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Polymorphisms in MMP-9 and TIMP-2 in Chinese Patients with Varicose Veins

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Background. **Varicose veins (VVs), a common vascular disease, are functionally characterized by dilation and tortuosity and are widely prevalent in the adult population. The pathophysiology and molecular mechanism of VVs are still unclear. A genetic risk for VVs has been demonstrated, although no genetic variant pertaining to VVs has been identified. Matrix metalloproteinases (MMPs) and their endogenous tissue inhibitors (TIMPs), which can prevent excessive extracellular matrix (ECM) degradation, greatly impact vascular remodeling and may play a vital role in patients with VVs. We evaluated a potential association between polymorphisms in the promoters of MMP-9 and TIMP-2 and the risk for VVs in the Chinese population.**

Materials and Methods. **Genotyping of the promoter region polymorphisms -1562C/T in MMP-9 and -418G/C in TIMP-2 was performed with PCR and restriction fragment length polymorphism (PCR-RFLP) assays with a group of 60 patients with VVs and 60 healthy controls. Purified PCR products were sequenced.**

Results. **A significant correlation was found between patients with VVs and controls at -1562C/T in MMP-9. The TIMP-2 gene polymorphism -418G/C was also associated with VVs.**

Conclusions. **Our results suggest that polymorphisms in the promoter region of MMP-9 and TIMP-2 are associated with VVs in the Chinese population**

Key Words: **MMP-9; TIMP-2; promoter polymorphism; varicose vein.**

INTRODUCTION

Varicose veins (VVs) commonly occur on the backs of the calves or on the inside of the leg and constitute a vascular disorder characterized by dilation and tortuosity [1]. Epidemiological statistics show that more than 25% of the adult population is affected by VVs [2]. Risk factors such as genetic determinants, age, female gender, obesity, pregnancies, standing for a long time, sedentary lifestyle, and family history have been identified [3]. However, explicit data on the pathophysiology and the molecular mechanisms underlying the pathogenesis of VVs are still scanty. Recently, Mellor *et al.* [4] demonstrated that mutations in FOXC2, which is involved in lymphoedema distichiasis, are associated with primary vein failure. It has been strongly suggested that genetic variation plays an important role in individuals who suffer from vascular diseases and, thus, research has concentrated on some candidate genes associated with VVs to identify the frequencies of specific genes in the general population.

Evidence has shown that matrix metalloproteinases (MMPs), a family of highly homologous protein-degrading zinc-dependent endopeptidases [5], can functionally degrade the extracellular matrix (ECM). The synthesis and degradation of the ECM have been demonstrated to be altered in vascular diseases [6]. Members of the MMP family include collagenases, proteoglycans, matrilysins, stromelysins, and membrane-type MMPs [7]. MMPs and their endogenous tissue inhibitors (TIMPs) can prevent excessive ECM degradation and influence the process of vascular remodeling by regulating the migration and proliferation of vascular smooth muscle cells [8]. Therefore, the balance between MMPs and TIMPs greatly impacts remodeling. In a patient with VVs, the balance breaks down, and

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the abnormal activity of MMPs increases, which destroys the integrity and contributes to pathologic alteration of the venous wall structure [9]. Kowalewski *et al.* [10] detected increased expression and activity of MMPs in the venous wall of patients with thrombophlebitic VVs.

Further studies have reported that polymorphisms exist in the genes for MMPs and TIMPs [11–13] and, thus, a potential relationship may exist between mutations in the genes for MMPs or TIMPs and VVs. Kurzawski *et al.* [11] studied promoter polymorphisms of MMP-1 (tissue collagenase I) and MMP-3 (stromelysin I). They showed that the genotype frequencies of the MMP1-1607dupG and MMP3-1171dupA alleles were similar between patients and controls, indicating that MMP-1 and MMP-3 promoter polymorphisms were not valuable markers of susceptibility to VVs. However, an association between other MMP genotypes and the risk for VVs cannot be definitively excluded [11], and other attractive candidates in the MMP family may provide positive hints. MMP-9 (gelatinase B), which breaks down the ECM by degrading type IV collagen [12], has several genetic variants in its gene. Zhang *et al.* [13] reported a substitution of cytosine (C) to thymidine (T) at nucleotide -1562 bp in the MMP-9 promoter region, which may influence transcription. TIMP metallopeptidase inhibitor 2 (TIMP-2), a member of the TIMP gene family that regulates the activity of MMPs [14], has a substitution of guanine (G) to C at position -418 bp in the promoter [15]. Because the molecular mechanisms of VV formation have not been clarified, we performed a case-control study in the Chinese population to examine whether the reported polymorphisms in MMP-9 and TIMP-2 are risk factors for VVs.

MATERIALS AND METHODS

Chinese patients consisted of those undergoing vascular surgery at Zhongshan Hospital, and the control group was from the Department of Forensic Medicine, Shanghai Medical College, Fudan University. Individuals in this study were selected at random following proper informed consent. All procedures were conducted according to the protocols approved by the Institutional Committee on Ethics of Fudan University.

DNA Samples

DNA samples were obtained from 60 unrelated patients. All the patients enrolled in this study were ethnically homogeneous and of the Han nationality. The diagnosis conformed to the criteria for VVs, which are defined as "dilated palpable veins $>$ 4 mm in diameter" or reticular "dilated and nonpalpable subdermal veins $\leq$ 4 mm" [16]. All 60 patients had the C3–C4 phenotype according to CEAP classification (Clinical-Etiology-Anatomy-Pathophysiology classification of Chronic Venous Disorders). The control group consisted of 60 healthy individuals who did not have VVs and who were recruited from the same geographic region. Genomic DNA was extracted from 1 mL of peripheral venous blood samples with a DNA isolation kit (TianGen-Biotech Co. Ltd., Shanghai, China).

Genotyping

PCR and restriction fragment length polymorphism (RFLP) were used to genotype the promoter polymorphisms of MMP-9 and TIMP-2 in each DNA sample. Primer sequences and conditions are shown in Table 1. Direct sequencing was performed with approximately 150 ng genomic DNA, 10 μM each primer, 0.2 mM dNTP, 2.0 mM $MgCl_2$, 1.0 U Taq DNA polymerase, and 10 $\times$ reaction buffer (TianGenBiotech Co. Ltd.).

MMP-9 Genotyping

PCR products were digested with one unit of SphI (New England BioLabs, Beverly, MA) at 37°C for 1 h and then separated on a 2.5% agarose gel containing ethidium bromide. PCR products were 435 bp in length. The T allele was cut into fragments of 247 and 188 bp, whereas the C allele remained uncut.

TIMP-2 Genotyping

Using one unit of AvaI (New England BioLabs) PCR products were digested at 37°C for 1 h. DNA fragments were separated on an 8% polyacrylamide gel and stained with ethidium bromide. The G allele possesses two restriction sites for AvaI, whereas the C allele has only one. DNA fragments for the allele G were 230, 51, and 23 bp, and those for the C allele were 253 and 51 bp.

DNA Sequencing

PCR products were isolated from a 2.5% agarose gel stained with ethidium bromide. Purified DNA samples were sent to Invitrogen Co. (Shanghai, China) for sequencing.

Data Analysis

The reference sequences of the genes for MMP-9 and TIMP-2 in GenBank (accession no.4318; 7077) were used to determine the observed genotype and allele frequencies. Concordance of the genotype

TABLE 1

Variants and PCR Conditions

	Primers 5′→3′ and PCR conditions	
Variants	Forward	Reverse
MMP-9	GCCTGGCACATAGTAGGCCC	CTTCCTAGCCAGCCGGCATC
	94°C, 4 min., 35 cycles; 94°C, 1 min.; 68°C, 1 min.; 72°C, 1 min	
TIMP-2	CGTCTCTTGTTGGCTGGTCA	CCTTCAGCTCGACTCTGGAG
	94°C, 4 min., 35 cycles; 94°C, 1 min.; 58°C, 45s.; 72°C, 45s	