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Promoter variants of *VTN* are associated with vascular disease

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ABSTRACT

Background: Vitronectin is involved in the whole process of atherosclerosis. Our aim is to determine the association of *VTN* functional promoter variants with different types of vascular disease, and conclude the roles of vitronectin involved in vascular disease.
Methods: Gel shift assays and luciferase reporter assays were used to determine the impact of variants on promoter activity. The correlation of plasma vitronectin levels with the variant was assessed in normal controls. The association of the variant with vascular disease was determined in 3 case–control studies.
Results: A strong linkage disequilibrium was found between rs2227721 and rs2227720 in *VTN* promoter in Chinese ($r^2 = 1.0$). Both variants resulted in a decreased transcription activity, and rs2227721 decreased the binding efficiency of transcription factor YY1 to the region. The rs2227721 was correlated with plasma vitronectin levels in normal controls ($r = -0.207$, $P = 0.028$). The rs2227721 was associated with susceptibility of vascular disease; the odds ratios among subjects carrying rs2227721-T allele were 1.298 (95% Confidence Interval—CI, 1.033–1.631) for non-MI CAD ($P<0.05$), 1.346 (95% CI, 1.068–1.695) for chronic MI ($P<0.05$), 1.486 (95% CI, 1.145–1.928) for acute MI ($P<0.001$), and 1.619 (95% CI, 1.108–2.366) for deep venous thrombosis ($P<0.05$).
Conclusion: VTN promoter haplotype would be a novel genetic marker for vascular disease.

1. Introduction

Vitronectin is a multifunctional plasma glycoprotein that participates in the regulation of coagulation, fibrinolysis, complement cascade [1,2], cell adhesion and pericellular proteolysis on surfaces of cells and extracellular matrices [3,4]. Vitronectin binds multiple ligands, including integrins, plasminogen activator inhibitor (PAI-1), the urokinase receptor, collagen, complement C5b-7, and heparin. PAI-1, a key regulator of fibrinolysis [3–7], is the major ligand of vitronectin involved in atherosclerosis. Interestingly, all of the active PAI-1 in blood circulates in complex with vitronectin [8].

Vitronectin expression is a critical determinant of intimal hyperplasia and the antiproliferative properties of PAI-1. PAI-1 can also inhibit smooth muscle cell (SMC) migration by binding to vitronectin in the extracellular matrix, thereby blocking vitronectin binding to integrin and nonintegrin receptors present on SMC [9]. In addition, vitronectin/PAI-1 inhibits thrombin [10]. Given that thrombin stimulates SMC proliferation and is hypothesized to stimulate intimal hyperplasia independently of its prothrombotic effects [11–13], it is possible that vitronectin/PAI-1 could regulate intimal hyperplasia by inhibiting thrombin. It is still controversial whether vitronectin/PAI-1 promotes or inhibits neointima formation in atherosclerosis and/or restenosis after vascular injury [13–15].

Vitronectin may also play a key role in the regulation of thrombosis–fibrinolysis system. But its role in thrombosis is also currently controversial. Vitronectin binds to platelet glycoproteins IIb/IIIa and $\alpha_v\beta_5$ [3]. It plays a dual role in platelet aggregation, the released platelet granule vitronectin enhances platelet aggregation while plasma vitronectin inhibits platelet aggregation [16]. In addition to its platelet interactions, vitronectin may control the clearance of vascular thrombi by binding and stabilizing PAI-1 [17,18] and the thrombotic response to vascular injury by regulating thrombin function [19]. Also, several studies reported that high plasma vitronectin levels are associated with coronary heart disease [20,21], and adverse cardiovascular outcomes following acute stenting [22].

The rupture of coronary plaques with subsequent thrombosis represents the principal pathophysiology underlying acute coronary syndromes [23]. Acute myocardial infarction (MI) is a typical acute coronary syndrome. A patient's vulnerability to an acute MI may be modulated by individual variations in the balance between coagulation and fibrinolysis. Similarly, the increased risk of venous thrombosis is the consequence of a hypercoagulable state explained by modifications in clotting factor levels and by a reduction in venous velocity flow [24]. Less thrombotic events occurred in stable CAD such as non-MI CAD

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and chronic MI. Evidence from angioscopic studies demonstrates that plaque rupture and thrombosis are present in around 20% of patients with stable CAD [25].

In this study, we tested whether the variants in *VTN* promoter are functional, then determined vitronectin roles in thrombosis or non-thrombotic atherosclerosis by assessing the relationship of the functional variants with susceptibility of chronic MI, non-MI CAD, acute MI, or deep venous thrombosis (DVT).

2. Materials and methods

The study was approved by the ethics committee of Fuwai Hospital. All of the subjects who participated in the study provided their written informed consent, self-reported as Han nationality and resided in north China. The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology.

2.1. Selection of single nucleotide variants

According to previous study [26], the promoter region from −491 to +210 (transcription start site as +1, cDNA sequence NM_000638 as reference) was analyzed. Two common variants (with minor allele frequency of at least 5%) rs2227720 and rs2227721 were included in the study, which are in a strong linkage disequilibrium block in *VTN* promoter in CHB population from the HapMap database (public release #28; NCBI build 36; dbSNP b126). The linkage disequilibrium was confirmed ($r^2 = 1.0$) in 100 subjects, then the rs2227721 was selected to analyze the association of *VTN* promoter with vascular disease.

2.2. Luciferase reporter assay

To determine VTN promoter activity, the promoter encompassing rs2227720 and rs2227721 (from −491 to +210) from patients carrying TT(rs2227720)–GG(rs2227721) or CC(rs2227720)–TT(rs2227721) genotypes was first cloned into the pGEM-T Easy vector with primers: 5′-CGCGGTACCGAGAAGAACCCAGAAATGAAACT (containing *Kpn*I site) and 5′-CGCAAGCTTGGTCAGCCAGAGCAACC (containing *Hind*III site). Then the fragment was released from the pGEM-T Easy vector by digesting with *Kpn*I and *Hind*III and sub-cloned into pGL3-basic vector. The vector containing the TT(rs2227720)–GG(rs2227721) genotype was designated as pGL3-T-G and the vector containing the CC(rs2227720)–TT(rs2227721) genotype as pGL3-C-T. Vectors pGL3-T-T and pGL3-C-G were obtained by introducing mutation with Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Cells were co-transfected with 5 ng of Renilla luciferase expression vector pRL-TK and 50 ng of the firefly luciferase expression vector. Transfections were performed in 94-well plates, and luciferase activity was quantified using a luminometer (SIRIUS, Pforzheim, Germany) and the Stop & Glo luciferase report system (Promega, Madison, WI, USA). Firefly luciferase activity was normalized against the Renilla luciferase activity.

2.3. Gel shift assay

Double-stranded DNA was prepared for use as a probe by annealing a biotin-labeled synthetic oligonucleotide, 5′-TCTGCCCTCCAGAT/CCCACGGTGCTAAT-3′ for rs2227720, and 5′-TATCTCCCAGCTCG/TCCCAGGCCCAGTGTT-3′ for rs2227721. Cells were grown in a 10-cm dish and washed with ice-cold phosphate buffered saline. Nuclear extracts were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents, according to the manufacturer's protocol (Pierce, Rockford, IL, USA). Finally, nuclear protein was extracted in 100 μL of NER. Nuclear extract (2 μL) and 22.5 fmol of biotin-labeled DNA were incubated at room temperature in the binding buffer containing 10% glycerol, 5 mM $MgCl_2$, 50 ng/μL poly(d*I*-dC), and 0.05% NP-40. For gel supershift assays, nuclear extract and an anti-Sp1 or anti-YY1 antibody (Santa Cruz, USA) were incubated at room temperature for 20 min before mixing with the binding buffer containing the biotin-labeled DNA. After 20 min of incubation, the mixtures were analyzed by electrophoresis on a 6% polyacrylamide gel at 175 V for 1.5 h in Bio-Rad Tris-Glycine Running buffer (Bio-Rad, Hercules, CA, USA). The gel contents were electrophoretically transferred to a nylon membrane at 390 mA for 40 min. The membrane was then cross-linked on a transilluminator for 10 min. Detection of biotin-labeled DNA by chemiluminescence was conducted using the LightShift Chemiluminescent EMSA kit, according to the manufacturer's protocol (Pierce). The unlabeled double-stranded oligonucleotides (100-fold) in the labeled probe were negative controls.

2.4. Plasma vitronectin measurements

To investigate the impact of the gene variants of *VTN*, we determined plasma vitronectin levels in 113 normal subjects aged 43–60 years from Benxi, Liaoning Province, China. To avoid potential confounding effects of risk factors on the plasma vitronectin levels, the subjects without conventional risk factors were selected, including 71 with homozygote, 34 with heterozygote for the G allele, and 8 with homozygote for the T allele of the rs2227721. The assay was performed with enzyme-linked immunoassay kits from Molecular Innovations (Novi, MI, USA).

2.5. Study population for non-MI CAD and chronic MI

The cases were recruited from the consecutive patients admitted to radiology department of Fuwai Hospital for multiple detector computed tomography (MDCT) scanning for suspected or known CAD from April 2007 to December 2008. A total of 661 unrelated non-MI CAD patients and 626 chronic MI patients were enrolled. The inclusion criteria for non-MI CAD were >70% narrowing of the lumina of at least 1 of the major coronary arteries. A detailed history of angina or myocardial infarction was obtained. Chronic MI was judged by typical electrocardiogram change (Minnesota Code 1.1 or 1.2 in ECG) and by changes in plasma enzymes (troponin T, troponin I, creatine kinase-MB, aspartate aminotransferase, and glutamic pyruvic transaminase) without previous acute symptoms. The scanning protocol was described previously [27]. The 661 controls were selected from the subjects admitted to the hospital for excluding CAD, whose major coronary artery had no stenosis, and did not have any other vascular disease.

2.6. Study population for acute MI and DVT

We identified patients with an initial clinical presentation of acute MI or DVT by screening 20,000 patients admitted to Fuwai Hospital from December 2003 to June 2007. A total of 752 patients with acute MI, and 194 patients with DVT were recruited, the clear diagnosis was taken from clinical history. Patients with hematological diseases, peptic ulcers, liver and kidney dysfunctions, infections, autoimmune diseases, and tumors were excluded. In patients with DVT, 80 subjects had pulmonary embolism. The matched controls were selected from the subjects admitted to the hospital for a general health evaluation, whose major coronary artery had no stenosis, and did not have any other vascular disease.

2.7. Biological variable determination and clinical data collection

Blood samples were collected after a 12-hour overnight fast before cardiovascular procedures. In subjects with acute event, the drawing of blood was delayed for at least 6 weeks. The plasma and cell buffet coat were kept at −70 °C. Genomic DNA was extracted and biological variables were determined within 3 months. A complete clinical history was obtained from all subjects. In addition to neurological history and family history of hypertension, CAD, and diabetes mellitus (DM), the following vascular risk factors were also recorded: history of vascular disease, cigarette smoking and alcohol consumption, body mass index, systolic blood pressure (SBP), diastolic blood pressure (DBP), blood glucose, high density lipoprotein-cholesterol (HDL-C), non-HDL-C lipids, total plasma cholesterol (TC), and triglycerides (TG). Plasma biochemical parameters were assayed by an automatic analyzer (Hitachi 7060, Hitachi, Japan). Non-HDL-C was calculated by the Friedewald formula. Hypertension was defined as a mean of 3 independent measures of blood pressure >140/90 mm Hg or the use of antihypertensive drugs. DM was diagnosed when the subject had a fasting glucose >7.8 mmol/L, or >11.1 mmol/L at 2 h after oral glucose challenge, or the use of glucose-lowering drugs. All lipids were determined in a CDC qualified laboratory in Fuwai hospital.

2.8. Genotype analysis

A DNA isolation kit, RelaxGene Blood DNA System (TianGen, Beijing, China), was used for preparing genomic DNA following the recommendations of the manufacturer. Genotyping was performed using MassARRAY high-throughput DNA analysis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Sequenom, Inc., San Diego, CA, USA). The primers were designed by MassARRAY Assay Design software (version 3.1). SNPs were genotyped using iPLEX Gold technology (Sequenom) followed by an automated data analysis with the TYPER® RT software version 4.0. Reproducibility of genotyping was confirmed by bidirectional sequencing in 500 samples and the reproducibility was 99.4%.

2.9. Statistical analysis

The distribution of quantitative variables was tested for normality using one-sample Kolmogorov–Smirnov test. As TG level was highly skewed, we compared the difference between cases and controls with Mann–Whitney non-parametric test. Quantitative variables were compared with the one-way ANOVA test, including age, body mass index, blood pressure, TC, HDL-C, non-HDL-C, and glucose. A chi-square test was used to test for qualitative variables, genotype/allele frequencies and the Hardy–Weinberg equilibrium of the variants. Association of variants with vascular disease was analyzed by logistic regression adjusted by conventional risk factors. The association was expressed as odds ratio. Student's *t*-test and Pearson correlation were used to assess the difference and correlation between vitronectin plasma levels with genotypes of rs2227721. Owing to the low frequency of the minor alleles analyzed in this study at the population level, we defined a genetic dominant model for this analysis, in which the carriers of the rare allele were compared with those homozygous for the wild-type allele. A two-tailed probability value of <0.05 was considered significant. Analyses were performed with SPSS 13.0 for Windows.