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MMP2 Gene Polymorphisms and *MMP2* mRNA Levels in Patients with Superficial Varices of Lower Extremities

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Abstract. *Background: Although superficial varices of lower extremities with high morbidity are common, their etiology has not been elucidated yet. Previously, it was thought that venous hypertension was responsible for such cases by causing valvular insufficiency, but recent findings indicate that the changes in the venous wall structure might be main initiating factors. Matrix metalloproteinase enzyme-2 (MMP2) is one of the enzymes known to have functions in remodelling of the extracellular matrix mainly in vascular structures. Materials and Methods: We studied two functional gene polymorphisms in -735 and -1306 regions of matrix metalloproteinase enzyme-2 (MMP2) gene, and their effects on mRNA expression of MMP2. We used a previously defined (PCR-RFLP) method for polymorphism analyses. Results: CC genotype and C allele for MMP2 -735 gene region were more common in the control group and there was no significant difference between groups for MMP2 -1306 gene polymorphisms. MMP2 mRNA levels were higher in the group that had both varices and coronary artery disease (CAD). Conclusion: There was no significant effect of MMP2 polymorphisms on mRNA expression. As MMP2 mRNA levels were higher in varices patients with CAD compared to the CAD only and varices only groups, it is necessary to make advanced researches to elucidate the relationship between CAD and varices.*

The elasticity and distensibility of venous walls are determined by extracellular matrix proteins, collagen and elastin (1). The extracellular matrix of veins contains several kinds of collagens, elastin, proteoglycans and glycoproteins that are important for vascular structures and cellular homeostasis (1, 2). Vascular modeling is known to be regulated by combined effects of several growth factors, cellular adhesion molecules and specific proteases (3).

Varicose veins are defined as "swollen, tortious veins" and can exist in different levels of seriousness, telangiectasia, valvular insufficiency, spider veins (2, 4, 5).

Varicose veins are supposed to be found in 40% of males, 51% of females; but in reality, those ratio are known to be variable in a large range, 2-56% for men, 1-73% in women (1, 2, 6). There are several risk factors as family history, advanced age, standing duration and history of deep vein thrombosis (DVT) (1, 4, 7). Smoking, oral contraceptive pills, hypertension and diabetes are important risk factors in development of varices (1, 4). Although the reasons for varices formation are not clear yet, the hypothesis is that individual structural extravascular matrix differences causing generation of varices (2).

Type-I collagen was found to be higher, while in contrast, type-III collagen was lower in varicose veins compared to healthy veins, and there were also damaged molecular interrelations between collagens and elastin (2). Normally, because of its elasticity, elastin provides strength against the volume load of the veins (2). In varicose veins elastin is found to be obviously decreased and fragmented, and irregular structures are created in proximal to the affected area (2, 8). These extracellular matrix differences are thought to result from the activity of remodelling enzymes, matrix metalloproteinases (MMPs) (2, 5, 9, 10).

Previously, it was thought that MMP1 and MMP2 activities might be responsible for the acceleration of matrix turnover in venous leg ulcers (9). It is also known to be responsible for degradation of gelatin, fibronectin, laminin and several kinds of collagens (11, 12). In another study, it was suggested that MMP2 causes venous dilatation, chronic

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Table I. *Primers and probe sequences used in the study of MMP2 RNA.*

	MMP2	HGPRT
Primer (left)	5-ATACCTGGATGCCGTCGT-3	5-TGACCTTGATTTATTTTGCATACC-3
Primer (right)	5-AGGCACCCTTGAAGAAGTAC-3	5-CGACAAGACGTTCAGTCCT-3
Probe	5-FAM-GGCGGCGG-3-dark quencher	5-FAM-GCTGAGGA-3-dark quencher

venous insufficiency and varicose veins (13). Normally, MMP2 is an enzyme known to be expressed constitutively (3, 14). More than one kind of polymorphism has been defined in the promoter region of the MMP2 gene; one is *MMP2* -1306 C/T polymorphism with its decremental effect on promoter activity and some RNA transcription (11, 15, 16, 17). Similarly, *MMP2* -735 C/T polymorphism was also found to provide lower promoter activity and decreased mRNA expression; DNA-protein complexes were reduced in T allele cases (18, 19). *MMP2* mRNA levels were also found to be decreased in varicose veins compared to normal veins (1), but in contrast, in some other studies MMP2 expression and activity were found to be increased (9, 20). In this study, we aimed to determine the effects of *MMP2* -1306 C/T and -735 C/T polymorphisms on the formation of varices and also evaluated the effects of these polymorphisms on *MMP2* mRNA expressions.

Materials and Methods

Participants. Patient and study groups were provided from Marmara University, Department of Cardiovascular Surgery. Patient group contained of the subjects with varicose veins, and control group without any varicose lesions on legs. The patient group was also divided into two separate groups: the first group contained cases operated for varicose veins on legs and the second group consisted of non-operated leg varicose vein patients. MMP2 mRNA levels were determined in the waste varicose vein material of this operated group and compared with a control group of coronary bypass patients using a part of their healthy saphenous vein used for bypass operation. For these *MMP2* mRNA studies, the tissue specimen were immediately placed in the liquid nitrogen containers in specimen tubes and mRNA was isolated as soon as possible after their arrival at the laboratory. Meanwhile, blood specimens were also taken from all subjects to isolate DNA materials immediately to evaluate *MMP2* polymorphisms. All study groups were informed about the study and agreed to participate.

Methods of genotyping. A previously described method was used for *MMP2* -735 gene region determination (21). The primers for polymerase chain reaction (PCR) for this region were: Forward: 5'-ATAGGGTAAACCTCCCCACATT-3' and reverse: 5'- GGTAAAAT GAGGCTGAGACCTG-3'. To amplify *MMP2* -735 region, 25 μl PCR mixture was prepared containing approximately 100 ng of template DNA, 0.5 μl of each primer, all four deoxyribonucleoside 5' triphosphates (each at 0.2 mM), 2.5 mM $MgCl_2$ and 1 U Taq polymerase in 1X reaction buffer (Fermentas, Lithuania). PCR conditions were: an initial melting step of 3 min at 95˚C; followed by 35 cycles of 45 s at 95˚C, 60 s at 60˚C and 80 s at 75˚C; and a final elongation step of 7 min at 72˚C. The restriction endonuclease HinfI was used to determine the *MMP2* -735 gene region polymorphism. The products of this reaction were separated on agarose gels containing ethidium bromide. *HinfI* digestion generated 300 bp fragment for *MMP2* -735C allele and two 254 bp and 46 bp fragments for *MMP2* -735 T allele.

A previously described method was used to amplify the *MMP2* -1306 gene region to evaluate the polymorphism in this region (18). The primers used for this procedure were: Forward: 5'-CTTCCTAGGCTGGTCCTTACTGA-3' and reverse: 5'-CTGAG ACCTGAAGAGCTAAAGAGCT-3'. Total reaction mixture for PCR amplification was 25 μl containing about 100 ng of template DNA, 0.5 μl of each primer, all four deoxyribonucleoside 5' triphosphates (each at 0.2 mM), 2.5 mM $MgCl_2$ and 1 U of Taq polymerase in 1X reaction buffer (Fermentas, Lithuania). The PCR reaction was carried out with an initial melting step of 5 min at 95˚C; followed by 35 cycles of 45 s at 95˚C, 45 s at 62˚C, 45 s at 72˚C; and a final elongation step of 7 min at 72˚C.

The restriction endonuclease *XspI* was used to determine the *MMP2* -1306 gene region polymorphism. The products of this reaction were separated on agarose gels containing ethidium bromide. *XspI* digestion generated an uncut 188 bp fragment for *MMP2* -1306C allele and two 162 bp and 26 bp fragments for *MMP2* -1306T allele.

MMP2 mRNA quantification. The varicose veins of the patient group and saphenous veins of the control group that were excised in the surgical operations were transferred to the laboratory in liquid nitrogen. A fatal of 25 mg of each specimen were placed in the buffer of RNA extraction kit immediately so as not to lose RNA material and homogenized carefully. Magnapure Compact RNA isolation Kit (Roche, Manheim, Germany) was used for RNA isolation. Using a commercial kit for cDNA synthesis (Transcriptor First Strand cDNA Synthesis kit; Roche, Manheim, Germany), mRNAs in the specimen were converted to cDNA according to the kit instructions. Hypoxanthine-guanine phosphoribosyl transferase (HGPRT) was used as expression control region for analysis of *MMP2*.

In the real-time PCR protocol, the solution contained 5 μl cDNA, 9.4 μl high quality distilled water, 4 μl buffer solution, 300 nM forward primer, 300 nM reverse primer, 10 μM probe (Table I). This mixture was amplified in a LightCycler (Light Cycler 1.5, Roche): 10 min preincubation at 95˚C, 45 cycles of 10 s denaturation at 95˚C, 30 s annealing at 60˚C, 10 s elongation at 72˚C and finally a single step of cooling for 30 s at 40˚C. The analyses were carried out by using the relative quantification $2^{-\Delta\Delta CT}$ method (22, 23).

Statistical analysis. SPSS 11.0 (SPSS Inc., Chicago, IL, USA) was used for this study. Chi-square and Fisher tests were used to determine genotype and allele distributions between groups.

Table II. *The genotype and allelic frequencies of study groups for MMP2 -1306 and -735 regions.*

MMP2 -735	Controls (n=65)	Patients (n=60)	Significance (*p*-value)
Genotype			
CC	44 (67.7%)	26 (43.3%)	0.005
CT	20 (30.8%)	32 (53.3%)	0.006
TT	1 (1.5%)	2 (3.3%)	>0.05
Allele			
C	108 (83.1%)	84 (70.0%)	>0.05
T	22 (16.9%)	36 (30.0%)	0.005
MMP2 -1306	Controls (n=82)	Patients (n=88)	Significance (*p*-value)
Genotype			
CC	53 (64.6%)	46 (52.3%)	>0.05
CT	24 (29.3%)	37 (42.0%)	>0.05
TT	5 (6.1%)	5 (5.7%)	>0.05
Allele			
C	130 (79.2%)	129 (73.3%)	>0.05
T	34 (20.7%)	47 (26.7%)	>0.05

Table III. *The MMP2 mRNA levels (as $2^{-\Delta\Delta CT}$ values) in related to MMP2 -735 and MMP2 -1306 genotype.*

	MMP-2 mRNA (mean±SD)	Significance (*p*-value)
MMP2 -735 Genotype		
CC (n=25)	0.98±0.77	>0.05
CT (n=15)	1.15±0.66	>0.05
MMP -1306 Genotype		
CC (n=39)	1.12±0.87	>0.05
CT (n=13)	0.77±0.34	>0.05
TT (n=3)	0.65±0.24	>0.05

Student's *t*-test and Annova were used to evaluate the effects of genotypes and alleles on mRNA levels. Allelic frequencies were calculated by using the gene counting method.

Results

As it is known that varices can develop in individuals of advanced age, we preferred to choose older control cases compared to varices patients to ensure the absence of varices in the control group; consequently the mean ages of these groups were significantly different from each other ($p<0.001$).

Table II shows the genotype and allelic frequencies of the study groups for *MMP2* -1306 and -735 regions. *MMP2* -735 C→T polymorphism was more frequent in varices patients than control cases ($p=0.005$).

The patient and control groups that were available to provide tissues for the mRNA studies were compared separately. The demographic information for patient and control groups for mRNA studies were similar, only the mean age of the patient group was higher than that of the control group ($p=0.034$). The MMP2 mRNA levels in relation to the *MMP2* -735 and *MMP2* -1306 genotypes are given in Table III. We preferred to compare several groups with each other. The group with varices contained both patients with and without CAD; when we compared these groups, it was found that *MMP2* mRNA expressions were significantly higher in varices patients with CAD ($p=0.015$) (Table IV).

Table IV. *MMP2 mRNA levels $2^{-\Delta\Delta CT}$ values in varices patient groups compared for CAD existence and CAD pateint groups compared for existence of varices.*

	MMP-2 mRNA (mean±SD)	Significance (*p*-value)
Varices patients with CAD (n=11)	1.38±1.10	0.015
Varices patients without CAD (n=14)	0.86±0.23	
CAD patients with varices (n=11)	1.38±1.10	0.116
CAD patients without varices (n=39)	0.95±0.67	

Discussion

Varicose veins can exist in different forms as telangiectasia, valvular insufficiency, and spider veins (2). They are not only innocent venous structural changes, but can also cause serious circulatory problems such as thrombophlebitis and thromboemboli (24). Although familial factors are believed to contribute to the creation of varicose veins, there is as yet no target gene to be evaluated. Despite some studies suggesting the effects of hemodynamic conditions, there is structural evidence proving the contribution of venous wall alterations to varices (1, 25). In recent studies, alterations were found in collagen types, elastin, laminin, tenascin and fibronectin of varicose vein walls, and the MMP family were thought to be responsible (2, 5, 9). In particular, MMP2 and MMP-9, two of the MMPs that are known as gelatinases, were thought to be effective in the remodelling of the extracellular matrix of vascular structures (26, 27). MMP2 was found to degrade gelatin, proteoglycans, elastin and laminin (11, 28). There are few studies about the effects of MMPs on varicose vein formation and the existing studies have conflicting results (29).

It was previously shown that the promoter region of the *MMP2* gene did not contain a TATA box and expression was constitutive for this region, it was not affected by growth factors and cytokines (14, 30). In our study, we found that the T allele and CT genotype were more frequent for the *MMP2* -735 region in the varices patient group than in the control group. But there were no significant difference

between any groups for the *MMP2* -1306 region. Several different polymorphisms have been found in this promoter region but two in particular have been shown to affect the expression and function of the *MMP2* enzyme, those were *MMP2* -1306C/T and *MMP2* -735C/T polymorphisms (18, 19, 30). It was claimed that *MMP2* -1306 C→T and MMP2 -735 C→T changes affect the Sp-1 binding region decreasing the promotor activity of the gene and so causing lower mRNA transcription (18, 19, 28, 30). In this study, we found no significant difference in *MMP2* mRNA levels neither for MMP2 -1306 C→T nor for MMP2 -735 C→T polymorphism; we conclude that these polymorphisms have no effect on mRNA expression.

Badier-Commander *et al*. found that MMP2 protein levels and activities were decreased in varicose veins compared to saphenous veins (25). We could not find a relationship between the varicose vein patient group and total control group for expression supporting their results, but *MMP2* mRNA levels were significantly higher in varices patients with CAD than in the patient group without CAD, but there were no difference in any of the alleles or genotypes. Interestingly, there were no such expression differences in the control group with or without CAD. Due to the necessity of the tissue specimen for such studies, it was impossible to check varicose patient groups with healthy control groups instead of coronary artery patients. These results must be evaluated with further studies that detect *MMP2* polymorphisms, mRNA and protein levels to find their effects on the formation of varicose veins and also CAD in larger patient groups.

In conclusion, we found that the *MMP2* -735 C→T polymorphism was more frequent in varices patients than control cases. We had to take the saphenous veins of coronary bypass patients to be able to have tissues to compare with varices specimens, so all of the control group cases for mRNA study were CAD patients. Some of our operated varices cases also had CAD. Hence, we had the opportunity to compare this group with the control group. We found that *MMP2* mRNA expressions were higher in varices patients with CAD compared to the CAD only and varices only groups.

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