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UČESTALOST PAI-1 4G/5G GENSKE VARIJANTE U SRPSKOJ POPULACIJI

Sažetak

Uvod: Inhibitor aktivatora plazminogena 1 (PAI-1) ima značajnu ulogu u procesu inhibicije fibrinolize i normalne hemostaze.

Prisustvo PAI-1 4G/4G genotipa uzrokuje povećanje ekspresije PAI-1. Povišen nivo PAI-1 u krvi povezan je sa brojnim bolestima kao što su tromboza, moždani udar, infarkt miokarda, spontani pobačaji, preeklampsija, insulinska rezistencija, dijabetes tipa 2, rak dojke i astma. U okviru ove studije određivana je učestalost PAI-1 4G/5G genske varijante kod zdravih ispitanika u srpskoj populaciji.

Metode: Studija je obuhvatala grupu od 210 zdravih ispitanika (105 žena i 105 muškaraca). Prisustvo PAI-1 4G/5G genske varijante detektovano je PCR-RFLP metodom.

Rezultati: Učestalost PAI-1 4G/4G genotipa iznosila je 34,76% i bila je povećana u odnosu na PAI-1 5G/5G genotip (19,05%), dok je najzastupljeniji genotip bio PAI-1 4G/5G (46,19%). Učestalost 4G alela bila je viša (0,58) u odnosu na 5G alel (0,42).

Zaključci: Učestalost PAI-1 4G/5G genske varijante u srpskoj populaciji slična je sa okolnim populacijama. Rezultati ove studije su značajni, jer predstavljaju prve podatke za srpsku populaciju što će omogućiti dalja istraživanja o ulozi PAI-1 4G/5G genske varijante u patogenezi brojnih bolesti.

Ključne reči: PAI-1 4G/5G, inhibitor aktivatora plazminogena

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Abstract

Introduction: Plasminogen activator inhibitor 1 (PAI-1) has a major role in inhibition of fibrinolysis and normal haemostasis.

The presence of the PAI-1 4G/4G genotype leads to increased expression of PAI-1. High bllod level of PAI-1 is associated with many diseases such as thrombosis, cerebral insult, myocardial infarction, pregnancy loss, preeclampsia, insulin resistance, type 2 diabetes, breast cancer and asthma. In this study, the prevalence of PAI-1 4G/5G gene variant was determined in healthy subjects from Serbian population.

Methods: The study was carried out in a group of 210 healthy subjects (105 women and 105 men). The presence of PAI-1 4G/5G gene variant was detected by PCR-RFLP analysis.

Results: The prevalence of PAI-1 4G/4G genotype was 34.76% and it was increased compared to PAI-1 5G/5G genotype (19.05%). The most frequent was PAI-1 4G/5G genotype (46.19%). Allelic frequency for 4G allele was higher (0.58) compared to 5G allele (0.42).

Conclusions: The prevalence of PAI-1 4G/5G gene variant in Serbian population is similar to the neighboring populations. Results of this study represent the first data for Serbian population. This study could be useful for further research where the role of PAI-1 4G/5G gene variant will be assessed in the pathogenesis of many diseases.

Key words: PAI-1 4G/5G, plasminogen activator inhibitor 1

Uvod

Hemostaza predstavlja jedan od najvažnijih mehanizama održavanja homeostaze čitavog organizma. Njena uloga je da obezbedi tečno stanje krvi i njen nesmetani protok kroz cirkulaciju, a u slučaju oštećenja krvnog suda da omogući stvaranje krvnog ugruška i zaustavljanje krvarenja. Radi se o veoma kompleksnoj i osetljivoj ravnoteži u čijem održavanju učestvuje više faktora: endotel krvnih sudova, trombociti, faktori koagulacije, inhibitori koagulacije i sistem fibrinolize (1).

Sistem fibrinolize omogućava razgradnju krvnog ugruška i uklanjanje fibrina iz cirkulacije. Centralni enzim ovog sistema je plazmin koji nastaje aktivacijom plazminogena. Aktivnost plazmina regulisana je kompleksnom mrežom aktivatora i inhibitora fibrinolize. Najznačajniji inhibitori fibrinolize su: inhibitori aktivatora plazminogena (PAI-1 i PAI-2) i α_2-antiplazmin (2).

Inhibitor aktivatora plazminogena tipa 1 (PAI-1) je glikoprotein molekulske mase 55 kD koji se sintetiše u endotelnim ćelijama, hepatocitima i megakariocitima (3). PAI-1 deluje kao inhibitor endogene fibrinolitičke aktivnosti, zbog svoje sposob-

nosti da inhibira aktivnost tkivnog tipa aktivatora plazminogena (t-PA), i urokinaza tipa aktivatora plazminogena (u-PA) (4-6). Gen za PAI-1 nalazi se na hromozomu 7 (7q21.3-q22.1) i sadrži 8 introna i 9 egzona (3). U promotoru PAI-1 gena na poziciji -675 bp opisana je insercija/delecija jednog guanozina koja je označena kao 4G/5G genska varijanta. Alelska varijanta sa 5 guanozina (5G) sadrži preklapajuća vezujuća mesta za aktivator i represor transkripcije, što dovodi do normalnog nivoa ekspresije PAI-1. Nasuprot tome, 4G alelska varijanta vezuje samo aktivator transkripcije, što dovodi do povećanja nivoa PAI-1 u krvi (3).

Povećana ekspresija PAI-1 dovodi do smanjene fibrinolize, zbog čega može predstavljati faktor rizika za niz kardiovaskularnih bolesti: infarkt miokarda (7,8), moždani udar (9), tromboze dubokih vena (3). Studije su pokazale da PAI-1 4G/5G genska varijanta može biti jedan od faktora rizika koji dovode do spontanog pobačaja (10, 11) i preeklampsije (12). Takođe, povišena ekspresija PAI-1 se povezuje sa insulinskom rezistencijom (13) i dijabetesom tipa 2 (14). Značajna uloga ove genske varijante pokazana je u patogenezi raka dojke (15) i astme (16).

Učestalost PAI-1 4G/5G genske varijante varira zavisno od etničke pripadnosti (17-22). Frekvenca ove genske varijante određivana je za veliki broj populacija, ali do sada nema objavljenih podataka za srpsku populaciju.

Cilj ove studije je da se odredi frekvenca PAI-1 4G/5G genske varijante u zdravoj populaciji sa područja Srbije.

Materijal i metode

U ovu studiju uključeno je 210 zdravih subjekata (105 žena i 105 muškaraca; starosti 39±11,25 godina) koji nisu imali nijedan trombotički događaj.

Za izolaciju DNK ispitanika korišćeni su limfociti periferne krvi i ćelije bukalne sluzokože. Krv ispitanika uzimana je sa 3,8% Na-citratom kao antikoagulansom, a uzorci bukalne sluzokože uzimani su sterilnim štapićem. Genomska DNK izolovana je upotrebom QIAamp DNA blood mini kit (QIAGEN, Germany), prema standardnom protokolu proizvođača.

Detekcija genske varijante PAI-1 4G/5G vršena je metodom PCR-RFLP. Reakcija lančanog umnožavanja DNK za analizu prisustva PAI-1 4G/5G genske varijante odvijala se u smeši finalne zapremine 25µL, sledećeg sastava: 1x pufer A (Kapa system, Boston, USA); 2,5mM $MgCl_2$; 200µM dNTP; 10pmol svakog od odgovarajućih graničnika; 1U Taq polimeraze i 200 ng DNK. Uslovi umnožavanja i korišćeni graničnici dati su u tabeli 1.

Produkti PCR reakcije proveravani su na 2% agaroznom gelu, a zatim su digerirani *BsellI* restrikcionim enzimom (*Biolabs, New England)*. Produkti digestije analizirani su vertikalnom elektroforezom na 10% poliakrilamidnom gelu. Na osnovu veličine

restrikcionih fragmenata razdvajani su normalni (77 i 21 bp) i mutirani (98 bp) aleli. Vizuelizacija DNK vršena je bojenjem gelova solima srebra (24).

Statistička analiza

Za određivanje Hardi-Vajnbergove ravnoteže (Hardy-Weinberg equilibrium) korišćen je softver Hardy-Weinberg equilibrium calculator (http://www.oege.org/software/hardy-weinberg.html).

Rezultati

U ovu studiju uključeno je 210 zdravih subjekata sa područja Srbije. Genotipizacija za PAI-1 4G/5G urađena je PCR-RFLP metodom (slika 1). Rezultati su pokazali da je kod 40 (19,05%) ispitanika bio prisutan PAI-1 5G/5G genotip, 97 (46,19%) su bili heterozigotni nosioci PAI-1 4G/5G genske varijante, dok je 73 (34,76%) ispitanika bilo nosilac PAI-1 4G alela u homozigotnom stanju. Alelska frekvenca za 4G alela iznosila je 0,58, a za 5G alel 0,42. Na osnovu dobijenih učestalosti 4G i 5G alela utvrđeno je da je grupa ispitanika bila u Hardi-Vajnbergovoj ravnoteži (χ^2=0.59).

Diskusija

U okviru ove studije određena je učestalost PAI-1 4G/5G genske varijante u zdravoj srpskoj populaciji. Rezultati su pokazali da učestalost 4G alela iznosi 0,58, što je u saglasnosti sa rezultatima dobijenim za okolne populacije (17–19). Ista učestalost za 4G alel utvrđena je u studiji Alfirevića i saradnika u hrvatskoj populaciji (17) (tabela 2). Slične rezultate dobili su Spiroski i saradnici za makedonsku populaciju u studiji koja je uključivala 82 zdrava ispitanika (40 žena i 42 muškarca; starosti 40,7±11,3 godina) (18). Nossikoff i saradnici su ispitivali učestalost PAI-1 4G/5G genske varijante kod pacijenata koji su imali infarkt miokarda (54 pacijenta) u poređenju sa zdravim ispitanicima (85 zdravih subjekata) u bugarskoj populaciji. Oni su pokazali da je u zdravoj bugarskoj populaciji frekvenca 4G alela nešto snižena (0,42) (19). U studiji koja je određivala učestalost PAI-1 4G/5G genske varijante kod pacijenata sa infarktom miokarda mlađih od 35 godina i kontrolne grupe zdravih ispitanika, pokazano je da je u zdravoj grčkoj populaciji učestalost 4G alela 0,52 (20). Sa druge strane, u populacijama Španije (21) i Italije (22) učestalost 4G alela je nešto niža (0,49; tj. 0,47).

Bez obzira na postojanje razlike u procentualnoj zastupljenosti, sve studije, uključujući i našu, pokazuju visoku učestalost 4G alela u različitim populacijama (17,

18, 20). Velika učestalost 4G alela u zdravoj populaciji može da ukazuje na njegovu potencijalnu protektivnu ulogu u nekim procesima.

Neke studije su pokazale da PAI-1 svojim protektivnim delovanjem može da smanji rizik za pojavu brojnih bolesti (5, 7). Tokom inflamatornih procesa u moždanom tkivu, prisustvo 4G alela može dovesti do povećanja nivoa PAI-1, što posledično dovodi do smanjenja proteolize i stabilizacije plaka. Lokalni mehanizmi unutar moždanog tkiva mogu biti uključeni u protektivno delovanje PAI-1 i time doprineti smanjenju rizika za pojavu moždanog udara (5). U holandskoj studiji, koja je obuhvatala osobe starosti od 65 do 84 godine, pokazano je da prisustvo PAI-1 4G/4G genotipa smanjuje rizik od moždanog udara, tranzijentnog ishemijskog napada i smrtnosti usled pojave kardiovaskularne bolesti (5). Inhibitorni efekat PAI-1 4G/4G genotipa na migraciju ćelija može da dovode do smanjenog rizika od bolesti koronarnih arterija (7).

Naša studija pokazuje da je učestalost PAI-1 4G/5G genske varijante u zdravoj srpskoj populaciji slična sa okolnim populacijama. Rezultati ove studije predstavljaju značajne podatke za buduće studije u kojima će biti određivana frekvenca PAI-1 4G/5G genske varijante kao potencijalnog faktora rizika povezanog sa brojnim bolestima.

Ovaj rad je finansiran od strane projekta broj 173008 Ministarstva za prosvetu, nauku i tehnološki razvoj Republike Srbije.

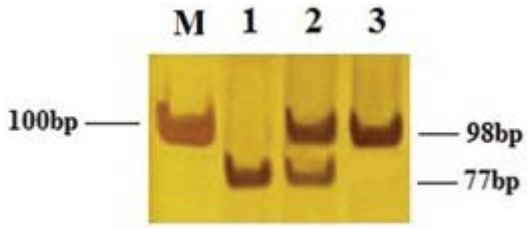
Tabela 1. Temperaturni profil i graničnici PCR reakcije

Graničnik Pa (23)	5' CACAGAGAGAGTTCTGGCCACGT 3'
Graničnik Pb (23)	5' CCAACAGAGGACTCTTGGTCT 3'
Temperaturni profil PCR reakcije	94 °C/5 min (94 °C/30 sec, 61 °C/30 sec, 72 °C/30 sec) 33 ciklusa 72 °C/10 min

Tabela 2. Genotipske i alelske učestalosti PAI-1 4G/5G genske varijante u različitim populacijama

Genotip	Srbija	Italija	Makedonija	Hrvatska	Bugarska	Grčka	Španija
4G/4G (%)	34,76	24,36	24,4	32	18	20,3	21
4G/5G (%)	46,19	50,26	62,2	52	49	63,3	52
5G/5G (%)	19,05	25,38	13,4	16	33	16,4	27
Alelska frekvenca	Srbija	Italija	Makedonija	Hrvatska	Bugarska	Grčka	Španija
4G alel	0,58	0,49	0,55	0,58	0,42	0,52	0,47
5G alel	0,42	0,51	0,45	0,42	0,58	0,48	0,53

Slika 1. Analiza genske varijante PAI-1 4G/5G PCR/RFLP metodom



M – Marker dužine (100bp)
1 – PAI-1 5G/5G genotip
2 – PAI-1 4G/5G genotip
3 – PAI-1 4G/4G genotip

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THE PREVALENCE OF PAI-1 4G/5G GENE VARIANT IN SERBIAN POPULATION

Abstract

Introduction: Plasminogen activator inhibitor 1 (PAI-1) has a major role in inhibition of fibrinolysis and normal haemostasis.

The presence of the PAI-1 4G/4G genotype leads to increased expression of PAI-1. High blood level of PAI-1 is associated with many diseases such as thrombosis, cerebral insult, myocardial infarction, pregnancy loss, preeclampsia, insulin resistance, type 2 diabetes, breast cancer and asthma.

In this study, the prevalence of PAI-1 4G/5G gene variant was determined in healthy subjects from Serbian population.

Methods: The study was carried out in a group of 210 healthy subjects (105 women and 105 men). The presence of PAI-1 4G/5G gene variant was detected by PCR-RFLP analysis.

Results: The prevalence of PAI-1 4G/4G genotype was 34.76% and it was increased compared to PAI-1 5G/5G genotype (19.05%). The most frequent was PAI-1 4G/5G genotype (46.19%). Allelic frequency for 4G allele was higher (0.58) compared to 5G allele (0.42).

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Conclusions: The prevalence of PAI-1 4G/5G gene variant in Serbian population is similar to the neighboring populations. Results of this study represent the first data for Serbian population. This study could be useful for further research where the role of PAI-1 4G/5G gene variant will be assessed in the pathogenesis of many diseases.

Keywords: PAI-1 4G/5G, plasminogen activator inhibitor 1

Introduction

Haemostasis represents one of the most important homeostasis mechanisms. It has major role in providing liquid state of blood and its normal flow through the circulation, while in the case of blood vessel damage it can ensure blood clot formation and preventing hemorrhage. In maintenance of this very complex balance several factors are included: the endothelium of blood vessels, platelets, coagulation factors, coagulation inhibitors and fibrinolytic system (1).

The fibrinolytic system has a role to prevent the pathological extension of the blood clots removing fibrin from the circulation. The central enzyme of this system is the plasmin generated by activation of plasminogen. Plasmin activity is regulated by a complex network of activators and inhibitors of fibrinolysis. The main inhibitors of fibrinolysis are: plasminogen activator inhibitor (PAI-1 and PAI-2) and α2-antiplasmin (2).

Plasminogen activator inhibitor type 1 (PAI-1) is a 55kD glycoprotein synthesized by endothelial cells, hepatocytes and megakaryocytes (3). PAI-1 acts as an inhibitor of endogenous fibrinolytic activity due to its ability to inhibit the activity of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (4-6). The gene for PAI-1 is located on chromosome 7 (7q21.3-q22.1) and contains 8 introns and 9 exons (3). At position -675bp in the promoter region of PAI-1 gene an insertion /deletion of a single guanosine is described, marked as 4G/5G gene variant. Allelic variant of 5 guanosines (5G) contains overlapping binding sites for activator and repressor of transcription, which leads to normal expression levels of PAI-1. In contrast, the 4G allelic variant, related only with a transcription activator, leads to increased level of PAI-1 in blood (3).

Increased expression of PAI-1 leads to reduced fibrinolysis, which may represent a risk factor for a number of cardiovascular diseases: myocardial infarction (7, 8), stroke (9), and deep vein thrombosis (3). Studies have shown that PAI-1 4G/5G gene variant may be one of the risk factors for spontaneous miscarriage (10, 11) and preeclampsia (12). Furthermore, increased expression of PAI-1 is associated with insulin resistance (13) and type 2 diabetes (14). Also, the important role of this genetic variant has been demonstrated in the pathogenesis of breast cancer (15) and asthma (16).

The prevalence of PAI-1 4G/5G gene variant varies depending on ethnicity (17-22). The frequency of this gene variant was determined for a number of populations, but there is no data observed for Serbian population.

The aim of this study was to determine the frequency of PAI-1 4G/5G gene variant in healthy Serbian population.

Materials and methods

Our study included 210 healthy subjects (105 women and 105 men; aged 39±11.25 years) with no history of thrombotic event. For the isolation of DNA peripheral blood lymphocytes and buccal mucosa cells were used. Blood samples from subjects were taken on 3.8% sodium citrate as anticoagulant, and buccal mucosa samples were taken with sterile swab. Genomic DNA was isolated using the QIAamp DNA Blood MiniKit (QIAGEN, Germany) according to manufacturer's standard protocol.

The PAI-1 4G/5G gene variant was detected by PCR-RFLP. Polymerase chain reaction was carried out in a 25μL reaction volume containing: 1x buffer A (Kapa Biosystems, Boston, USA), 2.5 mM $MgCl_2$; 200μM dNTP; 10pmol of primer Pa and Pb primer; 1U Kapa Taq polymerase (Kapa Biosystems, Boston, USA) and 200 ng of DNA.The thermal cycle profile and primers used in PCR are given in Table 1.

PCR products were digested by Bse*II* restriction enzyme (Biolabs, NewEngland) and analyzed on 10% polyacrylamide gel electrophoresis. Normal (77 and 21 bp) and mutant (98 bp) allele were separated based on the size of the restriction fragments. The DNA was visualized by silver staining (24).

Statistical analysis

The determination of Hardy-Weinberg equilibrium (Hardy-Weinberg equilibrium) was performed by using the online software, Hardy-Weinberg equilibrium calculator (http://www.oege.org/software/hardy-weinberg.html).

Results

Our study included 210 healthy subjects from the territory of Serbia. Genotyping of PAI-1 4G/5G was performed by PCR-RFLP method (Fig. 1). Our results showed that PAI-1 5G/5G genotype was present in 40 (19.05%) subjects, 97 (46.19%) were heterozygous carriers of PAI-1 4G/5G gene variant, while 73 (34.76%) subjects were carriers of homozygous PAI-1 4G allele. Allelic frequency of the 4G allele was 0.58 and frequency of the 5G allele was 0.42. The study group was in Hardy-Weinberg equilibrium (χ^2=0.59).

Discussion

In this study we determined the prevalence of PAI-1 4G/5G gene variant in healthy Serbian population. Our results showed that the frequency of 4G allele is 0.58, which is in concordance with the results obtained for the neighboring populations (17-19). In a study of Alfirevic et al. the same frequency of 4G allele was found in the Croatian population (17) (Table 2). Similar results were obtained by Spiroski et al. for the Macedonian population in a study involving 82 healthy subjects (40 women and 42 men; aged 40.7±11.3 years) (18). Nossikoff et al. examined the prevalence of PAI-1 4G/5G gene variant in Bulgarian patients with myocardial infarction (54 patients) and controls (85 healthy subjects).They showed that in a healthy Bulgarian population the frequency of 4G allele was slightly decreased (0.42) (19). In the Greek study, including patients suffered from premature myocardial infarction and healthy subjects, it has been shown that frequency of 4G allele is 0.52 in a healthy Greek population (20). On the other hand, in the population of Spain (21) and Italy (22) the 4G allele frequency was slightly lower (0.49 and 0.47, respectively).

All studies, including ours, have shown a high frequency of 4G allele in different populations (17, 18, 20). High frequency of 4G allele in the healthy population may indicate a potential protective role of this gene variant in certain disorders.

Some studies have shown that PAI-1 can reduce the diseases risk due to its protective action (5, 7).The presence of 4G allele may lead to increased levels of PAI-1 which can cause a reduction in proteolysis and plaque stabilization during the inflammatory process in the brain tissue. Local mechanisms within brain tissue may be involved in the protective effect of PAI-1 and thereby contribute to reduced risk of stroke (5). In the Dutch study, which included people aged 65 to 84 years, it has been shown that the presence of PAI-1 4G/4G genotype reduces the risk of stroke, transient ischemic attack, and death caused by the presence of cardiovascular disease (5). The inhibitory effect of PAI-1 4G/4G genotype on cell migration may lead to a reduced risk of coronary artery disease (7).

Our study shows that the prevalence of PAI-1 4G/5G gene variant in healthy Serbian population is similar to the neighboring populations. These results provide important information for future studies which will investigate potential role of PAI-1 4G/5G gene variant in pathogenesis of various disorders.

This work was supported by grant No 173008 of the Ministry of Education, Science and Technological Development of Republic of Serbia.

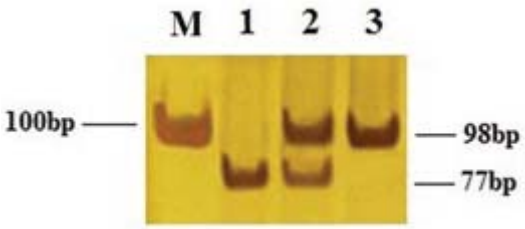
Table 1. The thermal cycle profile and primers used in PCR

Primer Pa (23)	5' CACAGAGAGAGTTCTGGCCACGT 3'
Primer Pb (23)	5' CCAACAGAGGACTCTTGGTCT 3'
Thermal cycle profile	94 °C/5 min (94 °C/30 sec, 61 °C/30 sec, 72 °C/30 sec) 33 ciklusa 72 °C/10 min

Table 2. Genotype and allelic frequency ofPAI-1 4G/5G gene variant in different populations

Genotype	Serbia	Italy	Macedonia	Croatia	Bulgaria	Greece	Spain
4G/4G (%)	34,76	24,36	24,4	32	18	20,3	21
4G/5G (%)	46,19	50,26	62,2	52	49	63,3	52
5G/5G (%)	19,05	25,38	13,4	16	33	16,4	27
Allelic frequency	Serbia	Italy	Macedonia	Croatia	Bulgaria	Greece	Spain
4G allel	0,58	0,49	0,55	0,58	0,42	0,52	0,47
5G allel	0,42	0,51	0,45	0,42	0,58	0,48	0,53

Figure 1. Polyacrylamide gel electrophoresis of the PAI-1 4G/5G gene variant



M- 100bp Ladder

1- PAI-1 5G/5G genotype

2- PAI-1 4G/5G genotype

3- PAI-1 4G/4G genotype

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