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ORIGINAL ARTICLE

MATRIX METALLOPROTEINASE-2 (*MMP-2*) AND-9 (*MMP-9*) GENE VARIANTS AND MICROVASCULAR COMPLICATIONS IN TYPE 2 DIABETES PATIENTS

Andjelic Jelic M*[1], Radojkovic D[2], Nikolic A[2], Rakicevic Lj[2], Babic T[2], Jelic D[3], Lalic NM[4]

*Corresponding Author: Marina Andjelic Jelic, Department of Endocrinology, Diabetes and Metabolic Diseases, Clinical Medical Centre Zvezdara, Dimitrija Tucovica 161, 11000 Belgrade, Serbia, Phone +381 64 122 2010 Fax +381 11 3088733, E mail: drmajelic@gmail.com

ABSTRACT

Vascular complications are the leading cause of increased morbidity and mortality of diabetic patients. It has been postulated that matrix metalloproteinases *MMP-2* and *MMP-9*, zinc-dependent endopeptidases through remodeling of the extracellular matrix, can contribute to the onset and progression of diabetic vascular complications. The aim of our study was to assess whether there is a major difference in single nucleotide polymorphisms in the *MMP-2* (at position -1306C>T) and *MMP-9* (at position -1562C>T) gene in type 2 diabetic patients and healthy controls and to determine whether there is an association of these gene variants with the presence of microvascular complications in diabetic patients. Our study included 102 type 2 diabetes patients and a control group which was comprised of 56 healthy controls. All diabetic patients were screened for microvascular diabetes complications. Genotypes were detected by polymerase chain reactions followed by restriction analyses with specific endonucleases and their frequencies were determined. The *MMP-2* variant -1306C>T showed a negative correlation with type 2 diabetes (p=0.028). It was also shown that the presence of the -1306C allele increases the probability of developing type 2 diabetes. This was a 2.2 fold increase and that the -1306 T allele has a protective role in regards to type 2 diabetes. The *MMP-2* variant -1306T showed a negative correlation with diabetic polyneuropathy (p=0.017), meaning that allele-1306T has a protective role in regards to diabetic polyneuropathy while the presence of allele -1306C increases the probability of developing diabetic polyneuropathy by 3.4 fold. Our study showed that the *MMP-2* gene variant (-1306C) doubles the risk of developing type 2 diabetes, and for the first time an association of this gene variant and the presence of diabetic polyneuropathy was shown.

Key words: diabetes mellitus; matrix metalloproteinase-2 and-9; single nucleotide polymorphism; diabetic polyneuropathy.

INTRODUCTION

Globally, diabetes mellitus (DM) is a growing medical and social problem. It is estimated that the number of people affected by diabetes will rise from 463 million in 2019 to 700 million by 2045 [1]. Vascular complications lead to increased morbidity and mortality of these patients. The incidence is growing of diabetic microvascular complications, including diabetic retinopathy (DR), diabetic polyneuropathy (DPN), and diabetic nephropathy (DN) [2-4]. The pathological mechanisms that are responsible for developing these complications are very complex and not fully understood. It seems that genetic susceptibility plays a role in developing such complications [5]. It has been postulated that zinc-dependent endopeptidases called matrix metalloproteinases (MMPs), are very important in remodelling the extracellular matrix, and therefore they have an impact on the onset and progression of diabetic vascular complications [6-7]. The regulation of MMPs is very complex, depending not only on the regulation of their tissue inhibitors, but also on the regulation of MMP gene expressions by transcriptional factors and epigenetic modifications [8]. Matrix metalloproteinase-2 (*MMP-2*)

[1] Department of Endocrinology, Diabetes and Metabolic Diseases, Clinical Medical Centre Zvezdara, Belgrade, Serbia

[2] Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia

[3] Faculty of Dentistry Pancevo, Serbia

[4] Clinic for Endocrinology, Diabetes and Metabolic Diseases, Clinical Centre of Serbia, Faculty of Medicine, University of Belgrade, Belgrade, Serbia

and matrix metalloproteinase-9 (*MMP-9*) are enzymes which are responsible for the degradation of the basal membrane through collagen IV cleveage and therefore have a role in angiogenesis.

Diabetic retinopathy is a very common microvascular diabetic complication, affecting up to one third of patients with diabetes [9-10]. It can lead to seriuous vision impairments and vision loss, especially in the adult working population [11]. The duration of diabetes and poor metabolic control, as well as elevated blood pressure can contribute to the development of DR [12-14]. Genetic factors also seem to have an important role, and they account for 25% to 50 % of the risk of developing DR [15]. The role of *MMP-2* and *MMP-9* is recognized even in the early phases of DR [16] as well as in the latter stages or in the proliferative retinopathy [17-18]. In the pathogenesis of DR, activated MMPs cause the damage of mitochondria and augment retinal capillary cell apoptosis [19]. Only a few studies regarding the MMPs gene single nucleotide polymorphism (SNP) studies have been completed, and the results of their correlation with DR are conflicting [20-21].

Diabetic polyneuropathy (DP) is the most prevalent diabetic complication which affects almost half of the patients with diabetes [22]. It can lead to major disability due to foot amputation, and DP significantly reduces the quality of life [23]. It has been shown that MMPs and their inhibitors may have a role in the process of the demyelization and the regeneration of axons [24-25]. *MMP-2* and *MMP-9* play a critical role in the pathogenesis of DP [26].

A single genetic study has shown that SNP -1562C>T in the promoter of the *MMP-9* gene is seen in patients with diabetic foot ulcers and that increased *MMP-9* production from the high expressing T allele may promote matrix degradation [27].

Diabetic nephropathy (DN) develops in 20-40 % patients with diabetes [28] and is the leading cause of end-stage renal disease [29]. Genetic studies regarding the association of MMP gene polymorphism and DN provide conflicting results. Some show that certain polymorphisms of *MMP-9* may be predictive factors in the development of diabetic nephropathy [30] and some suggest the protective role of the T allele SNP - 1562C>T of *MMP-9* against diabetic nephropathy [31, 32]. It has also been shown that *MMP-2* gene polymorphism is associated with susceptibility and disease progression of DN [33].

The objectives of our study were to assess the frequencies of the *MMP-2* gene variant NC_000016.9:g.55511806C>T (-1306C>T) and *MMP-9* variant NC_000020.10:g.44635976C>T (-1562C>T) in type 2 diabetes patients and healthy subjects and to study whether there is a possible association with the presence of microvascular complications in type 2 diabetes patients.

MATERIAL AND METHODS

Study design, time and place

This study contained 102 subjects with type 2 diabetes who were at the Zvezdara University Medical Center from February 2016 to April 2018. The diagnosis of DM was established using the criteria of the World Health Organization (WHO). The presence of microvascular diabetes complications was investigated in all patients on the basis of medical history, laboratory analysis and physical examination. Detailed ophthalmological examination and fundoscopy were done in order to confirm retinal changes due to diabetes. Classification was done to distinguish between nonproliferative retinopathy and proliferative retinopathy. The diagnosis of nephropathy was established based on urine albumin excretion in 24h urine. The cut off value was 30 mg/24h. A comprehensive diabetic foot examination comprising of visual inspection, monofilament examination, pinprick sensation, and ankle reflexes was performed in order to establish the diagnosis of diabetic polyneuropathy.

The control group was comprised of 56 healthy subjects who were recruited during their regular annual health assessments. The local Ethics Committee gave permission to conduct the study and each participant signed informed consent.

Variable

Apart from demographic variables (gender and age), additional information regarding the duration of diabetes, antidiabetic medications, and smoking history was collected. Anthropometric variables included measurements of height, weight, and body mass index (BMI). The BMI was calculated according to the following formula: BMI (kg/m2) = body weight (kg)/ height (m2).

Metabolic variables included measurements of fasting blood glucose (FBG), total cholesterol, high density lipoprotein cholesterol, low density lipoprotein cholesterol, total triglycerides, serum creatinine, and glycosylated hemoglobin (HbA1c). Peripheral blood samples were taken from the patients with type 2 diabetes and the control subjects after overnight fasting for at least 8 hours and metabolic parameters were analyzed using the biochemical analyzer Olimpus AU680. Detection of the *MMP-2* and *MMP-9* gene variants was performed using polymerase chain a reaction-restriction fragment length polymorphism (PCR-RFLP) analysis [34]. The region containing the *MMP-2* variant -1306C>T was amplified using a forward primer 5'-CTTCCTAGGCTGGTCCTTACTGA-3' and a reverse primer 5'-CTGAGACCTGAAGAGCTAAAGAGCT-3'. The region containing the *MMP-9* variant -1562C>T was amplified using a forward primer 5'-GCCTGGCACAT-

AGTAGGCCC-3' and a reverse primer 5'-CTTCCTAGC-CAGCCGGCATC-3'. Amplification of both regions was performed by PCR reaction directly from blood using the following program: 3 cycles at 98°C for 5 min and 55°C for 3 min; 95°C for 5 min; 35 cycles at 95°C for 30 sec; 58°C for 45 sec; and 72°C for 45 sec; and 72°C for 10 min. The PCR products obtained with primers for *MMP-2* (193bp) were incubated with the restriction endonuclease BfaI (Thermo Scientific, Waltham, MA, USA) at 37°C overnight, resulting in either a 193bp fragment containing the -1306C allele or 164bp and 29bp fragments in the presence of the -1306T allele. The PCR products obtained with primers for *MMP-9* (436bp) were incubated with the restriction endonuclease SphI (Thermo Scientific, Waltham, MA, USA) at 37°C overnight, resulting in a 436bp fragment containing the -1562C allele or 194bp and 242bp fragments in the presence of the -1562T allele. Alleles were separated on agarose gel electrophoresis and visualized with ethidium-bromide staining and UV transillumination.

STATISTICAL METHODS

Statistical analysis was performed using Statistical Package for Social Sciences 20.0 (SPSS Inc., Chicago, Illinois, USA). Descriptive statistics were computed as mean values and standard deviations for continuous variables, and as absolute frequencies and percentage values for categorical variables.

The association between the *MMP-2* (-1306 C>T) and *MMP-9* (-1562 C>T) variants with disease incidence was examined according to the dominant genetic model (carriers of *wild type* genotype vs. *heterozygous* and *homozygous* carriers), using Pearson's chi-squared test ($\chi 2$). The measure of the strength of the association between genotypes and disease incidence was expressed as an Odds Ratio with a confidence interval of 95% CI (Confidence Interval). A p-value less than 0.05 was considered statistically significant.

RESULTS

The study population included 102 diabetic patients, 65 men and 37 women, aged 61.4 ± 7.3 years with a mean BMI 28.8±4.8kg m2. The mean duration of diabetes was 10.1±5.8 years. Most of the patients were on metformin (86.3 %), 22.5 % on oral antidiabetic drug (OAD), 13.7 % on insulin and 63.7 % on an OAD/insulin combination. The basic demographic features of patients with diabetes are shown in the Table 1. The control group included 56 patients who did not differ in basic demographic characteristics.

Table 1. Demographic and clinical characteristics of patients.

Variable	Values
Age, mean± SD	61.4 ± 7.3
Gender, male n (%) female n (%)	65 (63.7) 37 (36.3)
Smokers, n (%)	23 (22.5)
Diabetes duration, mean± SD	10.1±5.8
BMI, mean± SD	28.8±4.8
HbA1c, mean± SD	9.40±2.22
FBG, mean± SD	6.90±1.94
Total cholesterol (mmol/l), mean± SD	5.20±1.35
LDLc (mmol/l), mean± SD	3.11±1.03
HDLc (mmol/l), mean± SD	1.15±0.30
Triglycerides (mmol/l), mean± SD	2.25±1.16
Albumin mg/24h/urine, mean± SD	12.54±2.79
Serum creatinine (μmol/l), mean± SD	87.81±42.75
OAD only, n (%)	23 (22.5)
Insulin only, n (%)	14 (13.7)
OAD + insulin, n (%)	65 (63.7)
Metformin, n (%)	88 (86.3)
Retinopathy non-proliferative, n (%)	21 (20.6)
Retinopathy proliferative, n (%)	9 (8.8)
Polyneuropathy, n (%)	40 (39.2)
Nephropathy, n (%)	11 (10.8)

In this study, the *MMP-2* variant -1306C>T and the *MMP-9* variant -1562C>T were analyzed in diabetic patients and in healthy controls. The distribution of genotypes obtained for both variants in the patients with diabetes and the controls is presented in Table 2.

Table 2. Genotype distrubution of the *MMP-2* and *MMP-9* gene variants in patients with diabetes and in the control group.

Genotype	Patients n (%)	Controls n (%)	P-value OR (95 % CI) patients vs. control *
MMP-2			
-1306CC	53 (67.1)	27 (48)	0.028
-1306CT	24 (30.4)	25 (45)	0.457 (0.226-0.932)
-1306TT	2 (2.5)	4 (7)	
MMP-9			
-1562CC	56 (71)	40 (71)	0.945
-1562CT	20 (25)	16 (29)	1.27 (0.482-2.187)
-1562TT	3 (4)	0 (0)	

* The dominant model of inheritance was examined (carriers of -1306CC genotype vs. carriers of -1306CT or -1306TT genotype; carriers of -1562CC genotype vs. carriers of -1562CT or -1562TT genotype).

Based on data given in table 2, the frequencies of the -1306C and -1306T alleles in the group of diabetic patients are 0.82 and 0.18, respectively. In the control group, the frequencies of these alleles are 0.71 for -1306C and 0.29 for -1306T. In the group of diabetic patients, the frequencies of -1562C and -1562T alleles are 0.84 and 0.16, respectively; in the control group their frequencies are 0.86 and 0.14, respectively.

For the analyzed alleles, Hardy-Weinberg equilibrium testing was performed. The testing showed that the p value of Pearson's Chi-Square test for *MMP-2* alleles was 0.71 in the group of patients and 0.58 in the control group. For *MMP-9* alleles, the p value of Pearson's Chi-Square test was 0.48 and 0.21 in the group of diabetic patients and in the control group, respectively.

The association of *MMP-2* and *MMP-9* alleles with diabetes and microvascular complications was investigated in a dominant model of inheritance.

Genotype frequencies of the *MMP-9* variant -1562C>T between the patients and control group were not significantly different, as was neither the difference in the C nor T allelic frequencies between the control group and patients with type 2 DM.

The *MMP-2* variant -1306C>T showed a negative correlation with type 2 diabetes (p=0.028). The presence of the -1306C allele increases the probability of developing type 2 diabetes 2.2 times, while the -1306 T allele a has protective role.

In order to assess the impact of both variants on microvascular complications, the alleles' frequencies were correlated with the presence of diabetic retinopathy, polyneuropathy, and nephropathy in patients with type 2 diabetes. The *MMP-9* variant 1562C>T showed no association with any microvascular complications. The *MMP-2* variant -1306T showed a negative correlation with diabetic polyneuropathy (p=0.017, Table 3). The presence of the -1306C allele increases the probability of developing diabetic polyneuropathy 3.4 times, while the -1306T allele has protective role.

The *MMP-2* variant 1306C>T showed no association with the presence of diabetic retinopathy or diabetic nephropathy.

DISCUSSION

In our study, our primary goal was to assess the possible differences in *MMP-2* and *MMP-9* gene polymorphism between patients with type 2 diabetes and healthy controls. Our results showed that the presence of the *MMP-2* allele -1306C nearly doubles the risk of developing type 2 diabetes mellitus, while the -1306T allele provides protection against this disease. This correlates with the results of Sarray et al., who, for the first time, showed an association of the *MMP-2*-1306C>T gene variant with the susceptibility to developing type 2 diabetes, therefore confirming the protective effect of the T allele. [35]

To our knowledge, this is the first study investigating *MMP-2* and *MMP-9* genetic variants and all three microvascular complications.

Regarding DR and a possible genetic background, Singh K. et al. postulated that the functional SNP -1562C>T in the promoter of the *MMP-9* gene plays a very important role in developing proliferative DR due to the elevated *MMP-9* production from the high expressing T allele leading to retinal angiogenesis [20].

A statistically significant difference was observed in both allele and genotype distributions, but only between patients with proliferative retinopathy versus healthy controls with no DM. There were, however, no significant differences between patients with DM, no matter if they have DR or not. This is the same result as our study. Due to the limited number of patients with proliferative retinopathy we were not able to establish if there is a significant difference between these patients and healthy controls.

In another study, Beranek M. et al. showed that plasma levels of *MMP-2* were significantly higher in patients with proliferative DR who were carriers of the -1306 CC or the -1306 CT genotypes [21]. In Beranek's study, no major differences were found among the groups (diabetics with non-proliferative DR, diabetics with proliferative DR, and healthy controls) when comparing the genotype distribution for MMP-2-1306C>T and MMP-9-1562 C>T variants. This is consistent with our results.

MMPs are regarded as very important players in the development of diabetic polyneuropathy, not only by caus-

Table 3. Correlation of the *MMP-2* variant -1306T with the presence of diabetic polyneuropathy.

Genotype	Patients n (%)	Controls n (%)	p-value or (95 % CI)
-1306CC	22 (75.86)	27 (48.21)	0.0170* 0.2962 (0.1091-0.8045)
-1306TC and -1306TT	7 (24.14)	29 (51.79)	

ing extracellular matrix abnormalities but also by causing neuronal injury which leads to the development of neuropathic pain. So far, no genetic study has been performed regarding MMP gene variants and the presence of DPN. Our results showed that the presence of the *MMP-2* allele -1306C increases the probability of developing diabetic polyneuropathy by a factor of 3.4 times. This can be potentially used as genetic marker for detecting those diabetic patients who are at risk of developing this threatening vascular complication. Of course, we need more extensive studies to confirm this finding.

We found no correlation between either *MMP-2* or *MMP-9* gene variants and diabetic nephropathy. This was probably due to the limited number of patients with this complication in the study group.

In conclusion, our study showed that the variant -1306C>T in the *MMP-2* gene doubles the risk of developing type 2 diabetes, and we showed, for the first time, an association of this gene variant and the presence of diabetic polyneuropathy. As matrix metalloproteinases play an important role in the development of vascular diabetic complications, future studies with a larger number of patients are needed so as to verify variants in the genes for MMPs as markers for diabetic complications .

Declaration of Interest. The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article.

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