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THR83ALA POLYMORPHISM OF MGP GENE EXON 4 NOT RELATED TO ISCHEMIC ATHEROTHROMBOTIC STROKE IN THE NORTHEASTERN REGION OF UKRAINE

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Abnormal calcium salts depositing in the arterial vessels is considered to be a novel marker of atherosclerosis and related to cerebrovascular disease. Matrix gamma–carboxyglutamic acid protein (MGP) is one of the most potent inhibitors of ectopic mineralization, so it may be associated with calcification of atheromatous plaques, their instability and rupture, and thrombi formation. The aim of the study was to analyse whether the MGP exon 4 Thr83Ala polymorphism (rs4236) is related to the risk of ischemic atherothrombotic stroke (IAS) in the Ukrainian population. 170 IAS patients and 124 healthy controls were recruited to the study. The MGP polymorphism was examined by PCR–RFLP methodology. The distribution of homozygous carriers of a major allelic variant (Thr/Thr), and heterozygous (Thr/Ala) and homozygous minor allele (Ala/Ala) variants in IAS patients was 39.4 %, 48.8 % and 11.8 %, respectively. The corresponding distributions of variants in the control group were 34.7 %, 53.2 % and 12.1 % ($P>0.05$ by χ^2- test). It was concluded that MGP exon 4 Thr83Ala polymorphism is not associated with the risk of IAS both in men and women in the Ukrainian population. Only in one subgroup which included nonhypertensive patients, the risk of IAS in heterozygotes was significantly lower as compered with major allele homozygotes. The mechanism of this is not clear and should be a subject of further investigation.

Key words*: arterial calcification, ischemic atherothrombotic stroke, matrix Gla protein, single nucleotide polymorphism, Ukrainian population.*

INTRODUCTION

Matrix gamma–carboxyglutamic acid protein (MGP) is a mineralbinding extracellular protein synthesized in the vascular tissue. It belongs to a family of proteins that contain glutamyl residues that are post-translationally modified by a vitamin K-dependent gamma-glutamyl carboxylase into gammacarboxyglutamyc acid (Gla) residues [1].

MGP is known to be one of the most potent natural inhibitors of ectopic mineralization. Homozygous MGP deficient mice were observed to die within 8 week as a result of arterial calcification that led to blood vessel rupture [2]. MGP is highly expressed on calcified atherosclerotic plaques in humans [3], and it can modify calcification in such plaques and the risk of cardiovascular disease [4, 5, 6]. It is thought that anticalcifying activity of MGP depends on Gla-residues, which are able to bind calcium [7].

The human MGP gene is located on chromosome 12p13.1–p12.3 and it consists of 4 exons [8]. Numerous single nucleotide polymorphisms (SNPs) were identified both in the coding and regulatory regions of MGP gene. Among those, three SNPs (T-138C, G-7A, Thr83Ala) was in a number of the studies under consideration [9, 10, 11, 12, 13, 14, 15]. There are some evidences that these variants of SNP are related to the level of serum MGP, arterial calcification, and coronary artery disease [9, 10, 11, 13], although these data are not consistent [14, 15].

Ischemic atherothrombotic stroke (IAS) is, in many instances, the consequence of a thrombus forming on a ruptured atherosclerotic plaque. Abnormal calcium salts depositing in the arterial vessels is considered to be a novel marker of atherosclerosis and to be related to cerebrovascular disease [16].

Considering the central role of MGP in vascular calcification and a similar pathogenesis between coronary artery disease and severe cerebrovascular events, we hypothesized that Thr83Ala polymorphism in the exon 4 of MGP gene might influence the risk of IAS. To validate the hypothesis we performed an analysis of Thr83Ala genotype in patients with IAS and control subjects which represent the north-eastern region of Ukraine.

RESEARCH GOAL

The aim of our investigation was to determine whether there is an association between the Thr83Ala polymorphism of MGP gene exon 4 and the risk of IAS development.

MATERIALS AND METHODS

Study subjects

Our study group consisted of 170 unrelated Ukrainian patients with a mean ± SD of 64.8±9.5 years who had IAS and were under medical observation and outpatient treatment at the 5th Sumy City Clinical Hospital from 2009 to 2011. A final diagnosis of IAS was established on the basis of clinical, computed tomography and magnetic resonance imaging examinations. Each case of IAS was assessed according to TOAST criteria [17]. The patients with ischemic stroke of cardioembolic origin and undetermined etiology were excluded from the study group. Clinical characteristics of patients included generally accepted parameters related to risk factors for atherosclerosis and IAS: body mass index (BMI), blood pressure (BP), fasting blood glucose (FBG), blood plasma lipids and lipoproteins, and some indices of blood coagulation (prothrombin time). According to these parameters, all patients were divided into the pairs of subgroups defined by (1) BMI (<25 kg/m^2 or ≥ 25 kg/m^2), (2) BP (non-hypertensive or hypertensive: systolic BP>140 mmHg, diastolic BP>90 mmHg), (3) FBG (non-diabetic or diabetic).

The control group consisted of 124 elderly Ukrainian subjects with a mean age of 76.6±10.2 years without a history of IAS and evidences of marked cardio- and cerebrovascular pathologies, as confirmed by general medical examination, electrocardiography, and routine blood and urine assays. Blood sampling for genotyping was performed under sterile conditions into 2.7 ml S–Monovette (Sarstedt, Germany) containing EDTA potassium salt as an anticoagulant, samples were frozen and stored at –20 °C.

Amplification and genotyping

DNA for genotyping was extracted from the venous blood using commercially available kits (Isogene Lab Ltd, Russia) according to the manufacturer's protocol. To identify MGP gene exon 4 Thr83Ala polymorphism (rs4236) the polymerase chain reaction (PCR) with subsequent restriction fragment length polymorphism (RFLP) analysis was performed. Specific region of the MGP gene was amplified using a pair of specific primers: upstream (sense) – 5'-TCAATAGGGAAGCCTGTGATG-3' and downstream (antisense) – 5'-TCAATAGGGAAGCCTGTGATG-3'. Primers were provided by Metabion (Germany). PCR was performed for 33 cycles in a 25 μl volume containing 50–100 ng of DNA, 5 μl 5X PCR–buffer, 1.5 mM magnesium sulfate, 200 μM of each dNTP, 20 pM of each primer and

0.5U of Taq DNA polymerase (Fermentas, Lithuania). PCR was carried out in a termocycler GeneAmp PCR System 2700 (Applied Biosystems, USA). Six microlitres (6 μL) of the PCR products (173 bp) were subjected to digestion with 3U Eco477 (Fermentas, Lithuania) and incubated at 37 °C for 18 h. The presence of adenine at position 3748 of MGP gene prevented restriction and, in the case of substitution for thymine Eco477, cleaved the amplified fragment of exon 4 into two fragments 127 bp and 46 bp in length (see Figure). The restriction fragments were separated by electrophoresis and analysed on an ethidium bromide–stained 2.5 % agarose gel visualized using ultraviolet transillumination.

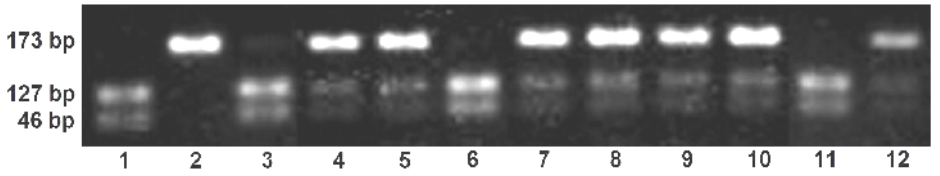

Figure – RFLP analysis of MGP exon 4 Thr83Ala polymorphism. Lanes 1, 3, 6, 11 (Thr/Thr genotype); lanes 4, 5, 7–10,12 (Thr/Ala genotype); lane 2 (Ala/Ala genotype)

Statistical analysis

The normal distribution and homogeneity of variances were tested before further statistical analyses. The χ^2–test was used to assess the deviations from the Hardy-Weinberg equilibrium for genotype frequencies, and it was also used for comparison of the allele and genotype frequencies between different study groups. The odds ratios (OR) and 95 % confidence intervals (CI) were calculated by logistic regression analysis. Since there are only a small number of individuals with the Ala/Ala genotype, we tested whether variable means differed significantly between subjects with and without the Thr variant (Thr/Thr vs. Thr/Ala and Ala/Ala). Separate analyses were performed in subgroups of IAS patients defined by BMI (<25 or ≥25), blood pressure (non-hypertensive or hypertensive), FBG (non-diabetic or diabetic). A comparison of variables between the groups of genotypes was performed using ANOVA or two-tailed Student's *t*-test. Differences were considered statistically significant with a p-value <0.05. All statistical analyses were performed using the Statistical Package for Social Science program (SPSS for Windows, version 17.0, SPSS Inc, Chicago, IL).

RESULTS

Genotypes of MGP exon 4 Thr83Ala polymorphism in IAS patients and controls are presented in Table 1. As shown, major allele homozygous and heterozygous, and minor allele homozygous variants of the MGP exon 4 were detected in 39.4 %, 48.8 % and 11.8 % of the IAS group, respectively. Corresponding date for control group were 34.7, 53.2 % and 12.1 %, respectively (P=0.701). Both in men and in women, the differences in genotype distribution in IAS patients and controls were not significant. None of the Thr83Ala genotype was associated with the risk for IS neither in man nor in women.

Among the known IAS risk factors there are increased BMI, hypertension, diabetes, i.e. factors concerning atherogenesis and thrombi formation. In Table 2, some clinical characteristics manifesting above–mentioned IAS risk factors are presented in the patients divided into two subgroups according to their genotype (Thr/Thr and Thr/Ala+Ala/Ala). It can be seen that there are no statistically significant differences between these two subgroups for all studied indices.

The division of patients into subgroups depending on the presence or absence of risk factors was done to evaluate the likelihood of IAS in persons with different MGP exon 4 Thr83Ala genotype (see Table 3). Only in one subgroup (non–hypertensive), risk for IAS (OR) was lower in heterozygotes (Thr/Ala) as compered with major allele homozygotes (Thr/Thr).

Table 1 – MGP gene Thr83Ala genotype distribution in control subjects and ischemic stroke patients

Genotype	Total		Female		Male	
	Controls n (%)	Cases n (%)	Controls n (%)	Cases n (%)	Controls n (%)	Cases n (%)
Thr/Thr	43 (34.7)	67 (39.4)	18 (40.0)	23 (31.9)	25 (31.6)	44 (44.9)
Thr/Ala	66 (53.2)	83 (48.8)	25 (55.6)	38 (52.8)	41 (51.9)	45 (45.9)
Ala/Ala	15 (12.1)	20 (11.8)	2 (4.4)	11 (15.3)	13 (16.5)	9 (9.2)
Total (n)	124	170	45	72	79	98
	χ^2=0.710, P=0.701		χ^2=3.477, P=0.176		χ^2=4.154, P=0.125	
OR						
Thr/Ala vs. Thr/Thr	0.807 (0.489–1.332) P=0.402		1.190 (0.536–2.639) P=1.190		0.624 (0.326–1.192) P=0.153	
Ala/Ala vs. Thr/Thr	0.856 (0.396–1.850) P=0.692		4.301 (0.845–21.925) P=0.079		0.393 (0.147–1.050) P=0.062	

Legend: n: number of subjects. OR: odds ratio. In parentheses – 95 % confidence interval

Table 2 – Clinical characteristics of ischemic stroke patients with different MGP gene Thr83Ala genotype

Parameter	Thr/Thr	Thr/Ala+Ala/Ala	Total	P
n	67	103	170	
Age, years	65.1±1.19	64.4±0.93	64.7±0.73	0.678
Gender, M/F	44/23	54/49	98/72	0.088
BMI (M), kg/m^2	27.6±0.47	27.7±0.64	27.6±0.41	0.917
BMI (F), kg/m^2	29.4±0.84	28.9±0.69	29.0±0.54	0.684
Systolic BP, mmHg	163±3.7	169±2.8	167±2.2	0.194
Diastolic BP, mmHg	95±1.8	96±1.6	95±1.2	0.590
Fasting glucose, mmol/L	5.86±0.2	5.96±0.15	5.92±0.12	0.677

Legend: Data are mean ± SE. n: number of subjects

Table 3 – MGP gene Thr83Ala genotype distribution in subgroups of ischemic stroke patients with different risk factors

Genotype	BMI<25 n (%)	BMI≥25 n (%)	Non-HT n (%)	HT n (%)	Non-Diabetes n (%)	Diabetes n (%)
Thr/Thr	12 (29.3)	55 (42.6)	22 (52.4)	45 (35.2)	57 (40.7)	10 (33.3)
Thr/Ala	25 (61.0)	58 (45.0)	14 (33.3)	69 (53.9)	66 (47.1)	17 (56.7)
Ala/Ala	4 (9.8)	16 (12.4)	6(14.3)	14 (10.9)	17 (12.1)	3 (10.0)
Total (n)	41	129	42	128	140	30
	P=0.199		P=0.066		P=0.639	
OR						
Thr/Ala vs. Thr/Thr	1.357 (0.617–2.985) P=0.447	0.687 (0.403–1.170) P=0.167	0.415 (0.192–0.898) P=0.025	0.999 (0.584–1.709) P=0.997	0.754 (0.447–1.272) P=0.291	1.108 (0.464–2.645) P=0.818
Ala/Ala vs. Thr/Thr	0.956 (0.267–3.420) P=0.944	0.834 (0.371–1.874) P=0.660	0.782 (0.266–2.296) P=0.654	0.892 (0.385–2.065) P=0.789	0.855 (0.384–1.901) P=0.701	0.860 (0.208–3.550) P=0.835

Legend: n: number of subjects in the subgroups. BMI: body mass index. Non-HT: non-hypertensive. HT: hypertensive. OR: odds ratio. In parentheses – 95 % confidence interval

DISCUSSION

Arterial wall calcification is a common pathological process that is of significance in its own right (Mцnckeberg's sclerosis) and can complicate the development of atherosclerotic plaques, contributing to their instability. Therefore, investigation of the factors related to the mechanisms of vascular calcification is of increased interest, as evidenced by the large number of literature sources [5, 18, 19, 20]. MGP is well known to play a pivotal role in preventing the blood vessels mineralization.

The exact mechanisms by which MGP inhibits the ectopic soft tissues calcification is not yet clear. The data on relationship between MGP and the calcifying vascular lesions are contradictory. Thus, Braam et al. [21] showed that the development of severe atherosclerosis is accompanied by an increase of serum MGP concentration. In contrast, Jono et al. [22] found that the serum MGP levels inversely correlated with the coronary artery calcification. Finally, O'Donell et al. [23], showing the relationship between the level of MGP and a number of risk factors, found no correlation between the serum MGP concentration and calcification of the coronary arteries.

With regard to the association between MGP gene polymorphisms and (a) the serum levels of this protein, (b) arterial calcification, and (c) the severe consequences of atherosclerosis, this problem was either not investigated (as for the brain vessels and ischemic stroke) or limited to studies of the coronary arteries and coronary artery disease complications (acute coronary syndrome, myocardial infarction). It should be mentioned that the results of a small number such studies is not consistent.

Recently, there are some works in which the relationship between Thr83Ala polymorphism, on the one hand, and MGP blood level, coronary arteries calcification, and myocardial infarction, on the other hand, was investigated. Thus, Crosier et al. revealed statistically significant association between this variant of SNP and MGP blood concentration in healthy men and women (USA) [10]. In the major allele homozygous patients, MGP blood level was the highest, in minor allele homozygotes it was the lowest, and in the heterozygotes intermediate values of this parameter were recorded.

In the same study [10], it was shown that in male subjects Thr83Ala polymorphism is related to coronary arteries calcification (CAC) both independently and in combination with other risk factors of atherosclerosis. With regard to female patients, the statistically significant association between this SNP and CAC was not detected. In men, the minor allele homozygotes were highly associated with CAC. In subjects with such genotype, the severity of CAC was significantly lower than in major allele homozygotes.

A very weak association between MGP gene Thr83Ala polymorphism and arterial calcification was found in the study of carotid and femoral arteries in healthy volunteers (AXA Study, France) [13]. Only in such patients in which calcified atheromatous plaques in femoral arteries was found by ultrasound examination, the minor allele was revealed more frequently than in subjects who had the plaques without signs of calcification.

In yet another study (ECTIM Study, Northern Ireland, France), it was shown that the allele frequency and genotype distribution for MGP gene Thr83Ala polymorphism did not differ in patients with myocardial infarction and in controls [13]. Only in one subgroup in which the patients and the controls were divided into subjects with high and low risk of coronary artery disease (CAD), the frequency of minor allele (83Ala) was higher in the infarction patients with low level of the CAD risk factors as compared with corresponding control subgroup.

In our previous study, we showed that the allele frequency and genotype distribution for MGP gene Thr83Ala polymorphism were similar in patients with acute coronary syndrome (unstable angina and myocardial infarction) and in control group [24].

It should be noted that the majority of studies cited here was devoted to the relation of MGP to coronary artery calcification and myocardial infarction. As to cerebral artery atherosclerosis and its severe events such as IAS, the role of arterial calcification in this disease and the association of MGP with cerebrovascular pathologies were the subject of investigation and discussion only in a few publications. In particular, Bos et al. [16] established a close relationship between calcification in the various vessel beds outside the brain and imaging markers of vascular brain disease. Calcification in each vessel bed was shown to be associated with the presence of cerebral infarcts and with larger volume of white matter lesions (WMLs). The most prominent associations were found between the intracranial carotid calcification and WML volume and between the extracranial carotid calcification and infarcts.

Acar et al. [25] studied a relationship of serum MGP levels to the development of intracerebral hemorrhages (ICH) and found that in patients with ICH, the serum MGP concentration was much lower than in control group. Moreover, in the non–survivors, the serum MGP levels were statistically significantly lower in comparison to the survivors. According to the authors, measurement of this parameter may be of value to estimate mortality.

At present, there are only a few publications concerning relation of the MGP SNPs to cerebrovascular disease. Del Rio–Espinola et al. [26] tried to find genetic predictors of reocclusion after successful fibrinolytic therapy during the acute phase of IAS. Analysing 236 polymorphisms, they revealed an association between MGP G-7A polymorphism and reocclusion risk. According to authors, the predictive scale that was generated permits the stratification of patients by their reocclusion risk with higher accuracy than clinical parameters alone.

In our study, we defined the distribution of MGP gene exon 4 Thr83Ala polymorphism in IAS patients and controls from the northeastern region of Ukraine. We did not find any association between this SNP and IAS risk neither in men nor in women. No association was also found between the Thr83Ala genotypes and IAS risk in the subgroups of IAS patients formed according to the presence or absence of some risk factors (BMI, hypertension, diabetes). Only in one subgroup which included non–hypertensive patients, the risk of IAS in heterozygotes was significantly lower as compered with major allele homozygotes.

CONCLUSION

We did not define any significant association between the exon 4 Thr83Ala polymorphism of the MGP gene and IAS risk neither in men nor in women which were representatives of the Ukrainian population. Non–hypertensive heterozygous IAS patients had significantly lower risk of IAS than the major allele homozygotes. The mechanisms of this is not clear and must be a subject of further investigation.

THR83ALA ПОЛІМОРФІЗМ 4–ГО ЕКЗОНА ГЕНА MGP, НЕ ПОВ'ЯЗАНИЙ ІЗ ІШЕМІЧНИМ АТЕРОТРОМБОТИЧНИМ ІНСУЛЬТОМ У ПІВНІЧНО–СХІДНОМУ РЕГІОНІ УКРАЇНИ

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Патологічне відкладання солей кальцію в артеріальні судини розглядають сьогодні як новітній маркер атеросклерозу і пов'язаної з ним цереброваскулярної патології. Матриксний білок, що містить гамма–карбоксиглутамінову кислоту (MGP), є одним із найпотужніших інгібіторів ектопічної мінералізації, а отже, він може мати відношення до кальцифікації атероматозних бляшок, їх нестабільності і розриву, а також утворення тромбів. Метою дослідження був аналіз можливого зв'язку Thr83Ala поліморфізму 4-го екзона гена MGP (rs4236) із ризиком розвитку ішемічного атеротромботичного інсульту (ІАІ) в українській популяції. Об'єктом дослідження були 170 пацієнтів з ІАІ і 124

практично здорові особи. Для визначення поліморфізму гена MGP послуговувалися методом PCR–RFLP. Розподіл гомозигот за основним алелем (Thr83Thr), гетерозигот (Thr83Ala) і гомозигот за мінорним алелем (Ala83Ala) у пацієнтів з ІАІ становив 39,4 , 48,8 і 11,8 %. Відповідний розподіл варіантів у контрольній групі був таким: 34,7 , 53,2 і 12,1 % (P>0,05 за χ^2-критерієм). Зроблено висновок, що в українській популяції Thr83Ala поліморфізм 4-го екзона гена MGP не асоційований із ризиком ІАІ ні у жінок, ні у чоловіків. Тільки в одній підгрупі, до якої входили пацієнти, які не мали артеріальної гіпертензії, ризик ІАІ у гетерозигот був істотно меншим, ніж у гомозигот за основним алелем. Механізм цього нез'ясований і повинен бути об'єктом подальших досліджень.

Ключові слова: *кальцифікація артерій, ішемічний атеротромботичний інсульт, матриксний Gla–протеїн, поліморфізм поодиноких нуклеотидів, українська популяція.*

THR83ALA ПОЛИМОРФИЗМ 4–ГО ЭКЗОНА ГЕНА MGP, НЕ СВЯЗАННЫЙ С ИШЕМИЧЕСКИМ АТЕРОТРОМБОТИЧЕСКИМ ИНСУЛЬТОМ В СЕВЕРО–ВОСТОЧНОМ РЕГИОНЕ УКРАИНЫ

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Патологическое отложение солей кальция в артериальные сосуды рассматривают сегодня как новейший маркер атеросклероза и связанной с ним цереброваскулярной патологии. Матриксный белок, содержащий гамма-карбоксиглутаминовую кислоту (MGP), является одним из самых мощных ингибиторов эктопической минерализации, а значит, он может иметь отношение к кальцификации атероматозных бляшек, их нестабильности и разрыву, а также образованию тромбов. Целью исследования был анализ возможной связи Thr83Ala полиморфизма 4-го экзона гена MGP (rs4236) с риском развития ишемического атеротромботического инсульта (ИАТИ) в украинской популяции. Объектом исследования были 170 пациентов с ИАТИ и 124 практически здоровых индивидуума. Для определения полиморфизма гена MGP использовали метод PCR–RFLP. Распределение гомозигот по основному аллелю (Thr83Thr), гетерозигот (Thr83Ala) и гомозигот по минорному аллелю (Ala83Ala) у пациентов с ИАТИ составляло: 39,4, 48,8 и 11,8 %. Соответствующее распределение вариантов в контрольной группе было таким: 34,7 , 53,2 и 12,1 % (P> 0,05 по χ^2-критерию). Сделан вывод о том, что в украинской популяции Thr83Ala полиморфизм 4-го экзона гена MGP не ассоциирован с риском ИАТИ ни у женщин, ни у мужчин. Только в одной подгруппе, в которую входили пациенты, не имевшие артериальной гипертензии, риск ИАТИ у гетерозигот был существенно меньше, чем у гомозигот по основному аллелю. Механизм этого неясен и должен быть объектом дальнейших исследований.

Ключевые слова: *кальцификация артерий, ишемический атеротромботический инсульт, матриксный Gla–протеин, полиморфизм одиночных нуклеотидов, украинская популяция.*

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