjati et al (38)
	5.5	3.1	Zeinali et al (39)
	4.1	3.07	Karimi et al (40)
Lebanon	16.42	2.99	Finan et al (41)
	5.5	4.5	Mahjoub et al (21)
Brazil	1.6	1	Souza et al (16)
Sweden	2.89	2.9	Wramsby et al (18)
Turkey	7	1.6	Altintas et al (32)
	4.6 -12	0.7	Irdem et al (42)
		1.2	Ayyildiz et al (43)
Greece	4	2	Foka et al (17)
	4.4		Antoniadi et al (44)
Saudi Arabia	2.5		Rees et al (45)
Cyprus	8.1		Antoniadi et al (44)
U.S.A.	3.7-4.2		Dizon-Townson et al (46), Ridker et al 1998 (47)
U.S.A.	2		Glueck et al (19)
Caucasian Americans	5.27		Ridker et al 1997 (48)
Hispanic Americans	2.21		Ridker et al 1997 (48)
African Americans	1.23		Ridker et al 1997 (48)
Asian Americans	0.45		Ridker et al 1997 (48)
Native Americans	1.25		Ridker et al 1997 (48)
U.K.	2.28	2.28	McCull et al (49)
Italy	3	3	Martinelli et al (10)
	1.9	4.2	Grandone et al (50)
	3.2	2.8	Sottilotta et al (20)
Germany	9.2		Pauer et al (51)
France	4.6	2.9	Reznikoff-Etiévan et al (14)
Ireland	1.36		Murphy et al (15)
Japan	0		Hashimoto et al (30)

TABLE 3. Distribution of Factor V Leiden G1691A and FII G20210A in different groups

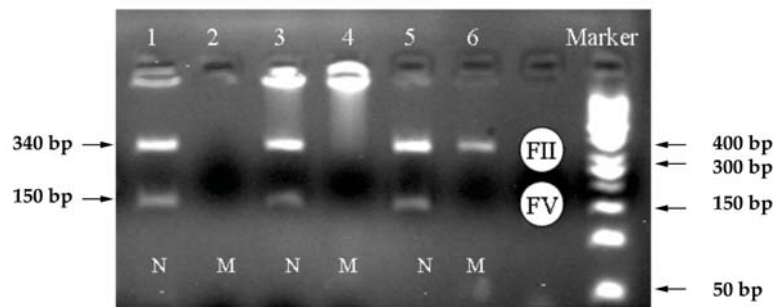


FIGURE 1. Determining of Factor V Leiden G1691A and FII G20210A mutations via MAS-PCR method in 3 samples
Factor V Leiden G1691A and FII G20210A mutations result in a PCR product of 150 bp and 340 bp fragments respectively. Two PCR reactions have been performed for each sample, first reaction with normal allele primers, and the second reaction with mutant allele primers. Electrophoresis analysis of PCR products from 3 samples have been shown in the figure. Lanes (1, 2) and (3, 4) indicate that samples 1 and 2 have two normal bands without any mutant band (Factor V 1691GG and FII 20210 GG genotypes), and lanes 5 and 6 are from a person who is heterozygote for FII (FII 20210GA genotypes), and normal for Factor V (Factor V 1691GG).
N = Normal; M = Mutant.

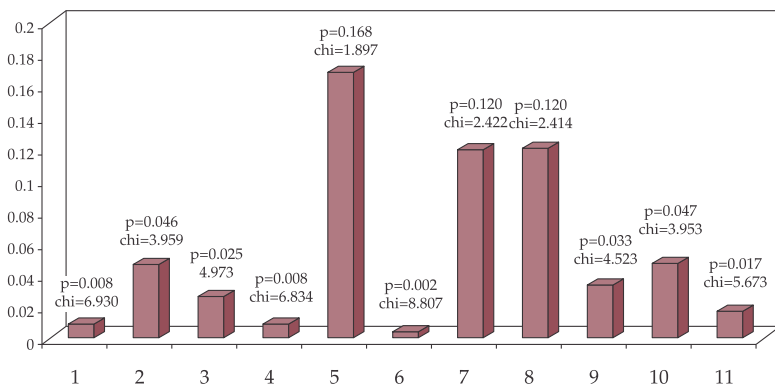


FIGURE 2. The prevalence of mutations in factor V Leiden in other groups in comparison with the present study
1: n=161 (Tehran, Iran [mixed ethnicity]), Rahimi et al (37), Zeinali et al (39); 2: n=404 (Kermanshah, Iran [Kurdish]), Rahimi et al (37); 3: n=198, Karimi et al (40); 4: n=200, Mahjoub et al (21); 5: n=384, Souza et al (16); 6: n=185, Altintas et al (32); 7: n=100, Foka et al (17); 8: n=50, Dizon-Townson et al (46); 9: n=437, Ridker et al 1998 (47); 10: n=217, Sottilotta et al (20); 11: n=87, Pauer et al (51); 12: n=240, Reznikoff-Etiévan et al (14).

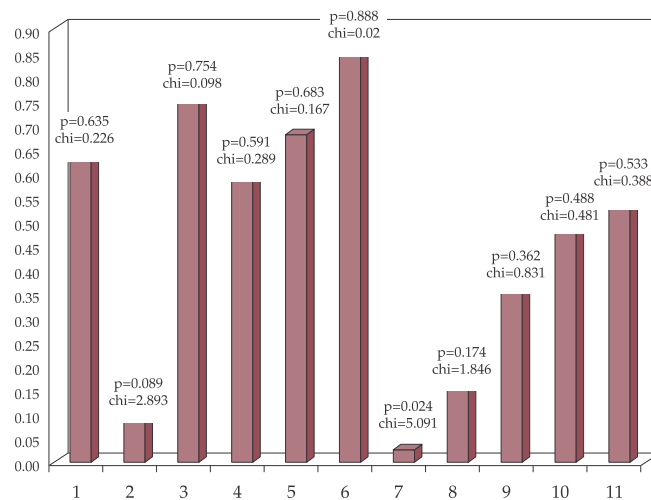


FIGURE 3. The prevalence of mutations in prothrombin G20210A in other groups in comparison with the present study
1: n=161 (Tehran, Iran [mixed ethnicity]), Rahimi et al (37), Zeinali et al (39); 2: n=434 (Kermanshah, Iran [Kurdish]), Rahimi et al (37); 3: n=62, Behjati et al (38); 4: n=198, Karimi et al (40); 5: n=67, Finan et al (41); 6: n=200, Mahjoub et al (21); 7: n=384, Souza et al (16); 8: n=185, Altintas et al (32); 9: n=100, Foka et al (17); 10: n=217, Sottilotta et al (20); 11: n=240, Reznikoff-Etiévan et al (14).

figure 3 shows the prevalence of prothrombin G20210A mutation in other groups compared with the present study and indicates that more studies such as Rahimi et al (37), Behjati et al (38), Zeinali et al (39), Karimi et al (40), Finan et al (41), Mahjoub et al (21), Altintas et al (32), Foka et al (17), Sottilotta et al (20), Reznikoff-Etiévan et al (14) are in agreement with the present study (p- value >0.05, for more details see figure 3); but, the only exception is Souza et al (16) (p- value =0.024 <0.05, χ^2 5.091, df=2). The findings of the present study are the first official report regarding frequency of factor V Leiden and prothrombin G20210A mutations in Iranian population with Azeri Turkish origin. Frequency of factor V Leiden and prothrombin G20210A mutations suggested that the latter was more frequent in our population,

which makes prothrombin G20210A mutation informative in the diagnosis of the high risk individuals (carriers). Our sample size was limited, so our findings cannot indicate that Iranian general population with Azeri Turkish origin never carries the factor V Leiden G1691A (R506Q, FV-Leiden). It can be concluded that the frequency of Factor V Leiden G1691A (R506Q, FV-Leiden) allele is extremely low but the prothrombin G20210A mutation is more frequent in an Iranian general population with Azeri Turkish origin. \square

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