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FGB -455 G>A and GP IIIa PIA1/A2 polymorphisms in a group of Romanian stroke patients

Implicațiile polimorfismelor genetice ale FGB -455 G>A și GP IIIa PIA1/A2 într-un grup de pacienți cu accident vascular cerebral din România

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Abstract

***Introduction**: Being a multifactorial disease, stroke is one of a major causes of death and disability worldwide. Several genetic polymorphisms have been associated with stroke etiophatology and FGB -455 G>A and GP IIIa PIA1/A2 are among them. In the present study, we investigated the association between FGB -455 G>A and GP IIIa PIA1A2 polymorphisms and the risk of ischemic stroke in a group of Romanian stroke patients.*

***Subjects and methods**: This case-control study included 148 patients with ischemic stroke and 150 healthy age, sex and ethnically matched unrelated controls. FGB -455G>A and GP IIIa PIA1A2 genotyping was carried out using PCR-RFLP. The association of FGB -455G>A and GP IIIa PIA1A2 polymorphisms and cardiovascular risk factors with ischemic stroke was tested using logistic regression analysis.*

***Results**: Molecular analysis did not reveal an increased frequency of the FGB -455 G>A variant allele and GP IIIa PIA1/A2 variant allele in the study group compared to the control group (p = 0.140, OR = 0.750, 95% CI = 0.522- 1.077; p = 0.823, OR = 0.944, 95% CI = 0.558 - 1.599 respectively). Furthermore, after performing logistic regression analysis adjusted for the known risk factors, a positive association with stroke was found in smokers (p = 0.026, OR = 1.800, 95% CI = 1.071- 3.024)*

***Conclusions**: No association was found between FGB -455 G>A and GP IIIa PIA1/A2 polymorphisms and ischemic stroke in the studied population.*

***Keywords**: ischemic stroke, fibrinogen, glycoprotein, polymorphism*

Rezumat

***Introducere**: Accidentul vascular cerebral (AVC), afecțiune cu caracter multifactorial recunoscut, constituie una dintre principalele cauze de deces și invaliditate din lume. Mai multe polimorfisme genetice au fost asociate cu etiopatogenia AVC, printre care FGB -455 G> A si GP IIIa PIA1/A2. În studiul de față, am investigat asocierea polimorfismelor FGB -455 G> A și GP IIIa PIA1/A2 cu riscul de aparitie a AVC ischemic într-un grup de pacienţi cu AVC din România.*

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***Subiecţi şi metode**: Studiul, de tip caz-control, a inclus 148 de pacienţi cu AVC ischemic şi 150 controale neînrudite, compatibile ca vârstă, sex şi etnie. Genotiparea polimorfismelor a fost realizată folosind tehnica PCR-RFLP. Prin regresie logistică s-a evaluat asocierea polimorfismelor FGB -455G> A şi GP IIIa PIA1A2 şi a factorilor de risc cardiovascular cu riscul de AVC ischemic.*

***Rezultate**: Frecvența alelelor variant a FGB -455 G> A și GP IIIa PIA1/A2 a fost similară în cele două loturi (p = 0.120, OR = 0,750, 95% CI = 0.522 - 1.077, respectiv p = 0.823, OR = 0.944, 95% CI = 0.558 - 1.599). În plus, după efectuarea, regresiei logistice ajustate pentru factorii de risc cunoscuți, la subiecții fumători a fost observată o asociere pozitivă cu AVC (p = 0,026, OR = 1.80, 95% CI = 1.07- 3.02).*

***Concluzii**: Nu a fost gasită nici o asociere între polimorfismele FGB -455 G> A și GP IIIa PIA1/A2 și AVC ischemic în populația studiată.*

***Cuvinte cheie**: accident vascular cerebral ischemic, fibrinogen, glicoproteină, polimorfism*

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Background

Ischemic stroke is one of the major public health issues, since it is among the main four causes of mortality in Europe (1) and one of the leading causes of disability and death worldwide (2). In Romania, the overall stroke has an increased prevalence, the risk factors being similar to those from other European Union (EU) countries (3).

The etiology of ischemic stroke is multifactorial, many genes with variable phenotypic expression being incriminated. Nowadays the major direction of investigation is the candidate-gene approach, based on case-control studies (4). During the past decade the identification of functional polymorphisms in prothrombotic factors encouraged the search for other possible involved genetic polymorphisms in acute-phase proteins and their receptors based on their well known involvement in arterial thrombotic disorders (5).

Fibrinogen is an acute-phase glycoprotein (GP) implicated in blood clotting that acts as a bridge between platelets by binding to their GP IIb/IIIa surface membrane proteins (6). According to epidemiological studies, elevated plasma fibrinogen levels induce atherothrombotic and inflammatory events that are associated with an increased risk of cardiovascular disorders (7, 8), including ischemic heart disease (IHD) (9), and stroke (10). Fibrinogen molecules contain two sets of disulfide-bridged Aα-, Bβ-, and γ-chains, which are encoded by fibrinogen gamma (FGG), fibrinogen alpha (FGA), and fibrinogen beta (FGB) genes, respectively (11). FGB gene has been more extensively studied in cerebrovascular diseases (CVD), because β-chain synthesis is the limiting step in the production of mature fibrinogen (6). The potential association between FGB gene polymorphisms (frequently FGB c.-455 G>A and c.-148 C>T) and ischemic stroke was investigated in previous studies (12, 13). The *FGB* –455G>A (rs1800790) polymorphism in the promoter region is strongly associated with elevated plasma fibrinogen levels and stroke (12, 14).

Mature fibrinogen receptors have two glycoprotein subunits: GP IIb and GP IIIa (15), that promote pro-coagulant activity and stabilize the growing platelet thrombus through interactions with fibrinogen and von Willebrand Factor (VWF) (16). These receptors have two major polymorphic sites, HPA-1 (or PI-A) and HPA-3, which are single base-pair substitutions resulting in amino acids replacement (p.Leu33Pro and p.Ile843Ser respectively) of the human platelet alloantigens (HPA) (17). If these polymorphisms were previously considered important only for inducing an immune response by producing alloantibodies that cause platelet-derived disorders

(18), it has recently become obvious that they might also influence receptor sensitivity, possibly by altering platelet activation and aggregation (19). Latest studies have shown that the p.Leu33Pro polymorphism (or PIA1/A2) might have such an effect (16, 17).

The aim of our study was to investigate the *FGB* –455G>A and *GP IIIa* PIA1/A2 (rs5918) polymorphisms in Romanian patients with ischemic stroke and controls, in order to assess a possible association between these polymorphisms and ischemic stroke.

Subjects and methods

Patients and controls

All patients and controls gave their written informed consent. This study was approved by the Ethics Committee of „Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania, and complies with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

The current study was designed as a cross-sectional, randomized case-control study on 298 individuals: 148 patients with ischemic stroke and 150 unrelated controls. Ischemic stroke was confirmed following neurological assessment and computed tomography scan evaluation in all cases. The controls enrolled in the study were age, sex and ethnically matched and had no medical history of ischemic stroke, or other thrombotic events. Demographic data and personal medical history information were collected for all participants.

Genotyping

Genomic DNA was extracted from 300 µl of peripheral blood using a Wizard Genomic DNA Purification Kit (Wizard® Genomic DNA Purification Kit, Promega, MA, USA) (20). The *FGB* -455G>A and *GP IIIa* PIA1/A2 genotypes were determined by PCR-RFLP assays as previously described, (5, 16) using the following primers: 5'-FW-AAG-AAT-TTG-GGA-ATG-CAA-TCT-CTG-CTA-CCT-3' and 5'-RV-CTC-CTC-ATT-GTC-GTT-GAC-ACC-TTG-GGA-C-3' for -455G>A and 5'-FW-ATA-AGC-TTA-GCT-ATT-GGG-AAG-TGG-TAG-GGC-CTG-3' and 5'-RV-CTT-CTG-ACT-CAA-GTC-CTA-ACG-3' for PIA1/A2. We analyzed genome building GRCh38.p2 starting from chromosome positions 154561824 for *FGB* -455G>A respectively 472832265 for *GP IIIa* PIA1/A2. Details for primer attachment, restriction sites and SNP (single nucleotide polymorphism) position can be seen in Figure 1. (21, 22)

For the specific genetic analysis, a total amount of 100ng of genomic DNA was amplified in a total volume of 25µl reaction mix (Thermo Fischer Scientific Inc., MA, USA) containing a reaction buffer of 1,5nM MgCl2, 20pmol of each primer, 200µm of each dNTPs and 0,5 U Taq polymerase. PCR was carried out using a commercial thermal cycler (Eppendorf Mastercycler Thermal Cycler). For the *FGB* -455 G>A polymorphism, the amplification protocol consisted of 3 minutes at 93°C followed by 30 cycles of 60 seconds at 93°C, 60 seconds at 55°C, and 2 minutes at 72°C, with a final elongation step at 72°C for 5 minutes. In the case of PIA1/A2 polymorphism, the protocol consisted of 5 minutes at 94°C followed by 35 cycles of 40 seconds at 94°C, 40 seconds at 52°C, and 40 seconds at 72°C with a final step at 72°C for 7 minutes.

The amplified DNA products, 1300 bp for *FGB* and 476 bp for *GP IIIa*, were digested overnight with 4U of HaeIII and MspI enzymes (Thermo Fischer Scientific Inc., MA, USA). The resulted fragments were then separated in a 3% agarose gel (MetaPhor® Agarose, Cambrex Bio Science Inc.). Electrophoretic analysis identified 4 distinct banding patterns for the PIA1/A2 polymorphism, corresponding to 3 possible genotypes: PIA1/A1 wild type homozygous

Figure 1. Schematic representation of FGB -455G>A and GP IIIa PIA1/A2 SNP analysis. 1-attachment site for Fw primer, 2- restriction site, 3- SNP location, 4- attachment site for Rev primer

genotype (279 and 197 bp fragments), PIA1/A2 heterozygous genotype (279,197,173 and 106 bp fragments) and PIA2/A2 mutant homozygous genotype (197, 173 and 106 bp); for the *FGB* -455G>A polymorphism, there were also 4 distinct banding patterns corresponding to 3 possible genotypes: G/G wild type homozygous genotype (575, 383 and 343 bp fragments), G/A heterozygous genotype (958, 575, 383 and 343 bp fragments) and A/A mutant homozygous genotype (958 and 343 bp). (Figures 2 and 3)

Statistical analysis

Statistical analysis was carried out using SPSS 18.0 for Windows software IBM SPSS Statistics 23 (IBM corp., Armonk, NY, USA). Student *t*-test for independent groups with unequal variances was used for comparing the mean age between the two groups. Chi-square test ($\chi 2$) was used for comparing differences in

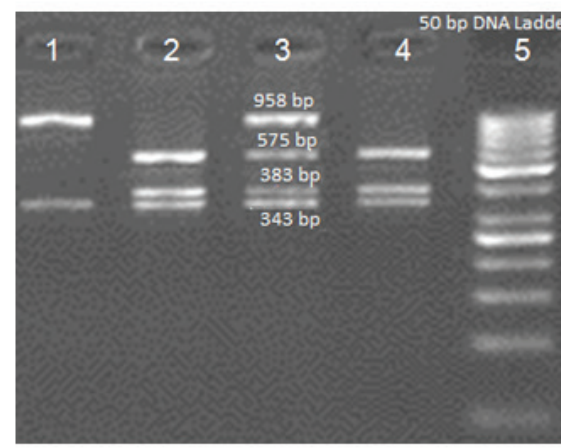

Figure 2. FGB -455 G>A electrophoresis: Lane 1- A/A, Lanes 2 and 4 –G/G, Lane 3- G/A, Lane 5- 50 bp DNA Ladder

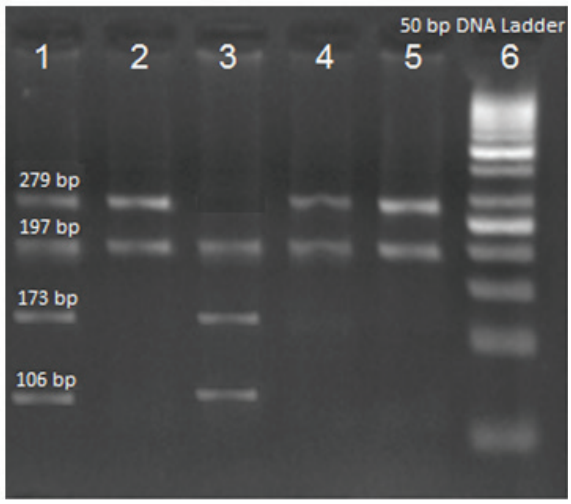

Figure 3. GP IIIa PIA1/A2 electrophoresis: Lane 1- PIA1/A2, Lanes 2,4 and 5 – PIA1/A1, Lane 3- PIA2/A2, Lane 5- 50bp DNA Ladder

sex and genotype frequencies between groups. Risk factors for ischemic stroke between case and control groups were analyzed with logistic regression and $\chi 2$ test. The genotype and allele frequency of each polymorphism between different groups was compared with the $\chi 2$ test. The same test was used to test Hardy-Weinberg equilibrium. We estimated the strength of association by calculating the odds ratio (OR) at 95% confidence interval (CI). A p-value less than 0.05, was considered statistically significant.

Results

The demographic data and cardiovascular risk factor status of the studied population are presented in Table 1.

Genotype distribution and allele frequency of the two polymorphisms among patients and controls are presented in Table 2. No deviation from the Hardy-Weinberg equilibrium was observed. There were no significant differences in

Table I. Demographic characteristics and risk factor profile of the study subjects

	Patients (n=148)	Controls (n=150)	OR (95%CI)**	p value
Age [years], mean (SD)*	70.13(±9.73)	68.57(±7.35)	-	-
Men (n), %	81 (54.7%)	85(56.6%)	1.081 (0.685-1.709)	0.815
Hypertension (n), %	85 (57.4%)	81 (54%)	0.870 (0.550-1.374)	0.561
Smokers (n), %	34(22.9%)	51(34%)	0.550 (0.334-0.929)	0.029

*- t Student's test; **$\chi 2$ test ; SD-standard deviation; OR- odds ratio; CI – 95% confidence interval

Table II. Genotype distribution and frequency of alleles of FGB -455 G>A and GP IIIa PIA1/A2 polymorphisms in patients vs. controls

Polymorphism	Variant	Patients n (%)	Controls n (%)	OR* (95%CI)	P* ($\chi 2$)
FGB -455 G>A	GG	89 (60.1)	72 (48.0)	1	0.067 (5.393)
	GA vs.GG	46 (31.1)	66 (44.0)	0.564(0.346-0.919)	
	AA vs. GG	13 (8.8)	12 (8.0)	0.876(0.377-2.038)	
	G allele frequency	224 (75.7)	210 (70.0)	1	0.120 (2.42)
	A allele frequency	72 (24.3)	90 (30.0)	0.750(0.522-1.077)	
GP IIIa PIA1/A2	A1A1	118 (79.7)	119 (79.3)	1	0.609 (0.991)
	A1A2 vs. A1A1	30(20.2)	30 (20.0)	1.008(0.572-1.777)	
	A2A2 vs. A1A1	0 (0.0)	1 (0.7)	-	
	A1 allele frequency	266 (89.9)	268 (89.3)	1	0.823 (0.05)
	A2 allele frequency	30 (10.1)	32 (10.7)	0.944(0.558- 1.599)	

*OR- odds ratio; CI – 95% confidence interval; *- χ^2 test; Statistical significant for p<0.05*

genotype and allele frequency for the -455 G>A and PIA1/A2 gene variants between the two groups.

For -455 G>A polymorphism, a higher frequency of the G/A+A/A genotypes was noted in the control group (OR 0.612, 95% CI 0.386-0.968, p = 0.037). Genotypes and alleles frequencies for the PIA1/A2 polymorphism had a similar distribution in patients and controls.

Logistic regression was performed to assess the impact of the two polymorphisms on stroke risk using stroke as the dependent variable, and the two studied polymorphisms as covariates. None of the gene variants were found to be independent predictors for increased risk of stroke. However, when we included hypertension and smoking as covariates, our logistic model was statistically significant, smoking being the strongest predictor for stroke (p = 0.026, OR = 1.800, 95% CI = 1.071- 3.024).

Table III. Estimated effects of FGB -455 G/A and GP IIIa PIA1/A2 logistic regression analysis of patients and controls, factor adjusted

	Patients vs. Controls		
	OR	(95% CI)	p value
FGB -455G>A	0.749	0.524-1.073	0.115
GP IIIa PIA1/A2	0.954	0.547-1.666	0.869
Hypertension	1.066	0.667-1.705	0.789
Smokers	1.800	1.071-3.024	0.026*

OR- odds ratio; CI – 95% confidence interval;
*- Statistical significant for p<0,050

Discussion

Fibrinogen plays a key role in platelet aggregation. By binding to their glycoprotein IIb-IIIa surface receptors, fibrinogen crosslinks platelets, essential for incorporating new platelets into the developing thrombus (13). Moreover, at the end of the coagulation cascade, fibrinogen is converted to fibrin, an insoluble protein which forms a network of fibers over the injury site; platelets and other blood cells get caught in this network and form a blood clot (23). Generating this haemostatic plug after vascular injury is essential and highly dependent on platelet function. In this context, thrombotic and hemorrhagic disorders might be tragic consequences of an altered platelet reactivity (24). Latest studies suggest that *FGB* -455 G>A and *GP IIIa* PIA1/A2 polymorphisms, might be implicated in the etiophatology of stroke (12, 17).

To our knowledge, this is the first study to assess the possible association between *FGB* -455G>A and *GP IIIa* PIA1/A2 polymorphisms in patients with ischemic stroke in an East European population.

Currently, in the scientific literature there is no available data regarding the distribution of these polymorphisms in Romanian population. The present study revealed a frequency of 46% for the -455 G>A variant allele of *FGB*, the highest data ever reported for the European population (20%) (25). As for the PIA1/A2 variant allele of *GP IIIa* the frequency was 20%, similar to the results reported in literature (15-25%) (26, 27). The results of the ***present research*** place our country among the populations with the highest frequency for -455 G>A variant allele of *FGB*. Furthermore, *GP IIIa* PIA1/A2 genotype frequencies in our study are similar to those reported in other Caucasian populations (26, 27).

In the present study we encountered no association between the studied gene variants and ischemic stroke. However, we found a significant inverse association for the G/A+A/A genotype correlation of *FGB* -455 G>A polymorphism (OR 0.612, 95% CI 0.386-0.968, p= 0.037). Although not a protective factor by itself, the A allele may interact with other gene polymorphisms or known risk factors and indirectly modulate disease susceptibility (28). These results are inconclusive and should be looked at carefully as no previous study in the literature

has mentioned such a finding. Previous studies from Hungarian (14), Dutch (29), Korean (30) and Polish (31) populations found no association between the A allele of the *FGB* -455 G>A polymorphism and ischemic stroke. In contrast, others studies found a higher frequency for the AA genotype of *FGB* -455 G>A polymorphism in patients with CVD (32). The A allele of the *FGB* -455 G>A polymorphism seems to be associated with an increased risk of developing recurrent lacunar stroke (12). Interestingly gender and age may play a modulating effect: Liu et. al. (33) described significant differences in genotype distribution when comparing male patients with elderly male controls. Consistent with our results, no relationship between ischemic stroke and the *GP IIIa* PIA1/A2 polymorphism was observed neither in US (34) nor Chinese populations (35). However, recent reports suggest that the PlA2 allele could be an additive risk factor for atherothrombosis and thus increase the risk of stroke (17). Moreover, the PlA2 allele associated risk might be of greater importance in younger stroke patients as seen in myocardial infarction, a medical condition sharing the same cardiovascular risk factors as stroke (36). In a meta-analysis Bentley et al. (9) found an association between the PIA1/A2 polymorphism and ischemic stroke in Caucasian population. Another recent meta-analysis conducted by Floyd et al. (17) on different populations supported the hypothesis that carriage of the PIA2 allele is a risk factor for ischemic stroke of cardioembolic and large vessel origin. In the same meta-analysis, when studies were stratified by age, sex and ethnicity the results indicated that neither sex nor age were major risk factors for stroke in association with *GP IIIa* PIA1/A2 polymorphisms. These findings could suggest that the impact of PIA2 allele presence in stroke may depend on ethnic differences.

Since stroke is a multifactorial pathology (4), we performed an adjusted logistic regression analysis with respect to the known risk factors. There was a significant dependence between smoking and the *FGB* −455 G<A and *GP IIIa* PIA1/A2 polymorphisms. However, hypertension did not show an association with the studied polymorphisms. A positive association with hypertension and smoking was previously reported by Martiskainen et al. (12) in lacunar stroke patients carriers of the *FGB* −455G>A A allele. In contrast, other studies (30, 31) failed to find any association between the *FGB* −455G>A polymorphism and smoking or hypertension in patients with stroke. Following the available literature data on the association of *GP IIIa* PIA1/A2 polymorphism with hypertension and smoking, we found conflicting results. According to Carter et al. (37) hypertension and smoking were independent predictors for acute stroke. Moreover, the *GP IIIa* PIA1/A2 polymorphism might be a genetic risk factor for ischemic stroke, as found in a selected high-risk hypertensive population where stroke patients had a higher PIA2 allele frequency than nonstroke hypertensive subjects (38). While independently analyzing the *GP IIIa* PIA1/A2 polymorphism in smokers with stroke some studies reported no link (27, 34), but significant association has been reported in smokers with lacunar infarcts (39). Our findings suggest that smoking in association with the studied genetic polymorphisms may be a risk factor for the development and progression of ischemic stroke.

An interesting finding is that the frequency of the *FGB* G>A variant allele in our study was two times higher than those reported in other European populations (25). Nevertheless, as currently there is no official data about the distribution of these genetic variants in the Romanian population we emphasize the need for larger epidemiological studies, to correctly assess these frequencies.

In the end, we wish to acknowledge our study limitations. A case-control study design is improper for the assessment of risk factors that

vary over time. In addition, the control group was recruited by random sampling from the general population in the same geographical region as the patients, thus inducing the possibility of spurious results because of the population stratification.

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Abbreviations

EU- European Union
GP- glycoprotein
IHD- ischemic heart disease
FGG- fibrinogen gamma
FGA- fibrinogen alpha
FGB- fibrinogen beta
CVD- cerebrovascular disease
vWF- von Willenbrand Factor
PCR-RFLP- polymerase chain reaction-restriction fragment length polymorphism
χ^2- Chi-square test
OR- odds ratio
CI- confidence interval
SNP- single nucleotide polymorphism

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