Clinica Chimica Acta 415 (2013) 233–238

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Association of vaspin gene polymorphisms with coronary artery disease in Chinese population and function study

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ARTICLE INFO

Article history:
Received 28 May 2012
Received in revised form 24 September 2012
Accepted 16 October 2012
Available online 31 October 2012

Keywords:
Adipokine
Coronary artery disease
Vaspin
Polymorphism

ABSTRACT

Background: Visceral adipose tissue-derived serine protease inhibitor (vaspin) is a recently identified adipokine. Studies suggest it is involved in many diseases such as obesity, diabetes and coronary artery disease (CAD). This study is to investigate the association of single nucleotide polymorphisms (SNPs) in vaspin with CAD and its potential mechanisms.

Methods: A total of 1570 consecutive patients undergoing coronary angiography were enrolled and the genotypes were determined by TaqMan allelic discrimination. Serum vaspin concentrations and mRNA expression levels were determined by ELISA and RT-PCR, respectively. Reporter gene assay was performed to investigate the effect of polymorphism on vaspin promoter function.

Results: After multivariate analysis, allele A of rs2236242 was found as an independent determinant of CAD (OR = 1.32, $p = 0.004$). Rs35262691 in vaspin promoter was associated with serum vaspin concentration and mRNA expression in peripheral blood mononuclear cells (PBMC) though no association had been found with CAD. Reporter gene assay further confirmed that CC genotype of rs35262691 had 2.1 ± 0.4-fold higher activities than TT genotype in facilitating gene expression.

Conclusions: Our results show that the variants of vaspin gene are associated with serum vaspin levels and risk for CAD in Chinese population.

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1. Introduction

Visceral adipose tissue-derived serine protease inhibitor (vaspin) was first identified from visceral adipose tissue of a rat model of type 2 diabetes [1]. In human being, increased vaspin expression in adipose tissue and higher serum vaspin level were found to be associated with insulin resistance [2–6]. It was suggested that vaspin increased as a compensation for insulin resistance, and the serum vaspin level would decrease when insulin resistance was improved by medication [7–10]. Ensuing animal studies confirmed that administration of vaspin could reduce food intake and insulin resistance [1,11].

Recent studies revealed that vaspin could target vascular cells, exerting anti-inflammation and anti-apoptotic effects besides improving insulin resistance [12]. Vaspin inhibited the secretion of inflammatory factors from vascular smooth muscle cells and antagonize apoptosis of endothelial cells induced by free fatty acid [13,14]. Interestingly, low serum vaspin concentrations correlated well with recent ischemic events in patients with carotid stenosis [15]; clinical studies including our study also found that serum vaspin levels decreased in patients with coronary artery disease (CAD) and were even lower in subjects with unstable angina than stable angina [16,17], yet another study found vaspin concentration was significantly higher in women with coronary artery stenosis than in those without stenosis [18]. The potential role of vaspin linking adverse fat distribution to cardiovascular disease is further supported by distinct vaspin mRNA expression profiles in periaortic, pericoronary, and apical epicardial adipose tissue which correlated with either aortic or coronary atherosclerosis, suggesting that vaspin may affect the atherosclerotic process [19,20].

The vaspin gene locates in chromosome 14q32.13 and consists of 6 exons and 5 introns. Kempf et al. had scanned vaspin gene and found one single nucleotide polymorphism (SNP) (rs2236242) in intron 4 having significant association with diabetes [21]. However, the study by Kempf et al. enrolled patients that are mainly middle-aged Germans, thus replication studies in other populations were needed to further prove the correlation of vaspin polymorphisms with diabetes or other diseases such as CAD.

Abbreviations: BMI, Body mass index; CAD, Coronary artery disease; CRP, C-reactive protein; HUVECs, Human umbilical vein endothelial cells; OR, Odds ratio; PBMC, Peripheral blood mononuclear cells; SNP, Single nucleotide polymorphism; Vaspin, Visceral adipose tissue-derived serine protease inhibitor; UTR, Untranslated regions.

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0009-8981/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.cca.2012.10.042

This study was designed to explore the association of CAD in Chinese population with polymorphisms located in or near vaspin gene function regions including its promoter, exons and untranslated regions (UTR), because polymorphisms located in these regions often affect gene function. Then the associations of vaspin gene variants with diabetes as well as serum inflammatory factors, glucose and lipid profiles were investigated. Moreover, we also explore the potential mechanisms of the impact of vaspin polymorphisms on the gene function.

2. Materials and methods

2.1. Subjects and study design

The clinical study protocol was approved by the Ethics Committees of Shanghai Tenth Hospital and Xinhua Hospital. All study participants gave their written informed consents. Participants were enrolled consecutively from January 2010 to May 2011. The study population was Shanghai residents undergoing coronary angiography to evaluate suspected or established CAD. Subjects with heart failure (left ventricular ejection fraction <30%), myocarditis, or cardiomyopathies were excluded. Exclusion criteria also included acute or chronic infections or inflammatory diseases, severe liver or renal function defect, malignant tumor, and hematologic disorders.

All study participants underwent a standard clinical examination. Body mass index (BMI) was calculated as weight divided by the square of height. Smoking was defined as having smoked for 1 year or more. Hypertension was defined as blood pressure ≥140/90 mmHg or the use of antihypertensive medications. Diabetes was diagnosed according to the WHO criteria [22]. And left ventricular ejection fraction was measured by cardiac ultrasonic testing.

2.2. Coronary angiography

Coronary angiography was performed using standard Judkins technique or through a radial approach. Significant CAD was defined as the presence of luminal diameter stenosis ≥50% in the left anterior descending artery, left circumflex artery, right coronary artery and their main branches. Left main trunk stenosis (≥50% luminal narrowing) was considered as a two-vessel disease. Severity of coronary atherosclerosis was further categorized according to number of coronary vessels with significant stenosis as 1-, 2-, or ≥3-vessel disease. All imaging analyses were performed by 2 experienced interventional cardiologists, who were blind to patients' clinical data.

2.3. ELISA assays and biochemical investigations

Blood samples were taken after a 10-h overnight fast before angiography and centrifuged at 1000 *g* for 10 min, then serum specimens were stored at −80 °C until analysis. Serum vaspin levels were measured by ELISA kit (Adipogen, Seoul, South Korea). C-reactive protein (CRP), serum levels of glucose, and lipid profiles, including total cholesterol, low-density lipoprotein cholesterol and high-density lipoprotein cholesterol, etc. were measured by colorimetric enzymatic assay systems (Roche MODULAR P-800, Swiss Confederation).

2.4. Analysis of human vaspin gene expression in PBMC

Reverse transcription real-time PCR was performed to determine vaspin mRNA expression in peripheral blood mononuclear cells (PBMC) as mentioned before [17]. To put it briefly, total RNA was extracted from PBMC using RNeasy Mini Kit (Qiagen, Hilden, Germany) and 1 μg RNA was reverse transcribed with PrimeScript® RT reagent Kit (Takara Bio inc., Otsu, Japan). Real-time PCR assays were performed using SYBR® Premix Ex Taq™ (Takara Bio Inc., Otsu, Japan) by Applied Biosystems 7900. The primers employed were as follows: vaspin: sense 5′ AGG GCT TCC ATT ACA TCA TCC A 3′; antisense 5′ AAC AGC GTG TTC CCA ATG CT 3′; and GAPDH: sense 5′ ACG GAT TTG GTC GTA TTG GG 3′; antisense 5′ TGA TTT TGG AGGGAT CTC GC 3′. Vaspin mRNA expression was calculated with GAPDH as internal control.

2.5. DNA sequencing and genotyping

DNA was extracted from peripheral blood using standard phenol-chloroform extraction. 40 patients without CAD were enrolled at random for scanning of vaspin promoter, 5′UTR, exon, and 3′UTR by PCR amplification and direct sequencing. The primers used for gene scanning and sequencing are shown in Supplement 1. Genotyping was performed with TaqMan allelic discrimination by means of an ABI 7900HT (Applied Biosystems, Foster City, CA, USA), in a 384-well format. The TaqMan assay kits as well as probes were purchased from Applied Biosystems (Applied Biosystems, Foster City, CA, USA). Data were analyzed using the ABI Prism SDS software version 2.3.

2.6. Cloning and reporter gene assay

To elucidate the possible effect of rs35262691 polymorphisms on the promoter activity, reporter gene assay was conducted. A 989 bp DNA fragment containing nucleotides −912 to +77 from the transcription starting site of the vaspin gene promoter including rs35262691 was amplified by PCR from each genotype using HS EX-Taq DNA polymerase (Takara Bio Inc., Otsu, Japan). The following primers, including the sites of restriction enzymes, were synthesized: forward: 5′-ACG CGTCCCTTCCCTAATGCAAAGGT-3′; and reverse: 5′-CTCGAGCCATGT TCAGTCCCCTCAGA-3′. The PCR products were cloned into the MluI and XhoI sites of the pGL3-basic vector to construct the pGL3-CC or pGL3-TT recombinant plasmid and then confirmed by sequencing.

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics Cell Discovery Systems (San Diego, CA, USA) and cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Transfection was done with Lipofectamine 2000 (Invitrogen, Paisley, UK) according to the manufacturer's manual. Briefly, cells were plated at the density of 1×10^5 cells per well in 12-well plates and were transfected 24 h later with 0.8 μg test plasmid and 0.08 μg of pRL-SV40 vector that served as an internal control to normalize luciferase activity. Cells were harvested after 24 h and the luciferase activities were assessed using the Dual-Luciferase reporter assay system (Promega, WI, USA) in a Synergy™ 2 Multi-Detection Microplate Reader (BioTek Instruments, USA). Five independent experiments were carried out, and each assay was performed in triplicate.

2.7. Statistical analysis

Continuous variables were expressed as mean ± SD. Categorical variables were presented as frequencies. Differences among groups were assessed using the chi-square or ANOVA tests, followed by post-hoc analysis (Bonferroni's correction) for comparison among groups. Odds ratios (ORs) of CAD were first estimated by chi-square test and then adjusted by traditional risk factors for CAD including gender, age, hypertension, hyperlipidemia, smoking and diabetes (all factors with $p<0.1$ between CAD and non-CAD in the study population). ORs of covariates determining CAD were estimated using a multivariate logistic regression model. The chi-square test for goodness of fit was used to verify the agreement with Hardy–Weinberg equilibrium. Haplotypes and linkage disequilibriums were estimated using the HapAnalyzer 1.0 software (http://hap.ngri.go.kr/). A p-value of ≤0.05 was taken as significant and ≤0.016 was taken as significant for post-hoc analysis. All analyses were performed with SPSS for Windows 13.0 (SPSS Inc, Chicago, Illinois, USA). Power calculations were

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