Original Article

Impact of Angiotensin-converting Enzyme and Matrix Metalloproteinase-3 Gene Polymorphisms on Risk for Developing Vascular Access Failure in Hemodialysis Patients — A Pilot Study

Aleksandar Jankovic[1], Jelena Tosic[1], Ivana Buzadzic[2], Petar Djuric[1], Ana Bulatovic[1], Dragana Marković[1], Jovan Popovic[1], Nada Dimkovic[1,3]

Departments of [1]Nephrology with Dialysis and [2]Human Genetics and Prenatal Diagnostics, University Medical Center Zvezdara, [3]Medical Faculty, University of Belgrade, Belgrade, Serbia

Abstract

For adequate hemodialysis, functional vascular access is obligatory. Neointimal hyperplasia (NIH) has a central role in stenosis and thrombosis development, which represent the most frequent causes of vascular access failure. Polymorphism of different genes that have a significant role in endothelial function may have an impact on NIH development. Therefore, the aim of our study is to determine the effect of angiotensin-converting enzyme (ACE) I/D and matrix metalloproteinase-3 (MMP3) 5A/6A polymorphism on risk for developing vascular access failure in hemodialysis patients. The study included 200 patients on regular hemodialysis at Nephrology Department, University Medical Center Zvezdara. Retrospective analysis included a collection of general and vascular access data from medical records. Genetic analysis was performed by using polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP). Patients were divided into two groups: Group 1—patients who have never experienced vascular access failure and Group 2—patients who have at least one spontaneous vascular access failure. There was no difference in age, gender, hemodialysis vintage, main diagnosis, presence of hypertension, and diabetes mellitus between the two groups. There were no statistically significant differences in the frequencies of ACE and MMP3 genotypes between the two groups. Without statistical significance, it was found that homozygotes for I allele had two times higher risk for developing vascular access failure than homozygotes for D allele (OR 2.00; 95%CI: 0.727–5.503; P = 0.180). In addition, patients with 5A allele have 1.7 times higher risk for developing vascular access failure compared with patients without this allele (OR 1.745; 95% CI: 0.868–3.507; P = 0.118). Patients with vascular access failure do not have different genotype distribution regarding ACE gene and MMP3 gene polymorphism as compared with patients without vascular access failure. Still, homozygotes for I allele and homozygotes for 5A allele have higher risk for developing vascular access failure compared with other patients.

Keywords: *Angiotensin-converting enzyme gene polymorphism, failure, matrix metalloproteinase-3 gene polymorphism, vascular access*

Introduction

Functional vascular access (VA) is obligatory for adequate and long-lasting hemodialysis (HD). Arteriovenous fistula (AVF) is considered as the most reliable vascular access compared to arteriovenous graft (AVG) and tunneled vascular catheter (TVC) in regard to lower infection rate and longer lifespan.[1] Unfortunately, only 60% of created AVFs are functional after 12 months.[2-4] As stenosis and thrombosis of vascular access represent the most frequent causes of vascular access failure, numerous studies have tried to identify the risk factors for these events. It was described that neointimal hyperplasia (NIH) have central role in VA stenosis and thrombosis. This process is caused by vascular smooth muscle cell (VSMC) proliferation, cell migration from the media to intima, and subsequent proliferation in the intima.[5]

It was assumed that polymorphisms of different genes have a significant role in endothelial function and may have an impact on the development of NIH and consequently vascular access failure. I/D polymorphism in gene for angiotensin-converting enzyme (ACE) is characterized by the presence/absence of 287-bp sequence of DNA in intron 16 of ACE gene, and it generates the following three genotypes: II and DD homozygotes and an ID heterozygote.[6] The DD genotype is associated with higher Angiotensin

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Address for correspondence:
Dr. Aleksandar Jankovic, Dimitrija Tucovica 161, Belgrade, Serbia.
E-mail: sashajan223@gmail.com

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II (Ang II) level.[7] It was experimentally proven that higher Ang II level is linked to enhanced intima proliferation in injured arterial walls.[8] Numerous studies have been conducted to prove the effects of ACE gene polymorphism on vascular access failure, but contradictory results had been found.[9-13]

Matrix- metalloproteinases (MMPs) include at least 25 secreted or surface-bound proteases, of which 14 have been identified in vascular cells.[14,15] 5A/6A polymorphism in gene for MMP3 is characterized by the presence of 6A allele (generating fragments of 130-bp length) or 5A allele (generating shorter fragments of 110-bp length). Heterozygote 5A/6A contains both fragments.[16] Studies have shown that 5A allele has greater activity in gene expression, which results in higher enzyme levels in 5A homozygotes, intermediate in heterozygotes, and lowest in 6A homozygotes.[17] One study was conducted to analyze the effect of 5A/6A polymorphism on AVF patency, and it was concluded that the presence of 6A homozygote is associated with increased AVF failure.[16]

The aim of this study was to determine the influence of ACE I/D polymorphism and MMP3 5A/6A polymorphism on vascular access failure in hemodialysis patients.

Materials and Methods

Patients

The study included 200 patients on regular hemodialysis, three times per week on polysulfone membranes for more than 6 months in Nephrology Department, University Medical Center Zvezdara. Retrospective analysis included data collection from the patients' records regarding age, gender, main diagnosis, hemodialysis vintage, presence of hypertension, and diabetes mellitus and the number of vascular access they have had till the moment of genetic analysis. Patients have been divided into two groups: Group 1—patients who have never experienced vascular access failure (No. =111) and Group 2—patients who have at least one spontaneous vascular access failure (No. = 89). For all patients, we have performed polymerase chain reaction (PCR) for determination of polymorphisms. Data from medical records and from genetic analysis were compared between these two groups. Informed consents were obtained from subjects enrolled in the study. Procedures were in accordance with The Declaration of Helsinki.

PCR-RFLP

For DNA extraction, the whole blood with ethylenediamine tetraacetic acid (EDTA) was used (5mL of whole blood stored at +4°C not longer than 4 days, or longer at −20°C). The extraction was performed by the macro-method of genomic DNA isolation.[18] The method is based on cell lysis, which is commonly achieved by chemical and physical methods—blending, grinding, or sonicating the sample. Then, the DNA is precipitated by ice-cold ethanol or isopropanol. Genotyping was performed by the method of PCR. The PCR was carried out in small reaction tubes (0.2 ml volumes) in a thermal cycler. One cycle includes denaturation of DNA by heating the reaction to 94–96°C; annealing the primers to the single-stranded DNA template at the temperature of 50–65°C, and primer elongation—DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand at the temperature of 72°C. PCR consists of a series of 25–45 repeated cycles, followed by final product extension. To check whether the PCR generated the anticipated DNA fragment, agarose gel electrophoresis is employed for size separation of the PCR products. Positive samples are then used for further analysis by restriction enzymes (RFLP). Restriction enzymes can recognize and cut DNA wherever a specific short sequence occurs, in a process known as a restriction digest. The DNA sample is broken into pieces by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis. The size of PCR products is determined by comparison with a DNA ladder, which contains DNA fragments of known size, run on the gel alongside the PCR products.

Genotypization of I/D polymorphism in ACE gene

DNA fragment of 287 pb, which represents deletion fragment in introne 16 was amplified by the PCR method. In a process of genotyping I/D polymorphism of ACE gene, PCR products were fragments of 490 kb and 190 kb length. Moreover, by using insert-specific set of primers, the products were fragments of 335-bp length.

Reaction content volume was 2.5μL PCR buffer solution, 0.75 μL $MgCl_2$, 0.5 μL dNTP, 1μL primer I, 1 μLprimer II, 0.2 μLDNA polymerase, and 5 μL genomic DNA in the whole volume of 25 μL. Reaction was performed in GeneAmp PCR System 2700, AB Applied Biosystem.

Amplification consisted of 30 cycles, including denaturation at 94°C for 1 min, primers annealing at 58°C for1 min, and DNA extension/elongation at 72°C for 2 min. Fragments with no insertion (D allele) and other with insertion (I allele) of 190bp i 490bp, respectively, were detected using 2% agarose gel electrophoresis with ethidium bromide.

To enhance DD genotyping specificity, amplification was performed by using insert-specific set of primers in PCR conditions: 1 min of denaturation at 94°C, followed by 30 cycles with 30 s at 94°C, 45 s at 67°C (annealing), and 2 min at 72°C (extension). Only the presence of I allele is generating fragments of 335-bp length, which were identified by 3% agarose gel electrophoresis. Amplified sample with *Alu* sequence insertion represents a fragment of 490 bp (genotype II); whereas DNA fragment of 190 bp represents sequence deletion in allele

(genotype DD). Appearance of both fragments represents heterozygote (genotype ID).

Genotypization of 5A/6A polymorphism in MMP3 gene

For genotypization of 5A/6A polymorphism the PCR-RFLP method was used. Reaction content volume was as follows: 2.5 μL of PCR buffer solution, 0.75 μL of $MgCl_2$, 0.5 μL of dNTP, 1 μL of primer I, 1 μL of primer II, 0.2 μL of DNA polymerase, and 5 μL of genomic DNK in the whole volume of 25 μL. The PCR product of 130 bp length was digested by Rsy I restricting enzyme, which can recognize 5′-GACN↓ NNGTC-3′ sequence of DNA, which contains 5A allele. The presence of 6A allele only generates fragments of 130-bp length; whereas 5A allele generates shorter fragments of 110 bp length. Heterozygote 5A/6A contains both fragments.

Statistical analysis

Statistical calculations were performed using the SPSS 20.0 software program. The Kolmogorov–Smirnov test was performed for making assumptions about the distribution of data that were expressed as percentages for categorical values and mean values for continuous variables. Chi-square test, Student's *t*-test, or MannWhitney test was used to analyze the differences in various baseline variables between the groups of patients. Binary logistic regression analysis was used to analyze the influence of gene polymorphisms on previous vascular access failure (dichotomous variable was used: yes/no previous vascular access failure). A two-tailed *P* value <0.05 was considered statistically significant.

Results

There was no difference in age, gender, hemodialysis vintage, main diagnosis, presence of hypertension, and diabetes mellitus between the two groups of patients [Table 1]. Among all patients, 173 (86.5%) have AVF as their first vascular access and other 27 (13.5%) have AVG. There were no patients with TVC as the first vascular access.

Among 200 patients, 110 (55%) were heterozygotes regarding I/D polymorphism in ACE gene; there were 20 (10%) I homozygotes and 70 (35%) D homozygotes. Analysis of MMP3 5A/6A polymorphism revealed that 144 patients (72%) were heterozygotes, 12 patients (6%) were 5A homozygotes, and 44 patients (22%) were 6A homozygotes. Distribution of ACE and MMP3 gene polymorphism is presented in Table 2, and no significant differences were found between the two groups.

Table 3 shows the influence of ACE gene polymorphism on VA failure, and it was found that I homozygotes had two times higher odds ratio for developing VA failure than D homozygotes (*P* = ns). Similar odds ratio was observed for comparison of I homozygotes versus both heterozygotes and D homozygotes (*P* = ns). Regarding MMP3 gene polymorphism, 6A homozygotes have smaller risk for developing vascular access failure compared to 5A homozygotes. The presence of 5A allele did not show significant influence on developing VA failure, but patients with 5A allele have 1.7 times higher odds ratio for VA failure compared with patients without this allele [Table 4].

Discussion

As the endothelial dysfunction have a central role in NIH formation, numerous experimental and clinical studies tried to identify possible causes of this process and Brahmbhatt *et al.* in their review paper suggest that inflammation, uremia, hypoxia, and disturbed hemodynamics are major contributors.[19] Still, this process is not fully understood. Polymorphisms of different genes that have significant roles in endothelial function were examined regarding their effect in NIH formation and consequently in stenosis and thrombosis of vascular access.

Table 1: Patients' characteristics

Variable	Group 1	Group 2	*P**
No. of patients	111	89	
Age (X±SD), years	62±12	64±11	0.236
Gender (male/female)	68/43 (61.3%/38.7%)	46/43 (51.7%/48.3%)	0.197
HD vintage (X±SD), years	7.0±5.3	6.9±5.1	0.927
Main diagnosis			0.352
HTN	54 (48.6%)	37 (41.6%)	
DM	16 (14.4%)	9 (10.1%)	
ADPKD	13 (11.7%)	8 (9.0%)	
UA	15 (13.5%)	17 (19.1%)	
CGN	11 (9.9%)	13 (14.6%)	
UK	2 (1.8%)	5 (5.6%)	
HTA presence (yes/no)	63/48 (56.8%/43.2%)	46/43 (51.7%/48.3%)	0.479
DM (yes/no)	19/92 (17.1%/82.9%)	11/78 (12.4%/87.6%)	0.427

HTN: Hypertensive nephropathy; DM: Diabetes mellitus; ADPKD: Autosomal dominant polycystic kidney disease; UA: Urological abnormalities; CGN: Chronic glomerulonepritides; UK: Unknown; X: Mean; SD: Standard deviation. *According to χ^2 test, Students' *t*-test or Mann-Whitney test where appropriate

Table 2: ACE and MMP3 gene genotypes in two observed groups

	Group 1 111	Group 2 89	*P*
ACE polymorphism (I/D)			0.339
I/I	8 (7.2%)	12 (13.5%)	
I/D	63 (56.8%)	47 (52.8%)	
D/D	40 (36.0%)	30 (33.7%)	
MMP3 polymorphism (5A/6A)			0.266
5A/5A	7 (6.3%)	5 (5.6%)	
5A/6A	75 (67.6%)	69 (77.5%)	
6A/6A	29 (26.1%)	15 (16.9%)	

Table 3: Influence of ACE gene polymorphism on developing vascular access failure

ACE polymorphisms	OR	CI 95%	*P*
I/I vs. D/D	2.000	0.727-5.503	0.180
I/I vs. I/D+D/D	1.963	0.924-4.168	0.079

Table 4: Influence of MMP3 polymorphism on developing vascular access failure

MMP3 polymorphisms	OR	CI 95%	*P*
6A/6A vs. 5A/5A	0.724	0.196-2.673	0.628
5A/5A + 5A/6A vs. 6A/6A	1.745	0.868-3.507	0.118

We retrospectively analyzed the influence of ACE gene polymorphism on vascular access failure. We have shown that there is no difference in distribution of II, ID, and DD genotypes among patients with/without vascular access failure. However, I homozygotes had two times higher odds ratio for developing vascular access failure than D homozygotes and heterozygotes. Other studies that have analyzed the effects of ACE gene polymorphism on vascular access failure have shown contradictory results. Sener *et al*.[9] analyzed the distribution of I/D polymorphisms in ACE gene in three groups of patients: 47 hemodialysis patients who had AVF failure, 51 hemodialysis patients who have never had AVF thrombosis, and 40 healthy control subjects. There was no statistically significant difference in gene polymorphism distribution between two hemodialysis groups. They concluded that I/D heterozygotes have 2.67-fold higher risk for developing AVF thrombosis.In our study, patients with II genotype have two times higher risk for vascular access failure, but both results did not reach statistical significance. Differences in results could be explained in different distribution of gene polymorphisms between our study groups. Namely, heterozygotes were the most frequent in our groups; whereas, in their group of patients, it was DD genotype (68% in thrombosis group and 8% in non thrombosis group). More similar to our distribution of ACE gene polymorphism was found in the study conducted by Heine *et al*.[11] They also did not find significant difference in ACE gene polymorphism between groups who experienced/not experienced AVF failure. Namely, they prospectively followed AVF failure in 137 incident hemodialysis patients with newly created AVF. During follow-up period, they registered 70 AVF failures. Moreover, AVFs in II homozygotes have the best 12-month and 24-month survival as compared with other genotypes but without statistical significance. This is not in accordance with our results where the presence of I allele leads to almost two times higher risk for developing VA failure also without statistical significance. This study and German study are different, because they have followed their patients prospectively and we have conducted retrospective analysis. Isbir *et al*.[10] followed thrombosis rate in femoral AVGs in the small group of patients (12 with thrombosis vs. 18 w/o thrombosis), and they stated that heterozygotes (ID genotype) in ACE gene experienced the most frequent and statistically significant thrombosis in comparison with other genotypes. These results are similar with those presented by Sener *et al*.[9] In the study performed by Moon *et al*.,[12] AVFs survival was followed in 155 hemodialysis patients for 58 months (mean). Genotype distribution was comparable to ours. Namely, they had 25% II, 57% ID, and 18% DD genotypes, but conclusion was completely different. This is because they revealed that D homozygotes have 2.45 higher hazard ratios for developing AVF failure with statistical significance. Similarly, statistically significant results were presented by Güngör *et al*.[19] This retrospective study with 520 hemodialysis patients revealed that D homozygotes have 4.25 times higher risk for developing AVF thrombosis compared to II and ID genotypes. The data by Moon *et al.* and by Grungor *et al*. are difficult to compare with our data due to different study designs. The most recent study performed by Chen *et al*.[13] with large study population (154 patients with AVF malfunction vs. 423 patients w/o AVF malfunction) have concluded that none of the analyzed genotypes (II, ID, DD) was linked to AVF malfunction. This study was similar to ours regarding the study design and patients' characteristics and results are in accordance.

Among the factors leading to endothelial dysfunction, MMPs are associated with VSMC migration and the degradation of extracellular matrix, which may contribute to the development of NIH.[15] The influence of 5A/6A polymorphism in MMP3 gene was examined by Lin *et al*.[15] In this retrospective study with 596 patients (426 w/o AVF failure vs. 170 with AVF failure), it was shown that 6A/6A genotype is the most frequent in both groups and these patients have statistically significant risk (hazard ratio HR 1.712) for developing AVF failure. Our results were different regarding distribution of gene polymorphisms and risk for developing vascular access failure. Namely, in our study group, 5A/6A genotype was the most frequent in both groups, and patients with 5A allele had 1.745 higher odds ratio for developing vascular access failure. This discordance could be explained in different distribution of genotypes. In addition, the results from both studies

could be explained with previous experimental and clinical data, as there is evidence that 5A homozygotes are more prone to experience acute cardiovascular events due to higher degradation of extracellular matrix, whereas 6A homozygotes have higher risk for accelerated atherosclerosis as lower enzyme activity results in high deposition of matrix proteins.[16,20] Vascular access failure represents specific combination of chronic process of NIH and acute thrombus formation.

Limitations of our study are its' retrospective design and limited number of patients. However, genetic milieu is constant since the birth. In addition, conclusion based on genetic analysis should involve higher number of patients. Still, the differences in the literature data show that further studies in the field are justified with aim to conclude the role of genetic milieu on VA failure. The findings may have potential impact on planning of suitable renal replacement therapy in those patients who have less desirable genotypes.

In conclusion, we have determined that patients with and without VA failure do not have different genotype distribution regarding I/D polymorphism in ACE gene and 5A/6A polymorphism in MMP3 gene. Without statistical significance, binary logistic regression revealed that I homozygotes have two times higher odds ratio than D homozygotes and patients with 5A allele have 1.7 times higher odds ratio for developing vascular access failure compared with patients without this allele.

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Conflicts of interest

There are no conflicts of interest.

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