

# *Thr83Ala* Gene Polymorphism Association with Arterial Calcification, Acute Coronary Syndrome and Ischemic Strokes in Older Adults

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## Abstract

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**BACKGROUND:** Calcification of the arteries of the lower extremities is a very common pathological process, which has an independent significance in the development of cardiovascular diseases. There is evidence that development of calcification of the arteries correlates with a mutation of the MGP protein gene representing by the *Thr83Ala* polymorphism.

**AIM:** The purpose of the study was to analyse the connection of *Thr83Ala* polymorphism of the MGP gene with the development of calcification of the arteries of the lower extremities.

**METHODS:** The study involved 80 patients. Half of them had signs of calcification of the arteries of the lower extremities. The allelic *Thr83Ala* polymorphism of the MGP protein gene was determined by polymerase chain reaction, establishing the presence of calcification of the arteries by radiological and dopplerographic methods.

**RESULTS:** The study aimed to analyse the association of the *Thr83Ala* polymorphism of the matrix Gla protein gene with the development of calcification of the arteries of the lower extremities. The data obtained suggest that the replacement of threonine by alanine in the 83rd position of the MGP molecule can affect the functional properties of the protein and in particular its anticarcinogenic properties. Although there was no difference in the distribution of different variants of the genotype by *Thr83Ala* to the MGP gene polymorphism in patients with CA and healthy patients, but in the distribution of genotypes in the comparison groups separated by sex, it was found that in women, carriage of the Ala allele in a homozygous state is a factor, which protects the development of arterial calcination in the elderly and senile.

**CONCLUSION:** Differences in the distribution of different variants of the genotype according to *Thr83Ala* to the polymorphism of the MGP gene between patients with CAD and healthy patients do not exceed the limits of statistical significance. In the distribution of genotypes in the comparison groups divided by sex, it was found that in women the carrier of the Ala-allele in the homozygous state is a factor that prevents the development of Menkeberg arteriosclerosis in the elderly and old age.

## Introduction

Calcification of the arteries of the lower extremities (CA) is a risk factor for associated acute cardiovascular events, as well as gangrene of the lower extremities [1] [2] [3]. According to modern studies, calcification of peripheral arteries in older age groups is very common and ranges from 33.3% [1] to 50.2% [4].

It is known that the matrix Gla protein (MGP) is a strong inhibitor of vascular calcification. It refers to

representatives of the group of vitamin K-dependent proteins containing the residues of  $\gamma$ -carboxyglutamic acid (Gla). The very same group also comprises proteins, participating in blood coagulation: prothrombin, factors VII, IX and X, proteins C, S and Z. The human MGP molecule (molecular weight 10kDa) includes 84 amino acid residues, five of which are represented by  $\gamma$ -carboxyglutamic acid (Gla) [5]. The newly synthesized MGP molecule consists of 103 amino acid residues (84 is a mature protein, 19 is a transmembrane signal peptide) and contains, starting from at the N-terminus, three functional fragments: a transmembrane signal peptide, a probable site that

recognizes  $\gamma$ -carboxylase (putative recognition site for  $\gamma$ -carboxylase), a domain, containing Gla (Gla-containing domain) residues [6].

The Formed in the cells MGP experiences undergo post-translational modification, which consists of the carboxylation of five glutamic acid residues (Glu) to form a  $\gamma$ -carboxyglutamic acid (Gla). This reaction is catalysed by the enzyme  $\gamma$ -glutamyl carboxylase (GGCX) and is conjugated with the oxidation of the reduced form of vitamin K (hydroquinone) to the 2,3-epoxide of vitamin K. Thus, without the oxidation of vitamin K, the carboxylation of the Glu residues of the MGP molecule cannot occur. In turn, an enough amount of vitamin K for the carboxylation reaction of MGP depends on the state of the reverse reaction of its reduction, which is carried out with the help of a vitamin K-epoxy-reductase complex (VKRC). In addition to  $\gamma$ -carboxylation, MGP can undergo other post-translational modifications, specific proteolytic splitting in the C-terminal fragment of the molecule [7] [8] and phosphorylation of three serine residues at the N-terminal tail [9]. The MGP gene in humans is represented by a single copy, which is containing in the short upper arm of the 12th chromosome (12p12.3-13.1) [6]. It encodes 84 amino acid residues of the mature protein and 19 residues of the transmembrane signal peptide. The length of the gene is 3900 nucleotides; it consists of 4 exons, separated by three large intermediate sequences (introns), containing for more than 80% of the total length of the gene.

The single nucleotide polymorphism of Thr83Ala (rs4236) is such rearrangement, when in the 4th exon of the MGP gene, and at 3748 positions, thymine is replaced by adenine. This leads to the fact that the 83rd amino acid of the MGP molecule of threonine is replaced by alanine. The change in the primary structure of protein molecules can request in a variety of functional disorders; in case of MGP, one should expect changes in these familiar protein effects that are associated with its anticarcinogenic properties. These include, for example, binding to calcium ions and hydroxyapatite crystals; binding to extracellular matrix components; interaction with the BMP-2 and elimination of the latter's effects; participation in the regulation of apoptosis [10] [11].

Thus, the purpose of the study was to analyse the connection of Thr83Ala polymorphism of the MGP gene with the development of calcification of the arteries of the lower extremities.

## Methods

Eighty patients, attending Sumy Region Veterans Affairs, were involved in this study. Half of

them (40 persons) had signs of calcification of the arteries of the lower extremities (main group), and the other 40 persons had no signs (control group). Patients with diagnosed diabetes mellitus were not added to any of the study groups that allowed it possible to exclude the effect of this factor on the studied connections. The control group and the main group of patients did not differ according to persons of different sexes ( $P = 0.369$ ), as per middle age ( $74.4 \pm 0.76$  vs.  $74.4 \pm 0.97$ ,  $P = 0.984$ ) as well as according to body mass index ( $26.02 \pm 0.22$  versus  $26.01 \pm 0.35$ ,  $P = 0.999$ ). The presence of SC was determined to base on X-ray and Doppler data [12].

The studies were carried out in compliance with the main provisions of the Council of Europe Convention on Human Rights and Biomedicine, as well as the Helsinki Declaration of the World Medical Association on the Ethical Principles of Scientific Medical Research with Human Participation (1964, with subsequent additions, including the 2000 version). All patients signed informed consent to participate in the studies with a subsequent collection of venous blood for genetic analysis.

Determination of gene polymorphism was performed using the polymerase chain reaction (PCR) method with subsequent by analysis of the length of the restriction fragments upon their detection using restriction fragment length polymorphism (RFLP).

For genotyping, venous blood was collected in sterile conditions in a mono volume of 2.7 ml with potassium salt of ethylenediaminetetraacetic acid ("Sarstedt", Germany), which served as an anticoagulant. The blood was frozen and stored at the temperature of  $-20^{\circ}\text{C}$ . DNA was extracted from it using the "Izogen" kits (Russia).

Amplification of the fragment of the gene containing the Thr83Ala site of polymorphism in the 4th exon of the MGP gene was performed using a pair of specific primers: sense-5'-TCAATAGGGAAGCCTGTGATG-3' and antisense-5'-AGGGGGATACAAAATCAGGTG-3. The amplification program was as follows: denaturation- $94^{\circ}\text{C}$  (50 c), hybridisation of primers,  $64.5^{\circ}\text{C}$  (45 c), elongation- $72^{\circ}\text{C}$  (1 min), totally 33 cycles.

Later, 6  $\mu\text{l}$  of the amplification product was incubated at the temperature of  $37^{\circ}\text{C}$  during 18 hours with 3 units of Eco477 restriction enzyme in R buffer of the following composition: 10mM Tris-HCl (pH 8.5), 10 mM magnesium chloride 100 mM potassium chloride and 0.1 mg/ml albumin. The presence of adenine in the 3748 position of the MGP gene prevents restriction, and upon substitution of adenine by thymine restriction, it splits the amplified area of the 4th exon (length-173 pairs of nitrogen bases) into two fragments: 127 and 46 base pairs (Figure 1).

Amplifications of the achieved fragment of the MGP gene after restriction were separated in a 2.5% agarose gel, containing ethidium bromide. Horizontal

electrophoresis (0.1 A; 140 V) was performing for 20 minutes. DNA visualisation after electrophoresis was performed using a transilluminator ("Biocom", Russia).

Statistical analysis was carried out by using the SPSS-17 program. In this case, the reliability of the differences was determined by the Pearson  $\chi^2$ -criterion and the Students t-criterion. A value of  $p < 0.05$  was considered reliable. The odds ratio (OR) and the 95% confidence interval were calculated using the logistic regression method.

## Results

There are three possible variants of the genotype for the *Thr83Ala*: *Thr/Thr* (homozygous for the main allele), *Thr/Ala* (heterozygotes) and *Ala/Ala* (homozygous for the minor allele) polymorphism (Figure 1).

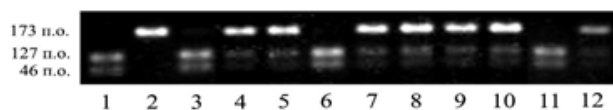


Figure 1: Results of the electrophoresis of fragments of the MGP gene after restriction for the detection of *Thr83Ala* polymorphism. Tracks 1, 3, 6, 11 correspond to the *Thr/Thr* genotype; 4, 5, 7, 8, 9, 10, 12-*Thr/Ala*-genotype; 2-*Ala/Ala* genotype

Genotyping patients with CA and patients of the control group for this SNP allowed establishing the frequency with which one can come across the certain variants of this gene meet and allow to compare them among the groups and according to gender. When checking the correspondence of the distribution of alleles to the Hardy-Weinberg law, it was established that the ratio of Thr and Ala alleles in both groups does not differ significantly from those expected (Table 1).

Table 1: The frequency of allelic variants and alleles according to *Thr83Ala* polymorphism of the MGP gene in the control group and patients with CA

	Control group	Patients with CA
Homozygotes <i>Thr/Thr</i> , n (%)	13 (32.5)	16 (40.0)
Heterozygotes <i>Thr/Ala</i> , n (%)	17 (42.5)	19 (47.5)
Homozygotes <i>Ala/Ala</i> , n (%)	10 (25.0)	5 (12.5)
Thr-allele	0.54	0.64
Ala-allele	0.46	0.36
$\chi^2$	0.84	0.03
P	> 0.05	> 0.05

Note: n-the number of patients,  $\chi^2$  and P reflect the deviation in each group from the Hardy-Weinberg equilibrium

Table 2 shows the results of the analysis of the frequencies of individual phenotypes according to the *Thr83Ala* polymorphism in the control group and patients with CA. They show that the ratio of homozygotes for the main allele (*Thr/Thr*), heterozygotes (*Thr/Ala*) and homozygotes for the minor allele (*Ala/Ala*) in patients with arteriosclerosis

comprises 40%, 47.5% and 12.5%, while in the control group the corresponding indices were 32.5%, 42.5% and 25%. Differences in the distribution of different variants of the genotype between patients with CA and healthy patients did not exceed the statistical significance.

Table 2: The Association of the *Thr83Ala* polymorphism of the MGP gene with the CA

		Control	CA	Totally
<i>Thr/Thr</i>	N	13	16	29
		32.5%	40.0%	
<i>Thr/Ala</i>	N	17	19	36
		42.5%	47.5%	
<i>Ala/Ala</i>	n	10	5	15
		25.0%	12.5%	
Totally	n	40	40	80
		100%	100%	

$$\chi^2 = 2.088; P = 0.352$$

Note. N is the number of patients.

However, the analysis of the distribution of genotypes in sex-disaggregated comparison groups allowed to identify certain significant differences. In general, the ratio of the two variants of homozygotes and heterozygotes (*Thr/Thr*: *Thr/Ala*: *Ala/Ala*) in women and men did not differ in individuals with signs and without signs of CA (Table 3). In the first group, it was 40%: 55%: 5% for women and 40%: 40%: 20% for men, and for control group 18.8%: 50%: 31.2% for women ( $P = 0.081$ ) and 41.7%: 37.5%: 20.8% for men ( $P = 0.986$ ). However, it is easy to see that for this indicator, female representatives were significantly closer to the level of statistical significance than men.

Table 3: The association of the *Thr83Ala* polymorphism of the MGP gene with CA in females and males

Gender	Genotype		Control	CA	Totally
Female	<i>Thr/Thr</i>	n	3	8	11
			18.8%	40.0%	
	<i>Thr/Ala</i>	n	8	11	19
			50.0%	55.0%	
	<i>Ala/Ala</i>	n	5	1	6
		31.3%	5.0%		
	Totally	n	16	20	36
			100%	100%	
			$\chi^2 = 5.031; P = 0.081$		
Male	<i>Thr/Thr</i>	n	10	8	18
			41.7%	40.0%	
	<i>Thr/Ala</i>	n	9	8	17
			37.5%	40.0%	
	<i>Ala/Ala</i>	n	5	4	9
		20.8%	20.0%		
	Totally	n	24	20	44
			100%	100%	
			$\chi^2 = 0.029; P = 0.986$		

Note: n - the number of patients.

In women, the difference between patients with CA and patients without signs of arteriosclerosis was found when comparing individual variants of the genotype with each other. Therefore, it was found that the frequency of homozygotes in the minor allele (*Ala/Ala*) was significantly higher in control than in patients with CA (31.3% vs 5%,  $P = 0.036$ ).

Using the method of logistic regression, it is shown (Table 4), that in women with the *Ala/Ala* genotype the risk of CA is 0.075 when comparing with homozygotes over the main allele ( $P = 0.044$ ). This

means that elderly and senile patients homozygous for the Ala allele have a CA risk more than 10 times lower than *Thr/Thr* homozygotes.

**Table 4: Results of the analysis of the *Thr83Ala* association of the MGP gene polymorphism with calcification of the lower extremities arteries in women and men (logistic regression method)**

	Genotype	CR	SE	WS	P	OR	95% CI for OR	
							Lower	Higher
Totally	<i>Thr/Ala</i>	-0.096	0.501	0.037	0.847	0.908	0.340	2.424
	<i>Ala/Ala</i>	-0.901	0.663	1.847	0.174	0.406	0.111	1.490
Women	<i>Thr/Ala</i>	-0.662	0.821	0.651	0.420	0.516	0.103	2.578
	<i>Ala/Ala</i>	-2.590	1.288	4.046	0.044	0.075	0.006	0.936
Men	<i>Thr/Ala</i>	0.105	0.679	0.024	0.877	1.111	0.294	4.205
	<i>Ala/Ala</i>	0	0.822	0	1	1	0.200	5.004

Note data on homozygous for the main allele (*Thr/Thr*). CR-coefficient of regression, SE-standard error, WS-Wald statistics, P-indicator of statistical significance, OR-odds ratio, CI-confidence interval.

Reduced risk of CA in women with the *Ala/Ala* genotype is also evident when comparing them with carriers of the Thr allele (*Thr/Thr+Thr/Ala*) and separately with heterozygotes (*Thr/Ala*). In the first case, the OR ratio is 0.116, and in the second case, 0.145. However, due to an inadequate number of observations, the statistical significance of P did not reach the generally accepted level of confidence and amounted to 0.063 and 0.105, respectively.

Therefore, there is a reason to state that in women the carriage of the Ala-allele in the homozygous state is a factor that prevents the development of arteriocalcification in the elderly and old age.

The results of molecular genetic studies, obtained by us in patients with calcification of arteries of the lower extremities, can be compared with the data, obtained in the study of the connection of the *Thr83Ala* polymorphism of the MGP gene with the most common complications of the atherosclerotic process-acute coronary syndrome (ACS) and ischemic atherothrombotic stroke (IATS). Data for this comparison are taken from the work of the laboratory of molecular genetic studies of Sumy State University [2] [13] and are presented in Table 5.

**Table 5: Comparative characteristics of the distribution of genotypes according to *Thr83Ala* polymorphism of the MGP gene in patients with arterial calcification, ACS [2] and IATS [5], %**

Genotype	CA		ACS		IATS	
	Disease (n = 40)	Control (n = 40)	Disease (n = 115)	Control (110)	Disease (170)	Control (124)
<i>Thr83Ala</i>						
<i>Thr/Thr</i>	40.0	32.5	4.6	43.9	39.4	34.7
<i>Thr/Ala</i>	47.5	42.5	43.5	45.9	48.8	53.2
<i>Ala/Ala</i>	12.5	25.0	13.9	10.2	11.8	12.1

Note: n - the number of patients.

The data indicate that the percentage of homozygotes in the minor allele (*Ala/Ala*) in the control group for the CA was more than twice as high (25%) when comparing with the control groups of the other two studies (10.2% and 12.1%). At the same time, the percentage of such homozygotes in patients with CA (12.5%) corresponded to the above data for control in studies with ACS and IATS.

Since the homozygous condition of the minor allele in women interferes with the development of CA, the assumption of the age (physiological) origin of arteriocalcification is quite attractive. Since in our work patients with diabetes mellitus, which is one of the main causes of CA, were excluded both from the main and control groups, the presence or absence of lesions of the arteries should occur due to some other factors. One of such factors may be a hereditary predisposition, including the *Thr83Ala* polymorphism of the MGP gene. According to our data, it can be assumed that women homozygous for the Ala-allele are "abnormal" or "sick on the contrary," because their genetic factor interferes with the physiological ageing of the arteries inherent in most people in the elderly and senile ages.

The substitution of threonine for alanine at the 83rd position of the MGP molecule can affect the functional characteristics of the protein and in particular its anticarcinogenic properties. The latter, as is known today, is extremely important for preventing calcification of the arteries, as evidenced by experiments with genetically knocked out MGP (-/-) mice [14] and warfarin-mediated aortic medication in rats [15].

In conclusion: (1) differences in the distribution of different variants of the genotype according to *Thr83Ala* to the polymorphism of the MGP gene between patients with CA and healthy patients do not exceed the limits of statistical significance; (2) in the distribution of genotypes in the comparison groups divided by gender, it was found that in case of women the carrier of the Ala-allele in the homozygous state is a factor that prevents the development of Menkeberg arteriosclerosis in the elderly and old ages.

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