

## EKSPERIMENTINIAI TYRIMAI

### Matrix metalloproteinase-3 gene polymorphism and dilatative pathology of ascending thoracic aorta

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**Key words:** thoracic aorta; matrix metalloproteinase-3; gene polymorphism; aneurysm.

**Summary.** Matrix metalloproteinase-3 (MMP-3) degrades extracellular matrix and may lead to development of dilatative pathology of ascending thoracic aorta. Expression of MMP-3 depends upon the 5A/6A polymorphism in the promoter region. An increased number of 5A alleles leads to high expression of MMP-3. Thus, objective of the study was to determine whether the 5A/6A polymorphism in the promoter region of MMP-3 gene is associated with the development of dilatative pathology of ascending thoracic aorta.

We studied 76 patients (age ranged from 31 to 81 years; median age, 64 years) who underwent aortic reconstruction surgery due to dilatative pathology of ascending thoracic aorta and a random sample of the population (n=604) aged 25–64 years, all from Lithuania. DNA was analyzed by using real-time polymerase chain reaction to genotype polymorphism 5A/6A at a position – 1171 of the MMP3 gene promoter. The prevalence of MMP-3 genotypes was similar in the group of dilatative pathology of ascending thoracic aorta and random sample of population. The frequency of 5A allele did not differ significantly between both groups and was 0.506 and 0.514, respectively. Male carriers of 5A/5A genotype were significantly younger compared with those with the 6A/6A genotype.

In conclusion, the frequency of MMP-3 promoter 5A/6A genotypes did not differ between the group of patients with dilatative pathology of ascending thoracic aorta and the random sample of population, but the males with dilatative pathology of ascending thoracic aorta and 5A/5A genotype required aortic reconstruction surgery at the younger age than the males carrying 6A/6A genotype in the MMP-3 promoter region.

#### Introduction

The etiology of thoracic aortic aneurysm formation mechanism remains uncertain. Only small part of them is caused by inflammation, as in giant cell arteritis and syphilitic aortitis (1); by hereditary connective tissue disorders, such as mutations in the fibrillin genes in Marfan syndrome and Ehlers-Danlos syndrome (2); or by atherosclerosis (3). However, it is recognized that disarrangement of smooth muscle cells (SMCs) and the extracellular matrix (ECM) appeared to be crucial in pathogenesis of aortic aneurysm formation (4). Recent studies indicate that SMCs participate in remodeling of aortic wall by localized production of

MMPs and their tissue inhibitors in aortic media (1, 4).

MMPs may play a key role in physiologic and pathologic remodeling of tissues, including embryogenesis and tissue morphogenesis, wound repair, inflammatory diseases, cancerogenesis, etc. (5, 6). These multiple actions of MMPs are due to their ability to degrade extracellular matrices, basement membranes, and other proteins. Functional regulation of MMPs is controlled primarily by gene transcription and synthesis, whereas the activity is regulated by posttranslational activation of zymogens and by interactions of secreted MMPs with tissue inhibitors of metalloproteinases (TIMPs) (7–9). A common adenine insertion/

deletion polymorphism (5A/6A) at a position –1171 of the *MMP3* (*stromelysin-1*) gene promoter (National Center for Biotechnology Information SNP Identification No. rs3025039) influences *MMP-3* promoter activity and transcription factor binding (10). It was shown that the increased number of 5A alleles leads to increased expression of *MMP-3* (11–16) and higher plasma *MMP-3* activity (15). The latter phenomenon suggests relation between *MMP-3* genotype and the development of pathological states caused by connective tissue degradation, for example rupture of atherosclerotic plaque leading to the acute myocardial infarction or unstable angina pectoris (13, 14), spreading of carcinoma (6).

Therefore, in the present study we investigated whether the 5A/6A polymorphism in the promoter region of *MMP-3* gene is associated with the development of DP ATA.

### Materials and methods

The study enrolled 76 patients (55 males, 21 females) who underwent aortic reconstruction due to DP ATA at the Clinic of Cardiac, Thoracic, and Vascular Surgery, Kaunas University of Medicine Hospital, Lithuania. The age of patients ranged from 31 to 81 years (median, 64 years). The patients were operated on due to dissection of the thoracic aorta ( $n=24$ , dissection subgroup), poststenotic dilatation of the ascending aorta due to aortic stenosis ( $n=21$ , valvular subgroup), and chronic aneurysm of the thoracic aorta ( $n=31$ , aneurysm subgroup). All patients underwent preoperative transthoracic echocardiography. Specimens of aortic tissue taken during aortic reconstruction surgery were fixed in 10% neutral buffered formalin for 24 hours and then processed for routine paraffin embedding.

The persons (244 males and 366 females) screened within the framework of the international CINDI (Countrywide Integrated Noncommunicable Diseases Intervention) study (17) were used for comparative analysis. A random sample of the population aged 25–64 years was taken from the inhabitants' lists of primary health care centers in three administrative regions of Lithuania (Kaišiadorys, Kretinga, and Varėna). The health examination of selected sample was performed in 2007. During the examination, venous blood for genetic investigation was taken in 3-mL K3EDTA vacutainers. They were stored at  $-80^{\circ}\text{C}$  for subsequent DNA extraction.

Ethical approval was obtained from the Ethics Committee of Kaunas University of Medicine and Lithuanian Bioethical Committee (March 23, 2006, No. 11).

### DNA extraction

Standard procedure described by Sepp *et al.* (18) and Shi *et al.* (19) was used to extract genomic DNA from aortic wall sections taken during aortic reconstruction surgery. Six- $\mu\text{m}$ -thick 150 sections from paraffin-embedded aortic wall were collected into an autoclaved plastic microtube and lysed with digestion buffer (100 mM NaCl; 10 mM Tris-Cl, pH8; 25 mM sodium dodecyl sulfate) and 20  $\mu\text{L}$  of proteinase K (20 mg/mL, Fermentas, Lithuania) by incubation overnight at  $55^{\circ}\text{C}$ . The samples were incubated at  $98^{\circ}\text{C}$  to inactivate proteinase K and centrifuged at  $5000\times g$  for 15 min (18). The lysis solution was transferred into a new test tube, mixed with equal amount of phenol/chloroform/isoamyl alcohol (Sigma) in a 25:24:1 ratio and centrifuged at  $13\,000\times g$  for 15 min at  $4^{\circ}\text{C}$ . The procedure was repeated twice with equal amount of chloroform (Sigma) to wash out phenol remains. The aqueous supernatant was transferred into a micro test tube, mixed with equal volume of isopropanol (Sigma), incubated for 1 hour at  $-20^{\circ}\text{C}$ , and centrifuged at  $13\,000\times g$  for 15 min. The precipitated total DNA was rinsed with 70% ethanol and then resuspended in RNase-free water (19).

Genomic DNA from blood was isolated by using Sorpoclean Genomic DNA Extraction Module kit (Sorpo Diagnostics, Vilnius, Lithuania) according to manufacturer's instructions.

DNA quality and concentration were estimated by spectrophotometrical analysis and by ethidium bromide-stained agarose gel under ultraviolet light. The latter method was used to evaluate DNA degradation level in the samples from paraffin-embedded aortic tissue. Photographs were documented through a video documentation system, the BioDocAnalyse 2.0 (Biometra, Göttingen, Germany).

### Primers and probes for real-time polymerase chain reaction (RT PCR)

Primer-probe set was made using the TibMolBiol design service (Berlin, Germany) for *MMP-3* genotyping. Two fluorogenic minor groove binder probes, 5'-6FAM AAG ACA TGG TTT TTC CCC CCA TCAA BBQ-3' and 5'-YAK AAG ACA TGG TTT TTT CCC CCC ATC AA BBQ-3', were used to detect the adenine insertion/deletion mutation. The dyes 6-carboxyfluorescein (FAM; excitation, 494 nm) and Yakima Yellow (YAK; excitation, 526 nm) are easily differentiated in the Applied Biosystems Prism 7900HT PCR system. The primers were the following: forward primer, 5'-GTG GCC AAA TAT TTT CCC TGT ATTT-3'; reverse primer, 5'-GGC ACC TGG

CCT AAA GAC ATT-3'. RT PCR was performed using 12.5 µL of TaqMan 2X Universal PCR master mix (Applied Biosystems, Darmstadt, Germany), 0.4 µL of primers (20 µM) (TibMolBiol, Berlin, Germany), 0.125 µL of probes (20 µM) (TibMolBiol, Berlin, Germany), 10.45 µL of RNase- and DNase-free water (Ambion), and 1 µL of sample DNA, in a total volume of 25 µL per single tube reaction. DNase-free water used as nontemplate control and DNA of known MMP-3 genotypes used as a positive control were included in each assay run. Assay conditions were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The SNP assay was set up using SDS, version 2.3, software (Applied Biosystems, USA).

### Statistics

Statistical analysis was done using the SPSS statistical package (version 12.0; Chicago, IL). The nonparametric Kruskal-Wallis test was used for comparison between groups. A P value of <0.05 was accepted as statistically significant.

### Results

The frequency of MMP-3 5A/6A genotypes and alleles did not differ between males and females in the group of DP ATA and random sample of population. Therefore, the data presented in Table are combined for both genders. The distributions of genotypes in the DP ATA and random sample of population were compatible with the Hardy-Weinberg equilibrium. The prevalence of MMP-3 genotypes and 5A/6A alleles was similar between group of DP ATA and random sample of population (Table). When we divided group of DP ATA into dissection, valvular,

and aneurysm subgroups, the distribution of genotypes in each subgroup did not differ significantly. However, it was tendency of higher prevalence of 5A/5A genotype in dissection subgroup (33.3%) as compared with valvular and aneurysm subgroups (19.05% and 25.81%, respectively).

Figure presents data on the minimal, maximal, and median age according to MMP-3 5A/6A genotypes in males and females with DP ATA and in random sample of population. Males carrying 5A/5A genotype from the group of DP ATA were significantly younger than those with 6A/6A genotype ( $P < 0.05$ ). There was no significant difference in age between MMP-3 genotypes in both genders from random sample of population and in females with DP ATA.

### Discussion

Previous understanding of the pathogenesis of aortic aneurysms based on the biomechanical factors, such as arterial hemodynamics and wall mechanics, has been increased by the role of biological factors (20). Destruction of the elastic media appears to depend on proteinases, which are members of the MMP family (4). The latter have been implicated in the etiopathogenesis of thoracic aneurysm formation (21, 22). It was shown that alterations at the genetic level contribute to the overexpression of MMPs (10, 21, 22).

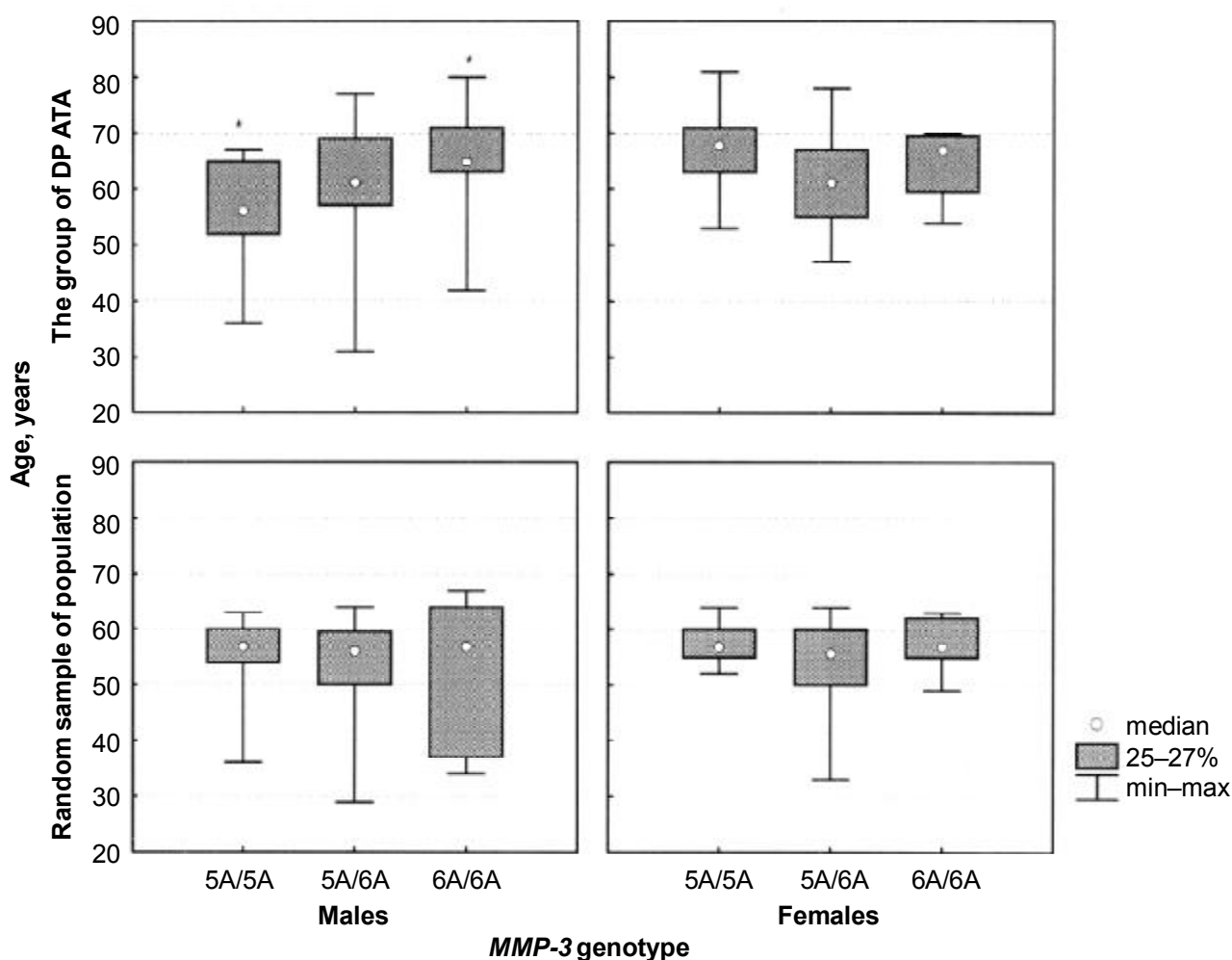
MMP-3 or stromelysin-1 is a member of stromelysin family. It is capable of degrading proteoglycan, fibronectin, laminin, and type IV collagen (9). *In vitro* studies of MMP-3 promoter strength showed that the 5A allele expressed higher activity than the 6A allele in both cultured fibroblasts and vascular smooth muscle cells (23). It is suggested that because of

**Table. Frequency of matrix metalloproteinase-3 (MMP-3) 5A/6A genotypes and alleles in patients with dilatative pathology of ascending thoracic aorta (DP ATA) and random sample of population (RSP)**

MMP-3	Patients with DP ATA, n (%) (n=76)	RSP, n (%) (n=604)	P value
<i>Genotype</i>			
5A/5A	20 (26.32)	157 (25.99)	0.90
5A/6A	37 (48.68)	307 (50.83)	0.73
6A/6A	19 (25.00)	140 (23.18)	0.80
<i>Allele</i>			
5A	77 (50.7)	621 (51.4)	0.87
6A	75 (49.3)	587 (48.6)	0.87
5A frequency	0.506	0.514	

5A allele frequency was calculated as follows:

$(2 \times \text{percentage of 5A/5A genotype frequency} + 1 \times \text{percentage of 5A/6A genotype frequency})/200$ .



**Fig.** Minimal, maximal, and median age according to *matrix metalloproteinase-3* (MMP-3) 5A/6A genotypes in males and females with dilatative pathology of ascending thoracic aorta (DP ATA) and random sample of population

\* $P < 0.05$  according to Kruskal-Wallis test.

reduced gene transcription, homozygosity for the 6A allele would be associated with lower stromelysin levels in arterial walls than other genotypes. Thus, in the present study, we investigated whether the 5A/6A polymorphism in the promoter region of *MMP-3* gene was associated with DP ATA. *MMP-3* 5A/6A polymorphism was estimated in 76 patients with DP ATA and random sample of population ( $n=604$ ), all from Lithuania. The frequency of genotypes and alleles was similar in both groups. We were not able to find publications on the prevalence of *MMP-3* 5A/6A polymorphism in patients with DP ATA in databases available for us (Medline (PubMed), ScienceDirect, Wiley InterScience, Blackwell Synergy, Oxford University Press: Oxford Journals). Some studies of the patients with abdominal aortic aneurysm indicate that presence of the 5A allele predisposes to aneurysmal disease (11, 24), while others do not find such asso-

ciation (25).

The frequency of the 5A allele in our random sample of Lithuanian population is 0.514. It is consistent with published *MMP-3* control population data for United Kingdom (24, 25), Belgium, Canada (26), Sweden (27), and Italy (12). The frequency of the 5A allele in French (28) and Austrian (29) control populations (0.46 and 0.469, respectively) was smaller than in random sample of Lithuanian population. It is of interest that the 5A allele frequency in the Japanese (14, 30), the South Korean (13), and in Taiwan control populations (15) is only 0.18, 0.15, and 0.18, respectively.

Kamijima *et al.* (4) showed that phenotypic modulation of SMCs in the aortic wall is a critical event in the enlargement of aortic abdominal aneurysm and *MMP* enhancement. Investigation of DP ATA demonstrated that transition of medial SMCs from the

contractile to the synthetic type led to the increased production of MMPs by SMCs, as well (1). A trigger inducing the transition of medial SMC phenotype remains obscure. Our results show that the frequency of *MMP-3* promoter 5A/6A genotypes did not differ between the group of patients with DP ATA and the random sample of population, but the male patients carrying 5A/5A genotype were significantly younger compared with those with the 6A/6A genotype. In addition, it was tendency of higher prevalence (33.3%) of 5A/5A genotype in dissection subgroup as compared to the random sample of population (25.99%). Our data lead to the hypothesis that *MMP-3* promoter 5A/6A genotypes are not associated with the transition of medial SMCs from the contractile to the synthetic phenotype in the thoracic aorta. On the other hand, when phenotypic modulation of SMCs leading to DP ATA occurs, males with 5A/5A genotype require aortic reconstruction surgery at the younger age than males carrying 6A/6A genotype at the *MMP-3* pro-

motor region. Our study is limited by the small number of patients in the subgroups of DP ATA. For example, dissection subgroup included only 24 patients. Therefore, we were not able to obtain significant results on the impact of 5A/5A genotype on aortic dissection. Second, we had no possibility to carry out transthoracic echocardiography for the randomly selected population. Because of this, we did not design a control group composed only of the persons free from any aortic dilatative pathology.

### Conclusions

The frequency of *MMP-3* promoter 5A/6A genotypes did not differ between the group of patients with dilatative pathology of ascending thoracic aorta and the random sample of population, but the males with dilatative pathology of ascending thoracic aorta and 5A/5A genotype require aortic reconstruction surgery at the significantly younger age than the males carrying 6A/6A genotype in the *MMP-3* promoter region.

## Matrikso metalo proteinazės-3 geno polimorfizmas ir kylančiosios krūtinės aortos dalies išsiplėtimas

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**Raktažodžiai:** krūtinės aorta, matrikso metalo proteinazė-3 (MMP-3), geno polimorfizmas, aneurizma.

**Santrauka.** Matrikso metalo proteinazė-3 (MMP-3), ardydama nelastelinį matriksą, gali sukelti kylančiosios krūtinės aortos dalies išsiplėtimą. Matrikso metalo proteinazės-3 ekspresija priklauso nuo geno promotoriaus 5A/6A polimorfizmo. Didesnis 5A alelių skaičius lemia didesnę matrikso metalo proteinazės-3 ekspresiją. Todėl šio tyrimo tikslas buvo nustatyti, ar yra ryšys tarp matrikso metalo proteinazės-3 geno promotoriaus 5A/6A polimorfizmo ir kylančiosios krūtinės aortos dalies išsiplėtimo susiformavimo. Ištyrėme 76 ligonius, kurių amžius nuo 31 iki 81 metų, mediana – 64 metai ir kurie buvo operuoti dėl kylančiosios krūtinės aortos dalies išsiplėtimo, atsitiktinės imties 25–64 metų asmenis (n=604). DNR buvo tirama atliekant tikrojo laiko polimerazės grandininę reakciją. Nustatytas matrikso metalo proteinazės-3 promotoriaus –1171 5A/6A polimorfizmo genotipas. Ligonų, kuriems diagnozuotas kylančiosios krūtinės aortos dalies išsiplėtimas, ir atsitiktinės imties asmenų matrikso metalo proteinazės-3 genotipų dažniai nesiskyrė. Taip pat statistiškai reikšmingai nesiskyrė 5A alelio dažnis. Jis buvo atitinkamai – 0,506 ir 0,514. Ligoniai vyrai, operuoti dėl kylančiosios krūtinės aortos dalies išsiplėtimo ir turintys 5A/5A genotipą, buvo statistiškai reikšmingai jaunesni nei turintys 6A/6A genotipą.

**Išvada.** Ligonų, kuriems buvo kylančiosios krūtinės aortos dalies išsiplėtimas, ir atsitiktinės imties asmenų matrikso metalo proteinazės-3 promotoriaus 5A/6A genotipų dažniai nesiskyrė. Ligoniams vyrams, turintiems 5A/5A genotipą, aortos rekonstrukcinės operacijos buvo atliekamos jaunesniame amžiuje nei vyrams, turintiems 6A/6A genotipą.

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