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The 5A Allele of the MMP3-Gene Promoter Polymorphism Is a Risk Factor for Poor Outcome of Hemodialysis Patients

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Cardiovascular morbidity is the leading cause of death in dialysis patients and many risk factors have been involved in its pathogenesis. Genetic susceptibility may be of importance including polymorphism for matrix metalloproteinase 3 (MMP3), which is an enzyme that catalyzes the degradation of collagen, proteoglycans, fibronectin, laminine and elastin. The MMP3 gene promoter contains an insertion/deletion polymorphism characterised by an array of 5 or 6 adenosine residues (5A/6A) at -1612 position. Literature data show that the 5A or 6A allele of the MMP3 gene shows different risk for cardiovascular and overall outcome in general population. The aim was to analyze the -1612 5A/6A promoter polymorphism in a group of hemodialysis patients and to correlate the findings with cardiovascular morbidity and 7-year all-cause and cardiovascular mortality. This study included 196 patients on hemodialysis for longer than six months at University Medical Center Zvezdara. The leading causes of end stage renal disease were hypertension and diabetes mellitus. Venous blood was collected on midweek dialysis session and genotype analysis was performed by using polymerase chain reaction-restriction fragment length polymorphism method. Among the 198 hemodialysis patients, there were 142 (72%) 5A/6A heterozygotes, 12 (6%) 5A/5A homozygotes, and 44 (22%) 6A/6A homozygotes. These data are consistent with Hardy-Weinberg equilibrium. After 7-year follow-up, the 5A homozygotes showed the lowest all-cause and cardiovascular survival, while the 6A homozygotes showed the highest cardiovascular survival. The 5A allele of the MMP3-gene promoter polymorphism is a potential risk factor in the poor outcome of hemodialysis patients.

Keywords: cardiovascular morbidity; gene polymorphism; hemodialysis; matrix metalloproteinase; mortality
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Introduction

Cardiovascular disease (CVD) is the leading cause of death in hemodialysis (HD) patients. The risk for developing cardiovascular (CV) event is 5-30 folds higher in patients with end-stage renal disease than in the general population (Foley and Parfrey 1998; Cheung et al. 2000; Locatelli et al. 2001; Pernod et al. 2006). Since traditional risk factors could not explain high prevalence of cardiovascular disease (Foley et al. 1996; Longenecker et al. 2002; Pernod et al. 2006), many reports have suggested a role of non-traditional risk factors in pathogenesis of CVD, including gene polymorphism of matrix metalloproteinase (MMP) (Humphries et al. 2002; Beyzade et al. 2003; Flex et al. 2004; Beilby et al. 2005; Koch et al. 2010).

Matrix metalloproteinase 3 is an enzyme which performs degradation of collagen type II, III, IV, IX and X, proteoglycans, fibronectin, laminine and elastin. Furthermore, this enzyme activates other metalloproteinases (MMP1, MMP7 and MMP9), with consequent remodeling of connective tissue. In the process of response to blood vessel injury MMP3 leads to activation of various cytokines, proteolytic enzymes, chemokines and inflammatory cells, which all leads to remodeling and forming an atheromatous plaque. Blood vessels remodeling is the basis of the formation of atherosclerosis and its complications. Activated T lymphocytes stimulate macrophages to produce more metalloproteinases which degrade matrix and thin fibrous cap, cause bleeding from vasa vasorum or lumen of the blood vessel itself, thus leading to destabilization of atherosclerotic plaque (Davies 1996; Gnasso et al. 2000; Humphries et al. 2002; Beyzade et al. 2003; Koch et al. 2010).

Promoter of human MMP3 gene contains an insertion/

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deletion (I/D) polymorphism characterised by an array of 5 to 6 adenosine bases (5A/6A) in the location of 1612 base pairs upstream from the initial place of transcription. It has been proven that 5A/6A polymorphism of this promoter affects the expression of gene for MMP3, leading to different cardiovascular events.

It was shown that 5A/6A polymorphism in the promoter of MMP3 gene has an effect on MMP3 concentration, that is associated with a number of cardiovascular consequences (Nagase and Woessner 1999; Ye 2006; Koch et al. 2010). So far, some studies have shown that the promoter of 5A allele has greater activity in gene expression, which results in higher enzyme level (5A homozygote), intermediate level (5A/6A heterozygote) and the lowest enzyme level (6A homozygote) (Beyzade et al. 2003). Therefore, 5A homozygotes are more prone to experience acute cardiovascular events due to higher degradation of extracellular matrix, while 6A homozygotes have higher risk for accelerated atherosclerosis as lower enzyme activity results in high deposition of matrix proteins (Ye et al. 1999; Newby 2005). Previous data are mainly related to the general population, whereas data for HD population are missing.

Therefore, the aim of this study was to analyze MMP3 gene polymorphism in group of HD patients and to correlate the findings with cardiovascular morbidity and all-cause and cardiovascular mortality.

Materials and Methods

This cross-sectional study included 198 patients, who were on regular hemodialysis at Zvezdara University Medical Center for more than six months. Patients were dialyzed three time per week on high-flux polysulfone membranes. Genetic analysis was performed by using polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP).

Retrospective analysis included data collection from the patients history including cardiovascular morbidity (myocardial infarction, cerebrovascular accident, coronary artery disease, heart arrhythmia, hypertension, left ventricular hypertrophy and peripheral artery disease). Collected data were correlated with genetic polymorphism for MMP3.

The prospective part of this study included 7-year follow up in order to analyse CV morbidity and all-cause and CV mortality regarding MMP3 gene polymorphism.

Heart arrhythmia was diagnosed by Electrocardiography (ECG) 24-hour holter monitoring, hypertension was defined as blood presure over 140/90 mmHg in more than two repeated measurments, while estimation of left ventricular hypertrophy was based on echosonography or ECG findings. Electrocardiography criteria included voltage criteria and repolarization abnormalities (S in V1 + R in V5-V6 $\geq$ 35 mm, R in aVL $\geq$ 11 mm), while echo criteria were based on measurment of left ventricular wall thickness at the end of diastole (LVEDd > 11 mm). Peripheral vascular desease was diagnosed performing doppler ehosonography or arteriography. Miocardial infarction was diagnosed by measuring the standard cardiospecific enzymes and cerebrovascular accident by computerized tomography.

The data from patients history included age, sex, causes of end stage renal disease (ESRD), duration of chronic heamodialysis programme, previous cardiovascular morbidity and the cause of death.

Genomic DNA was isolated from whole blood samples collected with EDTA by a salting out method (Miller et al. 1988). The 5A/6A promoter gene polymorphism at -1612 was detected by PCR-RFLP (Roden and Brown 2001). The MMP3 promoter region carrying the 5A/6A polymorphism was amplified from 100 ng of genomic DNA using oligonucleotide primers: forward, 5′-GAT TAC AGA CAT GGG TCAC-3′, and reverse 5′-TTT CAA TCA GGA CAA GAC GAA GTT T-3′. The PCR cycling conditions were as follow: initial denaturation for 2 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 57°C, and 30 s at 72°C with final extension at 72°C for 10 min. The reaction was performed with GeneAmp PCR System 2700, AB Applied Biosystem. The resulting 120-bp PCR product was digested with the fast digest restriction enzyme PdmI (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The digested product was subjected to gel electrophoresis (with 2% agarose gel) and visualized by ethidium bromide staining. The 5A allele possessing a recognition sequence 5′-GAA(N)4TTC-3′ for PdmI, cleaved the PCR products to 97-bp and 23-bp fragments. The PCR product derived from the 6A allele, containing 5′-GAA(N)5TTC-3′, was not digested with PdmI. The gels showed three PCR products after digestion of the restriction enzyme. As previously described, the 6A/6A genotype gave a 120-bp fragment, the 5A/5A genotype gave 97-bp and 23-bp fragments, and the heterozygous genotype gave three fragments.

This study was approved by the ethical committee of Zvezdara University Medical Center and patients gave written consent before participating in the study.

Statistical analysis has been performed using statistical power for social science (SPSS) software version 20 (IBM Corporation, New York, USA). Hardy-Weinberg equilibrium (HWE) was tested by Pearson's χ^2 test and all the data were consistent with HWE. Standard statistical analyses were performed in order to get measures of variability and central tendency. Association between genetic polymorphism and cardiovascular morbidity was tested by univariate and multivariate logistic regression analysis by χ^2 test and Fisher's exact test where appropriate. Odds ratio was calculated to estimate the risk for cardiovascular morbidity. Numeric values (patients' demographic characteristics) were analyzed by ANOVA. The 7-year overall and cardiovascular survival was analyzed by Kaplan Meier estimator. During the 7-year follow-up 12 patients were lost from the study, because of kidney transplantation, switch to peritoneal dialysis or changing the dialysis center. The results are shown in tables and graphs.

Results

Patients' general data are presented in Table 1, which shows that all patients of older age and had a high prevalence of cardiovascular diseases. The most frequent comorbid disease was hypertension, coronary artery disease and hyperlipoproteinemia, followed by left ventricular hypertrophy, heart arrhythmia, cerebrovascular disease and myocardial infarction.

Among the 198 hemodialysis patients, there were 142 (72%) 5A/6A heterozygotes, 12 (6%) 5A/5A homozygotes, and 44 (22%) 6A/6A homozygotes. Although there was no statistically significant difference, patients with 5A/5A genotype experienced higher risk for developing myocardial

Table 1. Patients' general data.

Age	62.3 ± 11.4
Sex	
Women	85 (42.9%)
Men	113 (57.1%)
Duration of hemodialysis in years	8.4 ± 5.2
Cause of kidney failure	
Hypertension	105 (53%)
Diabetes mellitus	26 (13%)
Glomerulonephritis	20 (9.5%)
Polycystic kidney disease	21 (10.5%)
Pyelonephritis chronica	16 (8%)
Endemic nephropathy	5 (2.5%)
Other	5 (2.5%)
Comorbidity	**N (%)**
Coronary artery disease	89 (45.4%)
Cerebrovascular accident	31 (15.8%)
Myocardial infarction	30 (15.3%)
Peripheral vascular disease	18 (9.2%)
Left ventricular hypertrophy	66 (33.8%)
Hyperlipoproteinemia	114 (58.2%)
Hypertension	178 (90.8%)
Heart arrhythmia	48 (24.5%)

infarction and cerebrovascular accident as compared with 6A homozygotes, and heterozygotes showed higher odds ratio for hypertension and peripheral vascular disease as compared with 6A homozygotes (Table 2). Odds ratio for the heterozygotes and homozygotes for 5A were determined in relation to the 6A/6A homozygotes whose OR has served as reference value (OR 1) in Tables 2 and 3. Protective effect of 5A allele of MMP3 gene was found for hyperlipoproteinemia, left ventricular hypertrophy and coronary artery disease (Table 3). There was no significant association between MMP3 gene polymorphism and cardiovascular morbidity (Table 4).

The all-cause mortality rate regarding MMP3 gene polymorphism is shown in Fig. 1. Heterozygotes had the highest 7-year survival rate (42%) and 5A/5A homozygotes had the lowest survival rate (33%). The CV mortality rate regarding MMP3 gene polymorphism during 7-year follow-up is shown in Fig. 2. The analysis showed that 6A/6A homozygotes had the highest CV survival rate (48%), while the lowest CV survival rate was found in 5A/5A homozygotes (33%).

Discussion

Results presented in this paper show that 5A/6A genotype is the most frequent in our group of hemodialysis patients. Patients with 5A allele of MMP3 gene have 1.3 folds higher risk to experience myocardial infarction, but 20% lower risk for coronary artery disease regarding 6A homozygotes. Several studies have shown that patients with 6A/6A genotype have higher incidence and more sig-

Table 2. High relative risk for developing cardiovascular morbidity regarding the 5A allele of MMP3 gene.

	5A/6A				5A/5A				6A/6A
	OR	95% CI		p	OR	95%CI		p	P
MI	0.779	0.158	3.831	0.758	1.324	0.245	7.149	0.754	0.494
CVA	0.919	0.342	2.474	0.868	3.425	0.763	15.397	0.108	0.146
PVD	1.784	0.193	16.447	0.610	0.938	0.111	7.954	0.953	0.488
HTA	1.333	0.125	14.219	0.812	0.985	0.117	8.313	0.989	0.901

The data were calculated in relation to 6A/6A genotype which was a reference value, OR 1.
MI, myocardial infarction; CVA, cerebrovascular accident; PVD, peripheral vascular disease; HTA, hypertension.

Table 3. Lower relative risk for developing cardiovascular morbidity regarding the 5A allele of MMP3 gene.

	5A/6A				5A/5A				6A/6A
	OR	95% CI		p	OR	95% CI		p	P
HLP	0.493	0.112	2.164	0.349	0.643	0.159	2.592	0.534	0.581
LVH	0.589	0.272	1.801	0.088	0.528	0.260	1.705	0.078	0.189
CAD	0.792	0.220	2.703	0.721	0.831	0.256	2.852	0.759	0.938

The data were calculated in relation to 6A/6A genotype which was a reference value, OR 1.
HLP, hyperlipoproteinemia; LVH, left ventricular hypertrophy; CAD, coronary artery disease.

Table 4. Incidence of cardiovascular disease regarding the MMP3 polymorphism.

CVD morbidity		MMP3		
		5A/5A	5A/6A	6A/6A
		N (%)	N (%)	N (%)
CAD	Yes	6 (50)	76 (53.5)	25(56.8)
	No	6 (50)	66 (46.5)	19 (43.2)
P		0.9		
MI	Yes	10 (83.3)	123 (86.6)	35 (79.5)
	No	2 (16.7)	19 (13.4)	9 (20.5)
P		0.6		
LVH	Yes	9 (75)	93 (65.5)	25 (56.8)
	No	3 (25)	49 (34.5)	19 (43.2)
P		0.3		
HLP	Yes	4 (33.3)	56 (39.2)	20 (45.5)
	No	8 (66.7)	86 (60.8)	24 (54.5)
P		0.6		
CVA	Yes	8 (66.7)	121 (85.2)	37 (84.1)
	No	4 (33.3)	21 (14.8)	7 (15.9)
P		0.3		
HTA	Yes	10 (83.3)	12 (8.5)	4 (9.1)
	No	2(16.7)	130 (91.5)	40(90.9)
P		0.9		

CVD, cardiovascular disease; MI, myocardial infarction; LVH, left ventricular hypertrophy; HLP, hyperlipoproteinemia; CVA, cerebrovascular accident; HTA, hypertension.

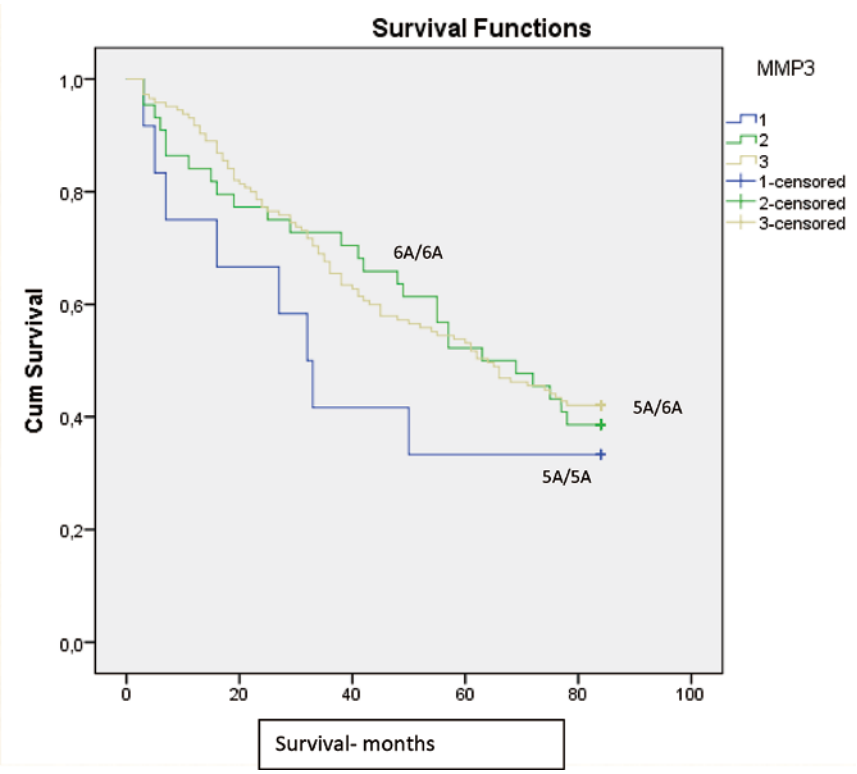

Fig. 1. Kaplan Meier analysis of overall outcome in hemodialysis population according to the MMP3 gene polymorphism. Line 1, 5A/5A; Line 2, 6A/6A; Line 3, 5A/6A.
Cum, cumulative; MMP3, matrix metalloproteinase 3.

nificant coronary artery stenosis than 5A homozygotes and heterozygotes (Ye et al. 1995, 1999; Beyzade et al. 2003). According to literature data, 6A homozygotes are more prone to develop coronary artery disease, while 5A homozygotes have the higher incidence of myocardial infarction. Similar studies have shown that the frequencies of the genotypes bearing the 5A allele are higher in patients with myocardial infarction (Terashima et al. 1999; Beyzade et al.

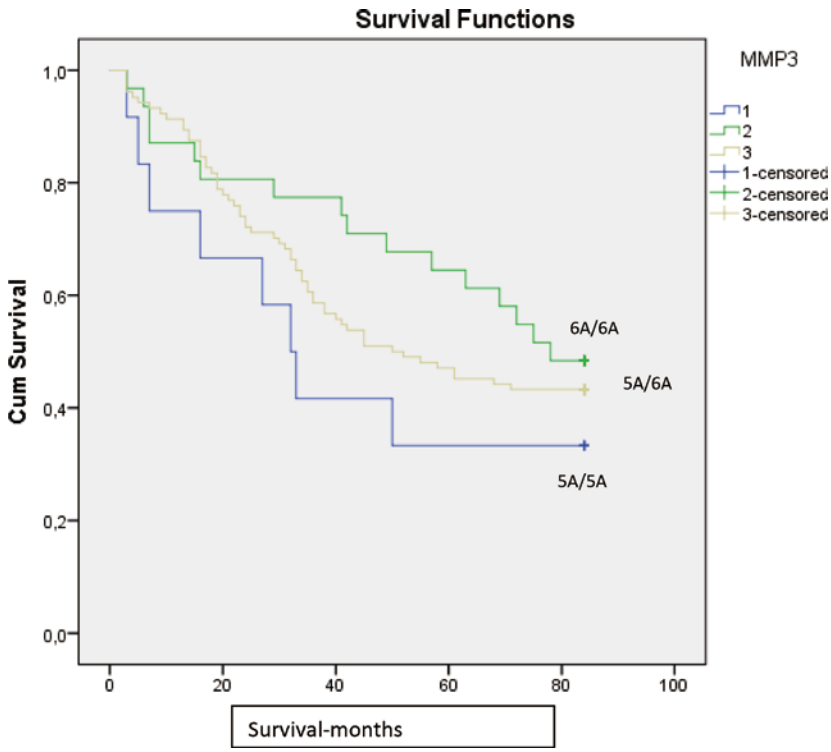

Fig. 2. Kaplan Meier analysis of CV outcome in hemodialysis population according to the MMP3 gene polymorphism. Line 1, 5A/5A; Line 2, 6A/6A; Line 3, 5A/6A.
Cum, cumulative; MMP3, matrix metalloproteinase 3.

2003; Ye 2006). Possible explanation is the different structure of atherosclerotic plaques. At one end of the spectrum are plaques rich in lipids and macrophages, which are commonly refered as lipid rich plaques, and at the other end of spectrum there are plaques rich in matrix proteins and smooth muscle cells, which are referred as fibrotic plaques. Plaques rich in lipids are prone to rupture causing myocardial infarction, while fibrotic plaques are more stable but bulkier (Davies 1996). Since MMP3 expression in vascular tissues is higher in individuals carrying 5A allele, a possible explanation for this finding is that in this individuals the higher enzyme activity causes lipid rich plaques, which are prone to rupture (Ye 2006). Individuals with 6A/6A genotype probably have higher rate of coronary artery stenosis due to lower enzyme activity and higher protein deposition which results in stable fibrotic plaques.

Humphries et al. (2002) studied MMP3 gene polymorphism in relation to restenosis in patients who underwent coronary balloon angioplasty and patients who had successful implantation of an endovascular stent. Patients with 6A/6A genotype showed higher incidence of restenosis after coronary balloon angioplasty, while the other genotypesdid not show this trend. The different findings in these two groups might reflect the different mechanisms underlying restenosis after baloon angioplasty and consequently instent restenosis. Arterial remodeling is the primary cause of restenosis after baloon angioplasty, whereas neointimal hyperplasia appears to be an important mechanism for instent restenosis (Mintz et al. 1996).

Our results show 3.4-fold higher risk for cerebrovascular accident in individuals with 5A allele. Three indipended studies have shown that 6A/6A genotype is associated with greater carotid intima-media thickness and higher rate of carotid stenosis (Nagase and Woessner 1999; Gnasso et al. 2000; Rauramaa et al. 2000). On the other hand, some studies have shown increased risk of stroke in 5A homozygotes (Flex et al. 2004). Although the mechanisms leading to the stroke are likely to be complex, the rupture of atherosclerotic plaque due to increased MMP3 expression may play a role.

There are similarities between the findings regarding coronary artery desease and cerebrovascular accidents. In both diseases 6A homozygotes are associated with higher rate of arterial stenosis, while 5A allele is associated with increased risk of acute incidents, such as stroke and myocardial infarction (Ye 2006).

Risk for development of peripheral artery disease in our group of patients was 1.8-fold higher in 5A allele carriers, which could be explained with the same model as in case of increased risk for myocardial infarction and cerebrovascular accident. Results also showed two-fold lower risk of hyperlipidemia in 5A carriers as compared with 6A homozygotes. Literature data did not show the influence of MMP3 gene on development of hyperlipidemia and this finding deserve further examination. Moreover, relative risk for left ventricular hypertrophy was two-fold lower in

individuals with 5A allele, which could be explained by higher deposition of proteins in extracellular matrix in 6A homozygote.

Our results showed that heterozygotes had 30% higher risk for hypertension. These findings are not in correlation with literature data about general population, which have shown the higher blood presure in 5A homozygotes. It was speculated that increased blood presure in individuals with 5A/5A genotype may be related to increased degradation of elastine in the blood wessel wall, leading to increased blood wessel stiffening (Foley et al. 1996; Beilby et al. 2005). Different findings in HD patients compared with the general population might be explained by multifactorial causes of high blood pressure in HD patients, including hypervolemia or inadequate salt excretion. In our study the 5A/5A homozygotes showed the lowest overall and CV survival rate, while 6A homozygotes showed the highest cardiovascular survival rate, which could be in correlation with all the findings that are addressed above.

This study suffers from several weaknesses. A better insight could be observed with incident but not prevalent patients. However, genetic milieu is constant and we believe that such analysis with prevalent patients is also justified. Despite revealed risk factor for particular cardiovascular abnormalities, we did not confirm any significant difference in cardiovascular morbidity according to MMP3 genetic polymorphism. We believe that it is the matter of numbers and this study needs to be confirmed in high number of dialysis patients which is usual for any conclusion concerning the interpretation of genetic influence on patients' outcome. In addition, the number of patients were limited, and we were also unable to include the control group for analyzing gene-gene interaction. Despite the mentioned shortcomings, however, the present study may enhance more detailed analyses in national (or regional)-based studies. In fact, the influence of genetic polymorphisms on cardiovascular mortality has not been sufficiently studied in dialysis patients, particularly in our region.

In conclusion, in this study, we show that MMP3 genetic polymorphism may play a role in the outcome of patients; 5A/5A homozygotes showed the lowest all-cause and cardiovascular survival. Identification of patients carrying high-risk genotypes may allow early preventive strategies and closer follow-up of appropriate target populations. We need longer follow-up in a larger group of patients to make a definitive conclusion about the influence of these gene polymorphisms on cardiovascular morbidity and its importance in daily clinical practice.

Conflict of Interest

The authors declare no conflict of interest.

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