Polymorphisms of MMP9 and TIMP2 in Patients with Varicose Veins

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Abstract. Background: Genetic predisposition is a suggested risk factor in the etiology of varicose veins. The matrix metalloproteinase (MMP) family degrades extracellular matrix (ECM) and may lead to disturbances in vein wall structure. The activity of MMPs in the ECM are controlled by specific tissue inhibitors of MMPs (TIMP). The present study aimed to investigate the relationship between MMP9 and TIMP2 gene polymorphisms and varicose vein risk. Materials and Methods: Genotyping of the polymorphisms of MMP9 (1562 C/T) and TIMP2 (418G/C) was performed using polymerase chain reaction and restriction-fragment length polymorphism assays in a group of patients with varicose veins (n=63) and healthy controls (n=70). Results: The frequencies of MMP9 alleles and genotypes did not differ significantly between patient and control groups. However, TIMP2 -418 C allele was associated with increased risk for varicose vein formation (p=0.007). It was also shown that the frequency of the GG genotype was also significantly higher in the control group than in the patient group (odds ratio=0.333, 95% confidence interval=0.14-0.78, p=0.012). Conclusion: TIMP2 -418 C allele is associated with susceptibility for varicose vein formation and individuals with GG genotype may have a lower risk for varicose vein formation.

Varicose veins and chronic venous insufficiency are the most common disorders of the lower extremities affecting nearly half of female and one quarter of male population in general (1). The major risk factors of venous diseases are increased age, ligamentous laxity, prolonged standing, pregnancy, obesity, leg trauma and previous venous thromboses (2-7). Family history and genetic predisposition are other important risk factors of varicose vein formation. It has been reported that the risk of developing varicose veins for an individual is 90% when both parents suffer from this disease, however, it is 20% when neither of parents were affected (8). Normally, the vein wall consists of three layers: the tunica adventitia, tunica media and tunica intima. The tunica intima, the inner layer of veins, is composed of a smooth endothelial lining surrounded by an extracellular matrix (ECM) consisting of elastin, collagen and proteoglycans to support the elastic recoil of veins. The tunica intima layer also contains valves to keep the blood flowing in a single upward direction towards the heart (9, 10).

Four mechanisms are considered to be responsible for the pathophysiology of varicose vein formation. One of these mechanisms is the loss of elasticity of the vein wall, mainly due to increasing age. The second is the absence or dysfunction of the venous valves; the third is dysfunction of muscular pumping system, and the last mechanism is the obstruction of the venous system. All these mechanisms lead to venous hypertension and dilated veins in the lower extremities (10, 11). As noted above, elastin and collagen are important elements of the ECM and responsible for maintaining the integrity of vein wall structure. It is reported that reduced elastin content leads to dilatation of the vein wall (12).

Matrix metalloproteinases (MMPs), with more than 24 members, are neutral endopeptidases that catalyze the degradation of the proteins of the ECM. This degradation of the ECM is a well-controlled process and is normally involved in many physiological conditions, such as angiogenesis, tissue remodeling and wound healing. However, exaggerated MMP activity may be implicated in...
MMP9 is a member of the gelatinase family. It is also known as gelatinase B. It degrades collagen types IV, V, and XI, N-telopeptides of type I collagen, as well as elastin. Like other MMPs, MMP9 is also produced in inactive zymogenic form and requires enzymatic activation. MMP activity is controlled by α2-macroglobulin and tissue inhibitors of matrix metalloproteinases (TIMPs). TIMPs regulate both the activation of the proenzyme and substrate degradation. The TIMP family has four members synthesized in vascular smooth muscle cells, endothelial cells and macrophages.

The expression of MMP1, -2, -3, -9 and -13 is increased in patients with varicose veins. The main cause of this increase is thought to be due to an imbalance between the activities of MMPs and TIMPs, resulting in ECM accumulation in the vein wall. The genetic polymorphisms of MMPs and TIMPs are thought to be important predisposing factors for this imbalance.

The purpose of this study was to investigate the effect of genetic polymorphisms of MMP9 (-1562) and TIMP2 (-418) on varicose vein formation and investigate whether those polymorphisms could be used in clinical area or not.

Materials and Methods

Patients. Sixty-three patients with varicose veins and 70 healthy controls were included in the study. The study protocol was approved by the local Ethical Committee (Approval number 2007/1297). The demographic properties of the groups are demonstrated in Table I. The patients were classified according to clinical, etiological, anatomical and pathophysiological (CEAP) classifications. High ligation with stripping with or without additional individual ligation and excision were performed for symptoms, complications or cosmetic needs. After written informed consent was given, 10 ml of EDTA blood was drawn for genetic analyses from both patients and controls.

<table>
<thead>
<tr>
<th>Clinical characteristic</th>
<th>Study group (n=63)</th>
<th>Control group (n=70)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age±SD, years</td>
<td>54.52±16.73</td>
<td>57.99±14.91</td>
<td>0.140</td>
</tr>
<tr>
<td>Female gender, n</td>
<td>34</td>
<td>22</td>
<td>0.007</td>
</tr>
<tr>
<td>Mean body mass index ± SD, kg/m²</td>
<td>26.90±4.19</td>
<td>25.84±2.60</td>
<td>0.133</td>
</tr>
<tr>
<td>Smoking, n</td>
<td>51</td>
<td>59</td>
<td>0.390</td>
</tr>
<tr>
<td>Positive family history, n</td>
<td>55</td>
<td>9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CEAP stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2 (%)</td>
<td>20 (36.4%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>C3 (%)</td>
<td>30 (54.5%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>C4 (%)</td>
<td>5 (9.1%)</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

CEAP: Clinical, Etiological, Anatomical and Pathophysiological classification.

Table II. Clinical, etiological, anatomical and pathophysiological (CEAP) classification (20).

<table>
<thead>
<tr>
<th>Clinical classification (C0-C6)</th>
<th>Etiologic classification (Ec, Ep, Es, En)</th>
<th>Anatomic classification (As, Ap, Ad, An)</th>
<th>Pathophysiologic classification (Pr, Po, Pr,o, Pn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C0: No visible or palpable signs of venous disease</td>
<td>Ec: Congenital</td>
<td>As: Superficial veins</td>
<td>Pr: Reflux</td>
</tr>
<tr>
<td>C1: Telangiectasies or reticular veins</td>
<td>Ep: Primary</td>
<td>Ap: Perforator veins</td>
<td>Po: Obstruction</td>
</tr>
<tr>
<td>C2: Varicose veins</td>
<td>Es: Secondary (post-thrombotic)</td>
<td>Ad: Deep veins</td>
<td>Pr,o: Reflux and obstruction</td>
</tr>
<tr>
<td>C3: Edema</td>
<td>En: No venous cause identified</td>
<td>An: No venous location identified</td>
<td>Pn: No venous pathophysiology identifiable</td>
</tr>
<tr>
<td>C4a: Pigmentation or eczema</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C4b: Lipodermatosclerosis or atrophie blanche</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C5: Healed venous ulcer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6: Active venous ulcer</td>
<td></td>
<td></td>
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</tbody>
</table>

Genetic analyses. Genomic DNA was isolated by a salting-out procedure. Genotyping of polymorphisms of MMP9 (-1562 C/T) and TIMP2 (-418 G/C) were performed using polymerase chain reaction and restriction-fragment length polymorphism assays (PCR-RFLP). Primers used for PCR analysis are given in Table III. The PCR reactions contained 2.5 μl PCR buffer (Fermentas Life Sciences, St. Leon-Rot, Germany), 3 μl dNTP-Mix (Fermentas Life Sciences) as well as 1 μl forward and 1 μl backward primers (Fermentas Life Sciences). The PCR comprised of an initial denaturation step for 5 min at 95°C, 35 cycles of 94°C for 30 sec,
63˚C for 30 seconds, 72˚C for 45 sec and one cycle at 72˚C for 7 min (final extension) for MMP9 (-1562 C/T) and an initial denaturation step 3 min at 95˚C, 35 cycles of 95˚C for 1 min, 58˚C for 1 min, 72˚C for 1 min and 1 cycle at 72˚C for 1 minute (final extension) for TIMP2 (-418 G/C). The restriction digests were prepared by the restriction enzymes Pae-I for MMP9 and Hga-I for TIMP2 (Fermentas Life Sciences). The digests were incubated overnight at 37˚C and analyzed by electrophoresis in a 2% agarose gel for allele discrimination.

Statistical analysis. All statistics were performed using SPSS version 17.0 for Windows (SPSS Inc. Chicago, IL, USA). Continuous variables were expressed as the mean±SD and were compared by unpaired Student’s t-test and ANOVA. Binary logistic regression analysis was performed to calculate the odds ratio and 95% confidence interval to estimate the risk of associations with genotypes of interest. Statistical significance was assumed if p-value was less than 0.05.

Results

Demographic data of the patients are summarized in Table I. Comparison of age, body mass index and smoking showed no statistically significant differences between the groups. However, comparison of gender and family history indicated a significant difference between groups. The patient group consisted of 34 (54%) females whereas the control group consisted of 22 (31.4%) females (OR=0.391, 95% CI=0.193-0.793, p=0.007). Family history of varicose veins were positive in 55 (87.3%) cases in the patient group, whereas it was positive in only nine (12.9%) cases in the control group (OR=46.59, 95% CI=16.80-129.18 p<0.0001).

Figures 1 and 2 show the PCR-RFLP analysis of the -1562C/T polymorphism of MMP9 and -418G/C polymorphism of TIMP2 gene promoters. The genotypic frequency distributions of MMP9 and TIMP2 did not show any significant deviation from those expected under the Hardy–Weinberg equilibrium, neither in the patient nor in the control group. Table IV shows the genotypic and allelic frequencies in the patient and control groups. The minor allelic frequency for MMP9 -1562C was 0.190 in patients and 0.164 in controls. The minor allelic frequency was 0.476 in patients versus 0.385 in controls for TIMP2 -418C. Frequencies of MMP9 -1562 genotypes and minor allelic frequency did not differ significantly between patients and controls, whereas the frequency of TIMP2 -418 GG genotype was significantly higher in the controls than in the patient group (p=0.012). Additionally, the frequency of the C allele was significantly higher in the patient group than in the control

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Table III. Primers used for PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP9</td>
<td>5’ GCCTGGCACATAGTAGGCCC 3’</td>
<td>5’ CTTCCTAGCCAGCCGGCATC 3’</td>
</tr>
<tr>
<td>TIMP2</td>
<td>5’ GATCCTGTCAGTTTCTCAA 3’</td>
<td>5’ TTTCCCCCTTAGCTGACTCT 3’</td>
</tr>
</tbody>
</table>
Allele TIMP2 -418 respectively).

statistical significance (OR=0.773, 95% CI=0.279-2.143
polymorphisms separately in women and men, revealed no
handy the ECM and increases cell permeability, leading to
leucocyte infiltration, smooth muscle proliferation and
increased venous hydrostatic pressure (22). Increased
hydrostatic pressure in the veins results in degeneration of
the cardiovascular system. It has been shown that MMP
expression was increased as a result of increased
mechanical stretch or pressure in tissues and this may
explain increased MMP levels in varicose veins after
handy gene transfers of TIMP1 and TIMP2 were performed (27).

Previous studies reported that C allele carriers of TIMP2 had
increased risk of several diseases, such as varicose veins, gastric
cancer, QT prolongation, generalized aggressive periodontitis
and chronic obstructive pulmonary disease (25, 28-31). It was
also reported that people with TIMP2 GG genotype might have
lower risk for varicose veins (25).

In conclusion, TIMP2 -418 C allele is associated with
susceptibility for varicose vein formation and the individuals
with GG genotype may have a lower risk for varicose vein
formation. However, polymorphisms of MMP9 -1562
alleles were not shown to be associated with varicose vein
formation in our study population.

References

1 Beebe-Dimmer JL, Pfeifer JR, Engle JS and Schottenfeld D: The
epidemiology of chronic venous insufficiency and varicose

2 Baker SR, Stacey MC, Jopp-McKay AG, Hoskin SE and

3 Criqui MH, Denenberg JO, Bergan J, Langer RD and Fronet A: Risk

### Table IV. Frequencies of (MMP9) -1562 and (TIMP2) -418 alleles and genotypes in patients with varicose veins and the control group.

<table>
<thead>
<tr>
<th>Genotype/ Allele</th>
<th>Study group (n=63)</th>
<th>Control group (n=70)</th>
<th>OR</th>
<th>95% CI</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP9 -1562</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1.00</td>
<td>0.41-2.43</td>
<td>0.352</td>
</tr>
<tr>
<td>CT</td>
<td>24 (38.1)</td>
<td>23 (32.9)</td>
<td>1.02</td>
<td>0.76-1.36</td>
<td>0.848</td>
</tr>
<tr>
<td>TT</td>
<td>39 (61.9)</td>
<td>47 (67.1)</td>
<td>0.94</td>
<td>0.81-1.08</td>
<td>0.389</td>
</tr>
<tr>
<td>MMP2 -418</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>4 (6.4)</td>
<td>5 (7.1)</td>
<td>0.80</td>
<td>0.21-2.97</td>
<td>0.739</td>
</tr>
<tr>
<td>CG</td>
<td>52 (82.5)</td>
<td>44 (62.9)</td>
<td>1.18</td>
<td>0.79-1.76</td>
<td>0.415</td>
</tr>
<tr>
<td>GG</td>
<td>7 (11.1)</td>
<td>21 (30.0)</td>
<td>0.33</td>
<td>0.14-0.78</td>
<td>0.012</td>
</tr>
<tr>
<td>Allele</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>24 (19.0)</td>
<td>23 (16.4)</td>
<td>1.04</td>
<td>0.58-1.84</td>
<td>0.884</td>
</tr>
<tr>
<td>T</td>
<td>102 (80.9)</td>
<td>117 (83.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CI: Confidence interval; OR: odds ratio.

**Discussion**

Varicose veins, although mostly considered a cosmetic
problem, usually result in a poor quality of life and even
severe morbidity due to ulcers of the lower extremity as a
result of chronic venous insufficiency. In the present study,
frequencies of MMP9 -1562 genotypes and alleles did not
statistically significantly differ between the patients with
varicose veins and healthy controls.

The pathophysiological mechanisms that result in varicose
veins are not fully understood. MMPs are the main
proteinases degrading the ECM (17). MMP9 digests
denatured collagens. MMPs have been demonstrated in many
tissues and in the cardiovascular system. It has been shown
that MMP expression was increased as a result of increased
mechanical stretch or pressure in tissues and this may
explain increased MMP levels in varicose veins after
increased venous hydrostatic pressure (22). Increased
hydrostatic pressure in the veins results in degeneration of
the ECM and increases cell permeability, leading to
leucocyte infiltration, smooth muscle proliferation and
ultimately in venous valvular degeneration (23, 24). It has
been suggested that polymorphisms in the promoter region
of MMP9 are associated with the varicose veins in the
Chinese population (25). Although our study also similarly
aimed to show the association between polymorphism of
MMP9 and presence of varicose veins, our Turkish study
population could differ from the Chinese population (25).
The Chinese study stated that those with CC genotype had a
higher risk for varicose veins, however, in our study neither
patients nor the healthy controls were carriers of the CC
genotype. Thus, this population difference and the difference
in the frequencies of the genotypes could explain this
difference between our study results and theirs (25).

The proteolytic activities of MMPs are primarily regulated by
TIMPs. TIMPs are small proteins of 21-28 kDa and they inhibit
MMP activities by binding to their active zinc-binding area (26).

In the present study, we showed that the C allele -418G
for TIMP2 was significantly associated with risk of varicose
veins (OR=3.429, 95% CI=1.34-8.75; p=0.010). It was also
shown that individuals with the GG genotype may have a
lower risk for varicose vein formation. (OR=0.333, 95%
CI=0.14-0.78; p=0.012). Additionally, the frequencies of the
C-carrying genotypes versus the GG genotype were
significantly higher in the patient group than the control
(p=0.007), suggesting that carriers of the C allele may have
a higher risk for varicose vein formation.

The anti-angiogenic activities of the endogenous TIMPs are
well-known. TIMP2 inhibits endothelial cell proliferation. It was
reported that formation of atherosclerotic plaques were reduced
when gene transfers of TIMP1 and TIMP2 were performed (27).

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