wJournal Homepage: - www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI:10.21474/IJAR01/4825
DOI URL: http://dx.doi.org/10.21474/IJAR01/4825
ISSN NO. 2320-5407

RESEARCH ARTICLE

FACTOR V LEIDEN G1691A AND PROTHROMBIN G20210A POLYMORPHISMS IN GEORGIAN ARTERIAL THROMBOSIS PATIENTS.

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Manuscript Info

Manuscript History

Received: 12 May 2017
Final Accepted: 14 June 2017
Published: July 2017

Key words:-
Arterial thrombosis, Georgia, FV Leiden, prothrombin, risk factors.

Abstract

Thrombosis plays a crucial role in the pathogenesis of acute myocardial infarction, stroke and venous thrombosis. Factor V Leiden (FVL) and prothrombin (PT) G20210A polymorphisms are the most frequent causes of inherited thrombophilia. In this work, we aimed to evaluate the prevalence of FVL and PT G20210A polymorphisms in a Georgian cohort of patients and healthy individuals, and its association with arterial thrombosis.

This study involved 214 individuals, 101 arterial thrombosis patients (71.3% males; 66.3 +/- 12.1 years old) and 113 healthy control subjects (67.3% males; 56.6 +/-11.3 years old). Genomic DNA was extracted from a dry blood spot on Whatman paper. Polymerase chain reaction was performed in order to determine the two genetic markers of thrombosis risk: FVL (G1691A) and PT G20210A polymorphisms.

The frequency of FVL allele polymorphism in the control group was 0.9%, which corresponds to a heterozygous stage frequency of 1.8%. In the patient group, an allelic frequency of 2% was found, which corresponds to the presence of 4% of heterozygous individuals. The frequency of heterozygosity for the PT G20210A polymorphism was 4.4% in the control group, which corresponds to an estimated allelic frequency of 2.2%. In the patient group, a frequency of heterozygosity of 3% was found, which corresponds to an estimated allelic frequency of 1.5%. Homozygosity for FVL and PT G20210A polymorphisms was not found in either group.

Our results suggests that FVL could be associated with arterial thrombosis as a double prevalence was found in the patient group when compared with the control group, although no significant differences were found. Moreover, our results also showed that the PTG20210A polymorphism seems to not be associated with arterial thrombosis in the Georgian population. More studies are required with a bigger sample in order to draw definitive conclusions.

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Introduction:-

Worldwide population projections indicate that the number of persons aged 60 or above will double by 2050 and triple by 2100 [1]. In Georgia, a change in age structure is expected, with an increasing rate of old people. According to the official data given by the Ministry of Labour, Health and Social Affairs of Georgia by 2014 the portion of 65-year and older population comprised 13.8% of the whole population [2]. This increased life expectancy is associated with an increased prevalence of chronic diseases, including cardiovascular diseases (CVD). The increasing prevalence of chronic diseases associated with ageing is associated with increasing use of health care services, a high number of individuals requiring special needs, and increased costs associated with health and social care [3].

CVD is the leading cause of death worldwide. A Global Burden of Disease Study in 2013, estimated that almost 30% of all deaths worldwide were caused by CVD. Moreover, CVD alone will be responsible for more deaths in low income countries than infectious diseases (including HIV/AIDS, tuberculosis, and malaria), maternal and perinatal conditions, and nutritional disorders combined [4]. Thus, CVD is currently the largest single contributor to global mortality and will continue to dominate mortality trends in the future. This is a very important problem in Georgia, due to the high death-rate associated with CVD (583.2 per 100 000 habitants) according to the Ministry of Labour, Health and Social Affairs of Georgia [5]. Thrombosis plays a crucial role in the pathogenesis of acute myocardial infarction, stroke and venous thrombosis. The recognition of thrombophilia as a potentially inheritable disease was first described 1965 [6] and later confirmed with evidence that coagulation proteins that increase the risk of thrombotic events, such as antithrombin, protein C and protein S, and dysfibrinogenemia, followed an autosomal dominant inheritance pattern [7].There is now evidence that familial thrombophilia followed a polygenic inheritance model, in which the co-segregation of one or more additional genetic factors increases the risk of thrombosis. Factor V Leiden (FVL) and prothrombin G20210A mutations are the most frequent causes of inherited thrombophilia [8].

Increases in coagulator potential is a common characteristic of the elderly, so age itself plays an important role as a risk factor in the onset of thromboembolic diseases, added to other risk factors such as smoking, hypertension or diabetes.The contribution of genetic polymorphisms, in particular of Leiden FV and FII G20210A, to the susceptibility to arterial thrombotic events in the elderly remains to be demonstrated. Moreover, there is a lack of information about FVL and PT G20210A polymorphisms in Georgia. For these reasons, we aimed to evaluate the prevalence of FVL and PT G20210A polymorphisms in a Georgian cohort of patients and of healthy individuals, and their associations with arterial thrombosis.

Material and Methods:-

Subjects:-

This study involved 214 individuals, 101 arterial thrombosis patients (71.3%males; 66.3 +/- 12.1 years old), 83% with myocardial infarction (MI) and 13% with ischemic stroke, and 113 healthy control subjects (67.3% males; 56.6 +/-11.3 years old). The mean age of the first MI and ischemic stroke was 62.6 +/- 12.6 and 71.5+/- 11.7 years, respectively. Moreover, 12 MI patients have had a recurrent MI episode. Troponin measurement and computed tomography were used for MI and ischemic stroke diagnosis of patients.

Dry blood samples were collected at the Heart Disease Department and Medical Ward of Batumi Hospital, Government of Autonomous Republic of Ajara, Georgia, and processed at the Faculty of Pharmacy, University of Porto, Portugal. All participants gave their written informed consent to participate in this study that was previously approved by the Ethics Committee from LTD UnimedAdjara (Georgia).

DNA Extraction:-

Genomic DNA was extracted from a dry blood spot on Whatman paper according to the instructions from KAPA express extract Kit (KAPA Biosystems). DNA samples were stored at -20ºC until use.

Polymerase Chain Reaction:-

Polymerase chain reaction (PCR) was performed in order to determine the two genetic markers of thrombosis risk, as previously described [9]: FVL (G1691A) and PT G20210A. To discriminate the single base changes between normal (N) and mutated (M) alleles, two reverse (R) primers (normal and mutated) were used and were paired with a common forward (F) primer. Primer sequences are provided in table 1. The PCR reactions were performed with an

initial denaturation of 95ºC for 3 min, followed by 39 and 36 cycles of 95ºC for 30 sec for FVL and PT G20210A, respectively; annealing temperatures of 58ºC and 56ºC for FV Leiden and PT G20210A, respectively, for 30 sec; 72ºC for 1 min for both polymorphisms; and a final extension at 72˚C for 5 min. Primers for the human factor (F) IX gene, used as an internal control, were FIX-forward 5'-CTCCTGCAGCATTGAGGGAGATGGACATT-3' and FIX-reverse 5'-CTCGAATTCGGCAAGCATACTCAATGTAT-3'.The amplification products were analyzed by electrophoresis in 2% agarose gel with ethidium bromide.

Table I:- Primers used for FVL (G1691A) and PT G20210A polymorphisms detection.

Factor V Leiden (G1691A)	F: 5'-TGTTATCACACTGGTGCTTAA-3'
	R-N: 5'-CAGATCCCTGGACAGACG -3'
	R-M: 5'-CAGATCCCTGGACAGACA-3'.
Prothrombin(G20210A)	F: 5'-TCTAGAAACAGTTGCCTGGC-3'
	R-N: 5'-CACTGGGAGCATTGAGGATC-3'
	R-M: 5'-CACTGGGAGCATTGAGGATT-3'.

F: forward primer, R-N: normal reverse primers; R-M: mutated reverse primer

Statistical Analysis:-

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, version 21.0) for Windows (SPSS Inc., Armonk, NY, USA). Results are presented as proportions. The association between categorical variables was analyzed using the chi-squared test or Fisher's exact test. Significance was accepted at $p<0.05$.

Results:-

Tables II and III showed FVL and PTG20210A genotypes and allelic frequencies of patients and control groups.

The frequency of FVL allele polymorphism in the control group was 0.9%, which corresponds to a heterozygous stage frequency of 1.8%. In the patient group, an allelic frequency of 2% was found, which corresponds to the presence of 4% of heterozygous individuals (table II). Homozygosity for FVL was not found in either group. Although this frequency was higher in the group of patients, no statistically significant differences were found in the allelic frequency between groups ($p>0.05$). When patients were stratified according to MI and Ischemic stroke status, no significant differences in prevalence of FVL were found between the MI and Ischemic stroke groups of patients and control groups, nor between recurrent MI patients and control groups.

Table II:- Genotype and allelic frequencies of FVL polymorphism in patients and control groups.

	n	**Genotype frequencies**			***p* value**	**Allele frequencies**	
		G/G n (%)	**G/A n (%)**	**A/A n (%)**		**G (%)**	**A (%)**
Controls	113	111 (98.2)	2 (1.8)	0	-	99.1	0.9
All Patients	101	97 (96)	4 (4)	0	0.424*	98	2
Myocardial infarction patients	84	80 (95.2)	4 (4.8)	0	0.405*	97.6	2.4
Recurrent myocardial infarction patients	12	11 (91.7)	1 (8.3)	0	0.263*		
Ischemic stroke patients	17	17	0	0	-	100	0

*p value vs. controls

Table III:-Genotype and allelic frequencies of PT G20210A polymorphism in patients and control groups.

	n	**Genotype frequencies**			***p* value**	**Allele frequencies**	
		G/G n (%)	**G/A n (%)**	**A/A n (%)**		**G (%)**	**A (%)**
Controls	113	108 (95.6)	5 (4.4)	0	-	97.8	2.2
All Patients	101	98 (97)	3 (3)	0	0.725*	98.5	1.5
Myocardial infarction patients	84	81 (96.4)	3 (3.6)	0	0.532*	98.2	1.8

Recurrent myocardial infarction patients	12	12 (100)	0	0	-	100	0
Ischemic stroke patients	17	17	0	0	-	100	0

*p value vs. controls

The frequency of heterozygosity for the PT G20210A polymorphism was 4.4% in the control group, which corresponds to an estimated allelic frequency of 2.2%. In the patient group, a frequency of heterozygosity of 3% was found, which corresponds to an estimated allelic frequency of 1.5% (Table III). Homozygosity for the PTG20210A polymorphism was not found in either group. No differences were found in the allelic frequency between control and patient groups. When patients were stratified according to MI and Ischemic stroke status, no significant differences were found in the prevalence of the PT G20210A polymorphism between the MI and Ischemic stroke group of patients and the control group, nor between recurrent myocardial infarction patient and control groups.

Discussion:-

There is evidence that both arterial and venous thrombotic events exponentially increase with age. Increasing life expectancy is the major cause of the current epidemic of both arterial and venous thrombosis [10-12], which should be related to the cumulative effects of risk factors on the arterial wall, increasing systemic activation of blood coagulation and immobility resulting in venous stasis, and decreased regular exercise.

Arterial disorders are associated with the presence of clinical and environmental risk factors, such as dyslipidemia, smoking, diabetes, hypertension, and others [13]. Moreover, several inherited risk factors were also described as associated with arterial disorders; however, its association with FVL and PT G20210A polymorphisms is debated.

FVL is associated with a point mutation in the gene encoding for circulating plasmatic vitamin-K-dependent glycoprotein produced by the liver, the factor V. It was described for the first time as activated protein C resistance (APC-R) syndrome in 1993 [14]. Factor V binds to factor X to form the prothrombin complex, which in turn activates factor II to cross-link fibrin complexes. As part of normal feedback inhibition, factor V activated (FVa) is deactivated by activated protein C (APC) by cleavage at amino acid 506, thus limiting the extent of clot formation. This polymorphism causes the production of FV protein that is resistant to the action of APC, resulting in over 90% of APC resistance [15]. FVL is the most common inherited form of thrombophilia, which accounts for 40–50% of cases. The worldwide prevalence of FVL is very heterogeneous, with a prevalence of 1–15% in European white populations [16] and is extremely rare in Asian and African populations [17]. In this work, a prevalence of 1.8% (mutant heterozygote) was found in our control sample from Georgia, which is in accordance to that found in European population. Moreover, in our group of arterial thrombosis patients, a prevalence of 4% (mutant heterozygote) and 4.8% for the MI patient group were found, which is double the prevalence found in the control group, suggesting a possible association between FVL and MI in the elderly. However, no statistical differences were found between the two groups, which makes it difficult to draw a definitive conclusion about this association in our population, since this statistic result may be related to the reduced number of cases found.

PT G20210A polymorphism was described in 1996 [18], and is associated with higher levels of the clotting factor prothrombin in the blood of carriers, which creates a higher tendency towards blood clotting. Indeed, carriers become at higher risk of developing thromboembolic events [19]. PT G20210A polymorphism is the second most common inherited thrombophilia after the FVL. The prevalence of PT G20210A polymorphism differs between countries and ethnic groups and is higher in Southern Europe and the Mediterranean regions [20]. Also, the prevalence of PT G20210A polymorphism in European Caucasians ranges between 1% and 8% [20]. In this work, a prevalence of 4.4% (mutant heterozygote) was found in the control sample population, which is in accordance with that found in other European populations. A lower prevalence (3%) of the mutant allele was found in arterial thrombosis patients, when compared with the control group (4.4%). These results suggest that the PT G20210A polymorphism is not associated with arterial thrombosis, including MI, in the Georgian population.

In conclusion, our results suggest that FVL could be associated with MI as a double prevalence was found in the patient group, although no significant difference was found. Moreover, we also found that the PT G20210A polymorphism seems to not be associated with arterial thrombosis in the Georgian population. More studies are required with a bigger sample in order to draw definitive conclusions.

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